Open access • Journal Article • DOI:10.1016/J.CBPC.2009.11.007

Direct and transgenerational impact on Daphnia magna of chemicals with a known effect on DNA methylation — Source link

Michiel B. Vandegehuchte, Filip Lemière, Lynn Vanhaecke, Wim Vanden Berghe ...+1 more authors

Institutions: Ghent University, University of Antwerp

Published on: 01 Apr 2010 - Comparative Biochemistry and Physiology C-toxicology & Pharmacology (Comp Biochem Physiol C Toxicol Pharmacol)

Topics: DNA methylation, Epigenetics, Vinclozolin and Daphnia magna

Direct and transgenerational impact on *Daphnia magna* of chemicals with a known effect on DNA methylation

Michiel B. Vandegehuchte, Filip Lemière, Lynn Vanhaecke, Wim Vanden Berghe, Colin R. Janssen

PII: S1532-0456(09)00244-0
DOI: doi: 10.1016/j.cbpc.2009.11.007
Reference: CBC 7594

To appear in: *Comparative Biochemistry and Physiology*

Received date: 16 September 2009
Revised date: 18 November 2009
Accepted date: 19 November 2009

Please cite this article as: Vandegehuchte, Michiel B., Lemière, Filip, Vanhaecke, Lynn, Vanden Berghe, Wim, Janssen, Colin R., Direct and transgenerational impact on *Daphnia magna* of chemicals with a known effect on DNA methylation, *Comparative Biochemistry and Physiology* (2009), doi: 10.1016/j.cbpc.2009.11.007

CBP ms.17570 Revised – part C

Direct and transgenerational impact on *Daphnia magna* of chemicals with a known effect on DNA methylation

Vandegehuchte, Michiel B.[a], Lemière, Filip[b], Vanhaecke, Lynn[c], Vanden Berghe, Wim[d], Janssen, Colin R.[a]

[a] *Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, J. Plateaustraat 22, B-9000 Ghent, Belgium, Michiel.Vandegehuchte@UGent.be and Colin.Janssen@UGent.be*

[b] *Nucleoside Research & Mass Spectrometry Unit, Centre for Proteome Analysis and Mass Spectrometry (CeProMa), Department of Chemistry, University of Antwerp, B-2020 Antwerp, Belgium, filip.lemiere@ua.ac.be*

[c] *Department of Veterinary Public Health and Food Safety, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium, Lynn.Vanhaecke@UGent.be*

[d] *Laboratory for Eukaryotic Gene Expression and Signal Transduction, Department of Physiology, Ghent University, K.L. Ledeganckstraat 35 - 11 HB, B-9000 Ghent, Belgium,* w.vandenberghe@UGent.be

Corresponding author: Michiel B. Vandegehuchte
J. Plateaustraat 22, B-9000 Ghent, Belgium
Tel.: +32 9 264 37 07
Fax: +32 9 264 37 66
Email: Michiel.Vandegehuchte@UGent.be

Abstract

The purpose of this study is to investigate (1) the induction of epigenetic effects in the crustacean *Daphnia magna* using DNA methylation as an epigenetic mark and (2) the potential stable transfer of such an epigenetic effect to non-exposed subsequent generations. Daphnids were exposed to chemical substances known to affect DNA methylation in mammals: vinclozolin, 5-azacytidine, 2'-deoxy-5-azacytidine, genistein and biochanin A. Effects on overall DNA cytosine methylation, body length and reproduction were evaluated in 21 day experiments. Using a multi-generational experimental design these endpoints were also evaluated in the F_1 and F_2 generation of both exposed and non-exposed offspring from F_0 daphnids exposed to 5-azacytidine, genistein or vinclozolin. A reduction in DNA methylation was consistently observed in daphnids exposed to vinclozolin and 5-azacytidine. Only in organisms exposed to 5-azacytidine was this effect transferred to the two subsequent non-exposed generations. A concurrent reduction in body length at day 7 was observed in these treatments. For the first time, exposure to environmental chemicals was shown to affect DNA methylation in the parental generation of *D. magna*. We also demonstrated a transgenerational alteration in an epigenetic system in *D. magna*, which indicates the possibility of transgenerational inheritance of environment-induced epigenetic changes in non-exposed subsequent generations.

Keywords. 2'-deoxy-5-azacytidine, 5-azacytidine, biochanin A, ecotoxicology, epigenetics, genistein, inheritance, vinclozolin

1 Introduction

Epigenetics has been defined as the inheritance of DNA activity that does not depend on the naked DNA nucleotide sequence (Esteller 2008b). Three mechanisms involved in epigenetic control are: DNA methylation, Polycomb and Trithorax group proteins in association with histone modifications and non-coding RNA molecules (Feil 2008). Numerous forms of interplay between these mechanisms have been reported (Chuang et al. 2007; Guil et al. 2009).

Exposure to environmental toxicants can induce epigenetic changes (Reamon-Buettner et

al. 2008). A recent review lists several environmental chemicals - such as metals, peroxisome proliferators, air pollutants and endocrine-disrupting chemicals - that are capable of modifying epigenetic marks (Baccarelli et al. 2009). In most cases DNA methylation is affected, but also histone modifications and microRNA expression can be altered by toxic stress. Different mechanisms may underlie the interaction between environmental toxicants and epigenetic changes. Tributyltin and triphenyltin for example have been shown to induce hypomethylation in the liver of the false kelpfish *Sebastiscus marmoratus* (Wang et al. 2009). This was attributed to imbalances in the transmethylation reaction between DNA and S-adenosylmethionine (SAM) / S-adenosylhomocysteine.

The inheritance of epigenetic factors can be mitotic, i.e. between cells of one organism or between different organisms in case of mitotic parthenogenesis, or meiotic, i.e. between different generations of sexually reproducing organisms. Although most studies on transgenerational epigenetic inheritance deal with plants or mammals, it has also been reported in insects (Youngson et al. 2008). Transgenerational activation of a polycomb/trithorax response element and histone H4 hyperacetylation have been demonstrated in *Drosophila* (Cavalli et al. 1998). Transgenerational transfer of chromosome sets with hypomethylated DNA has been reported in the mealybug *Planococcus citri* (Bongiorni et al. 1999; Bongiorni et al. 2009).

An interesting aspect of epigenetics for the field of environmental sciences is that environment-induced epigenetic changes can be transferred to subsequent generations even if the triggering environmental factor is removed. Mice fed with a methyl donor supplemented diet during gestation resulted in a shift in fenotypes up to two generations later, demonstrating a germ-line epigenetic change in a specific allele (Cropley et al. 2006). Anway et al. (2005) reported that non-exposed offspring of gestating female rats transiently exposed to vinclozolin and methoxychlor, exhibited reduced reproduction and altered DNA methylation patterns. If wide-spread epigenetic effects of environmental exposure are transferred to non-exposed future generations, this may have major consequences for the way ecological risk assessments of chemicals are performed as temporary exposures to contaminants may then compromise the future status of ecosystem structure and functioning.

DNA methylation, which is the addition of a methyl group on the 5 position of DNA

cytosines, is one of the best studied epigenetic marks (Clark et al. 1994; Oakeley 1999; Bird 2002; Watson et al. 2002). It is hypothesized that DNA methylation at CpG sites represses transcriptional initiation, but not necessarily represses transcription as such (Bird 1995). Recent research supports this hypothesis for infrequently transcribed genes (Mandrioli 2007; Suzuki et al. 2007). This implies that the presence or absence of DNA methylation at transcription start sites may have important consequences for various cellular processes.

Recently, DNA methylation in CpG sites has been detected in the waterflea *Daphnia magna*, an important species in many aquatic ecosystems and a model organism used in aquatic toxicology and environmental risk assessment (Vandegehuchte et al. 2009a). The total amount of cytosine methylation in *D. magna* DNA is lower than in mammals and plants, but was shown to differ in daphnids with different exposure histories. Although local hypo- or hypermethylation could not be measured with the LC-MS based technique used, it was shown that one generation exposure to a sublethal Zn concentration caused an overall reduction in DNA methylation in the F_1 offspring, which, however, was not passed on to the next generation (Vandegehuchte et al. 2009b). *Daphnia* has an interesting life cycle. It reproduces mainly through female parthenogenesis. However, certain environmental triggers (e.g. light, food) induce the production of males resulting in sexual reproduction (Zaffagnini 1987). In the laboratory, daphnids are maintained in their parthenogenetic state, in which diploid eggs develop into adult females. Oogenesis is in this case not fully meiotic nor strictly mitotic. However, no recombination occurs during parthenogenesis and as such parthenogenetic offspring are genetically identical to their mother (Hebert 1987). This makes *Daphnia* an ideal model organism for studying epigenetic transgenerational changes. It has been suggested that transgenerational effects in *Daphnia*, such as differences in the size of defensive helmets in offspring of females exposed to different predator kairomone concentrations, are based on gametic epigenetic inheritance (Agrawal et al. 1999; Youngson et al. 2008).

The aim of this study is to investigate whether DNA methylation in *D. magna* is affected by exposure to substances with a well-known effect on DNA methylation in mammals. Second it is hypothesized that such an epigenetic effect can be transferred to multiple non-exposed generations of *D. magna*. Effects on global DNA methylation levels are measured, as well

as effects on length and reproduction of the daphnids. Two active pharmaceutical compounds that are known to inhibit DNA methyltransferases (DNMTs) were examined: 5-azacytidine and 2'-deoxy-5-azacytidine (Piekarz et al. 2009). The isoflavones genistein and biochanin A were also evaluated as they have been associated with DNA hypermethylation. These substances were also shown to reduce DNMT activity and directly or indirectly alter DNA methylation (Fang et al. 2005; Dolinoy 2006; Dolinoy 2007). Finally the endocrine disrupting fungicide vinclozolin was tested as this compound induces aberrant methylation patterns after intraperitoneal injection in rats (Anway et al. 2005).

2 Materials and Methods

2.1 Daphnia cultures and experimental design

Daphnia 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. The culture medium used in all experiments consisted of aerated carbon filtered tap-water, enriched with selenium and vitamins (Elendt et al. 1990).

Preliminary acute tests were performed according to OECD 202 guideline (OECD 1994). A series of five concentrations was made based on concentrated stock solutions of 5-azacytidine (7000 mg/L in culture medium), 5-aza-2'-deoxycytidine (2333 mg/L in culture medium), Biochanin A (28426 mg/L in DMSO), Genistein (27 024 mg/L in DMSO) or Vinclozolin (350 g/L in acetone). All chemicals were purchased from Sigma-Aldrich, Bornem, Belgium. The actual test concentrations are given in the electronic supplementary material. Solvent controls were prepared for acetone and DMSO. Three replicate glass vessels were used with ten neonate daphnids in 25 mL test medium. Immobility was assessed after 48 hours as the number of daphnids that remained immobile for ≥ 10 s after test vessel swirling.

Chronic tests were performed according to OECD guideline 211 (OECD 1998). Concentrated stock solutions of 5-azacytidine (50000 mg/L in DMSO), 5-aza-2'-deoxycytidine (27000 mg/L in DMSO), Biochanin A (28426 mg/L in DMSO), Genistein (27 024 mg/L in DMSO) or Vinclozolin (71980 mg/L in DMSO), which were stored at -

20°C, were used to make up 4 test concentrations of each substance. The actual test concentrations are given in the electronic supplementary material. For this test and for the multigeneration experiment, 5-azacytidine and 5-aza-2'-deoxycytidine were purchased from Carbosynth, Compton, UK. To ensure minimal mortality, test concentrations were all lower than the lowest concentration which caused a significant effect in the acute test. Solvent controls with 0.0176% and 0.05% DMSO were also tested. Ten replicate glass vessels with a single neonate daphnid in 50 mL test medium were maintained for 21 days at 20°C ± 1 °C under a 16h/8h light/dark cycle. Media were renewed three times per week during which the number of living offspring were counted and discarded. Daphnids were fed daily with an algae mix consisting of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3/1 cell number ratio. The amount fed increased during the test: 250 µg/day in the first week, 500 µg/day in the second week and 750 µg/day in the third week. The length of each daphnid from the top of the head to the base of the spine was measured on day 7 and day 21 by analyzing a microscopic image with UTHSCSA Image Tool 3.0 (San Antonio, TX, USA).

A multigeneration experiment was performed with daphnids exposed to 5-azacytidine, genistein and vinclozolin. Based on the results of the chronic test, concentrations were selected which were shown to have an effect on DNA methylation or on reproduction, but a limited or no effect on mortality. Nominal concentrations were 7.4 mg/L 5-azacytidine, 4.4 mg/L genistein and 3.6 mg/L vinclozolin. Measured concentrations in freshly prepared medium were 2.9 ± 0.4 mg/L in F_0, 2.3 ± 0.3 mg/L in F_1 for 5-azacytidine; 4.7 ± 0.7 mg/L in F_0-F_2 for genistein and 0.54 ± 0.19 mg/L in F_0, 0.45 ± 0.16 mg/L in F_1, 0.18 ± 0.15 mg/L in F_2 for vinclozolin. Organisms were cultured in a semi-static manner in glass vessels, using 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. For each treatment, ten individual daphnids were maintained in parallel as described above. The length of these daphnids was measured on day 7 and 15.

The culturing scheme is represented in Fig. 1. Neonates from the laboratory culture were divided into four batches. One batch of thirty daphnids was transferred into standard medium with 0.015% DMSO and cultured in this medium for three generations as a control (F_0C-F_2C). A second batch of organisms was transferred to a medium containing 5-

azacytidine (A^+), a third batch to medium spiked with genistein (G^+) and the fourth batch to medium containing vinclozolin (V^+). Third brood F_1 neonates born from this F_0A^+, F_0G^+ or F_0V^+ generation were divided into two batches, of which one was transferred to the control medium (F_1A^-, F_1G^- or F_1V^-). These daphnids were thus only briefly exposed to the test substances during the first hours of their life cycle. The other batch was kept in the medium containing 5-azacytidine (F_1A^+), genistein (F_1G^+) or vinclozolin (F_1V^+). F_1A^-, F_1G^- and F_1V^- third brood, non-exposed offspring were further cultured in the control medium (F_2A^-, F_2G^- and F_2V^-), while offspring from F_1A^+, F_1G^+ and F_1V^+ were cultured in the same medium as their parents (F_2A^+, F_2G^+ and F_2V^+). Organisms were fed daily with an algae mix consisting of *P. subcapitata* and *C. reinhardtii* in a 3/1 cell number ratio. The amount fed increased during the test: 119 µg/org/day in the first week and 250 µg/org/day from the second week onwards.

2.2 Chemical analysis

Samples from the different treatments were taken at the beginning and end of the acute test, just before and after medium renewals in the chronic test and in each generation of the multigeneration experiments. Samples were stored in glass tubes at -20°C prior to analysis. The (deoxy)nucleoside analogues 5-azacytidine and 5-aza-2'-deoxycytidine were analyzed, after filtration of the incubation medium over a 0.45 µm filter, using LC-MS/MS with an external standard series in methanol. Chromatography was carried out on a Thermo Finnigan Surveyor LC system (San Jose, CA, USA) comprising a quaternary pump and an autosampler, equipped with a 5 µm 2.5 x 450 mm Sphinx C_{18} column obtained from Macherey-Nagel (Düren, Germany). Compounds were eluted at a flow rate of 400 µL/min using a linear gradient starting with a mixture of 50% A (0.01% aqueous formic acid) and 50% B (acetonitrile) for 5 min. The methanol percentage was increased from 50 to 100 % during a 5 minute period. Analytes were detected with an LTQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) in the MS-MS positive ion mode using a Heated Electrospray Ionisation (HESI) interface at 180°C. Mass 245 ($[M+H]^+$) was isolated for 5-azacytidine or mass 229 ($[M+H]^+$) for 5-aza-2'-deoxycytidine. The precursor isolation width was set to 2 Da, the activation Q to 0.25, and the collision energy to 40 %.

The isoflavones genistein and biochanin A were extracted from the incubation media (2

mL) by solid phase extraction using Isolute C18 columns (500 mg). Prior to extraction, chrysene (200 ng) was supplemented as an internal standard in both the samples and in the biochanin A and genistein standard series. The Isolute cartridges were preconditioned with 4 mL methanol and 4 mL water. After passing the eluate and washing the cartridges with 4 mL water and 2 mL hexane, elution was performed with 4 mL methanol. Subsequently, the extracts were evaporated to dryness under a stream of nitrogen and redissolved in 120 µL of methanol- 0.5% formic acid (50:50). Finally the extracts were centrifuged during 10 min at 2500xg and 4°C and injected into the LC-MS/MS in a volume of 30 µL. The HPLC apparatus consisted of a HP 1100 series pump, an AS3000 autosampler and HP vacuum degasser (Agilent, Palo Alto, USA), equipped with a Symmetry C_{18} column (5 µm, 150 x 2.1 mm, Waters, Milford, USA). For separation of the different compounds, a linear gradient was used starting with a mixture of 50% A (0.5% aqeous formic acid) and 50% B (methanol). The methanol percentage was increased from 50 to 100 % during a 15 minute period. The flow rate was set at 300 µL/min. Between each sample the column was allowed to equilibrate at initial conditions (10 minutes). Analysis was carried out using an LCQ^{DECA} Ion Trap Mass Analyzer (Thermo Electron, San Jose, USA) with an electrospray ionization (ESI) interface (Thermo Electron). The compounds were detected in the MS-MS positive ion mode. Alternating scans were used to isolate $[M+H]^+$ ions at masses 269.30 for genistein and 283.20 for biochanin A. The precursor isolation width was set to 2 Da, the activation Q to 0.25, and the collision energy to 45 %.

Vinclozolin was extracted from the incubation medium (1 or 5 mL) by liquid /liquid extraction using three sequential extraction steps with equal volumes of hexane/diethylether (50:50). Prior to extraction, heptachlor was supplemented to the incubation medium (50 µL of 20 mg/L) to serve as internal standard. After centrifugation of the solvent-incubation medium mixture at 2500xg for 5 min, the different solvent fractions were pooled and dried under a nitrogen stream at 40°C. Finally, the extract was redissolved in hexane and subsequently measured by GC-MS/MS. These analyses were performed using a Trace Gas Chromatograph 2000 fitted with a Polaris ion trap mass spectrometer (Thermo Fisher, Austin, TX, USA) and a Carlo Erba AS2000 Autosampler (Thermo Fisher). Helium (99.99% purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 mL min^{-1} and perfluorotributylamine (FC43) was used as calibration gas. A sample volume of 1 µL

242 was injected (split flow 60 mL min^{-1}, splitless time 1 min). Chromatographic separation of
243 the analytes and internal standard was performed on a BPX5 capillary column (25 m x 0.22
244 mm ID) with a 5% phenyl-polysilphenylene-siloxane phase (0.25 µm film) (SGE
245 Analytical Science Pty. Ltd., Victoria, Australia). The temperature program started at an
246 initial temperature of 80°C. Temperature was increased to 140°C applying a ramp of 50°C
247 min^{-1}. Subsequently, an increase to 260°C was assessed using a ramp of 5°C min^{-1}, holding
248 this temperature for 3 minutes. Spectra were obtained in positive electron impact ionisation
249 (EI) mode MS-MS scan. Mass range depended on the selected precursor ion, and the
250 collision energy ranged from 1.15 to 1.30 V.
251 For all analyses data processing was performed using Xcalibur® 2.0 software (Thermo
252 Electron).
253
254 *2.3 DNA methylation analysis*
255 DNA was extracted from 21 day-old daphnids at the end of the chronic test and from
256 daphnids on the first day the third brood was observed (day 14 to day 16) in the
257 multigeneration experiment. This was not possible in the F_1A^+ treatment, in which no
258 reproduction was observed up to day 21. Here DNA extraction of the 21 day-old daphnids
259 was performed. The MasterPure™ kit (Epicentre, Madison, WI, USA) was used following
260 the protocol for DNA extraction from tissue as provided by the manufacturer. Four to six
261 adult organisms per replicate were rinsed with deionized water, blotted dry and shock
262 frozen in liquid nitrogen prior to extraction. Hydrolysis of DNA was performed following
263 Crain (Crain 1990). A sample of 1.3 to 4.25 µg DNA was adjusted to 16.8 µL with Tris-
264 HCl (1 mM, pH 7.4). The DNA was denatured by heating at 100 °C for 3 min in a warm
265 water bath. The denatured DNA was hydrolyzed by adding 0.75 µL (1.5 units) nuclease P1
266 (Sigma-Aldrich, Bornem, Belgium) and 1/10 volume of 0.1 M NH_4OAc (pH 5.3). This was
267 incubated at 45°C for 2 h. Subsequently, 0.002 units phosphodiesterase I (Sigma-Aldrich,
268 Bornem, Belgium) and 1/10 volume of 1 M NH_4HCO_3 at pH 7.8 were added to the sample.
269 This was incubated at 37 °C for 2 h. Phosphates were removed by adding 0.5 units alkaline
270 phosphatase (Fermentas, St. Leon-Rot, Germany) and 1/10 volume phosphatase buffer and
271 this mixture was incubated at 37 °C for 1 h, after which it was stored at -20°C prior to
272 analysis.

Hydrolyzed DNA samples were analyzed for the detection of 5-methyl-2'-deoxycytidine on a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system with a Tandem Quadrupole (TQ) detector (Waters, Zellik, Belgium). The system was controlled by MassLynx software (version 4.1, Waters). LC separation was performed on a Waters Acquity UPLC HSS T3 1.8 µm column of 2.1 x 100 mm at a flow rate of 300 µL/min. A binary solvent system was used: 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Inlet method, gradient, mass spectrometric methods and conditions, standard curves and monitored transition pairs were as described before (Vandegehuchte et al. 2009b) For a number of samples the solvent flow was 350 µL/min and for the samples from the multigeneration experiment a different inlet method was used to optimize system stability. From t = 0 min to t = 2 min elution remained isocratic at 300 µL/min and 99% of water, after which a gradient was created to 70.9 % aqueous at t = 4.40 min. This dropped to 65% aqueaous at t = 4.50 min and was set to a washing step of 90% organic from t= 4.51 min to t = 5.51 min. Subsequently an equilibration step at initial conditions but with a flow of 500 µL/min followed from t = 5.52 min to t = 7.51 min, after which the flow was set back to the initial 300 µL at the end of the run at t = 7.52 min.

The relative 5-methyl-2'-deoxycytidine (mdC) content is expressed as a fraction of the total measured dG concentration or as % [mdC]/[dG] (Song et al. 2005). Both [mdC] and [dG] were quantified using an external standard series prepared with commercially available mdC (US Biological, Swampscott, MA, USA) and dG (Aldrich, Bornem, Belgium).

It should be clear that this method measures overall cytosine methylation, implying that effects on the DNA methylation at specific loci may go undetected, e.g. when hypomethylation in a certain region of the genome is accompanied by hypermethylation in another region.

2.4 Statistical analysis

EC_{50}s (Effective Concentration causing immobility in 50% of the daphnids) for the acute tests were calculated with the trimmed Spearman-Karber method using the US EPA software (http://www.epa.gov/nerleerd/stat2.htm) (Hamilton et al. 1977). All other statistics were performed with Statistica (Statistica, Tulsa, USA) or with Excel (Microsoft, Redmond, USA). Differences in reproduction (total number of juveniles per surviving

female adult), length or DNA methylation between treatments in the chronic and multigeneration experiment were assessed using Dunnett's test based on the pooled residual standard deviation, which was calculated with ANOVA. If an increase or decrease in reproduction, length or DNA methylation could be expected *a priori*, a one-tailed Dunnett's test was used. In all other cases a two-tailed Dunnett's test was performed. For DNA methylation as % [mdC]/[dG], a bootstrapping method was used to incorporate the error due to the uncertainty of the standard curves of mdC and dG (Vandegehuchte et al. 2009b). The method (either with or without the bootstrapping) resulting in the largest standard deviation was used for assessing differences between treatments. For reproduction in the multigeneration experiment, F_0A^+ and F_1A^+ were treated as outliers due to the large number of replicates with zero reproduction (which caused the variance in these treatments to be very low). Assumptions of normality and homoscedasticity were tested with Shapiro-Wilk's test and Bartlett's test, respectively. When the homoscedasticity assumption was not met, a Kruskal-Wallis non parametric test was used. If differences between treatments were detected with Kruskal-Wallis, treatments were compared with controls using Mann-Whitney U tests. When the DNA methylation between a treatment and a control was compared with Mann-Whitney U, a bootstrapping method was also used to incorporate the uncertainties on the standard curves of mdC and dG. For both treatments with r replicates, a random replicate was sampled r times (with replacement). For each selected replicate, a random value was selected from the t distribution associated with the uncertainty of the regression curves. This was repeated 2000 times. On these 2000 sets of two treatments, a Mann-Whitney U test was performed, with an associated p-value. The average of these 2000 p-values was taken as the final p-value for this Mann-Whitney U test. In all tests, the limit of significance was set at $p = 0.05$.

3 Results

3.1 Acute tests

Control immobility was 0 % in all controls, including the solvent controls. EC_{50}s are summarized in Table 1. For 5-aza-2'-deoxycytidine, no immobility was observed in any of the concentrations tested, while for vinclozolin, only 2 out of 30 daphnids were immobile

335 after 48 h exposure to the highest concentration. The EC_{50}s are based on concentrations
336 measured at the beginning of the test. The concentrations generally decreased during the
337 48h tests. At the end of the test, the concentrations of 5-azacytidine, 5-aza-2'-deoxycytidine
338 and vinclozolin were reduced to the following fractions of the initial concentrations: 6 to 77
339 %, 29% and 1 to 15%, respectively (see electronic supplementary material). The isoflavone
340 concentrations remained rather constant throughout the test. From nominal concentration of
341 ≥ 3.6 mg/L (measured concentration 0.182 ± 0.099 mg/L) vinclozolin, small non-dissolved
342 particles could be observed in the test medium. This is in accordance with the water
343 solubility of 3.5 mg/L vinclozolin at 20 °C (Vallero et al. 2003).
344
345 *3.2 Chronic experiments*
346 Differences between treatments will only be discussed when they are statistically
347 significant ($p < 0.05$).
348 LOECs are expressed as average measured concentrations in freshly prepared medium
349 (Table 2). The concentration of some compounds decreased considerably between two
350 medium renewals. 5-azacytidine and 5-aza-2'-deoxycytidine were not detectable after three
351 days, while after two days on average 22% and 49% (respectively) of the original
352 concentration was present in the medium. For biochanin A and genistein, there was no
353 consistent trend. Vinclozolin concentrations decreased to approximately 0.4 to 1.5% of the
354 initial concentration after three days.
355 Two quality controls (QCs) for DNA methylation were measured in triplicate, resulting in
356 relative standard deviations (RSDs) of 2.5% and 7.4% for mdC and 1.3% and 1.6% for dG.
357 Relative Errors (REs) were -0.1% for mdC for both QCs and 5.2 and 0.3% for dG.
358 No difference was detected in reproduction, length or DNA methylation between controls
359 with 0, 0.0176 and 0.05% DMSO that were started with the same batch of daphnids. These
360 controls were pooled for the calculation of the pooled residual standard deviation with
361 ANOVA for the experiments with biochanin A, genistein and vinclozolin.
362 For all test substances, an effect on at least one of the endpoints (reproduction, length and
363 overall DNA methylation) could be observed. Vinclozolin did not elicit an effect on
364 reproduction at the tested concentrations. 5-aza-2'-deoxycytidine did not induce an effect
365 on body length at any of the tested concentrations, while biochanin A, 5-aza-2'-

deoxycytidine and genistein did not affect overall DNA cytosine methylation.
The initial two highest concentrations of the 5-azacytidine test caused 100% mortality after two days. Therefore, two lower 5-azacytidine concentrations and a new control were introduced into the design. Reproduction was determined as the number of living juvenile daphnids per surviving female adult. In the 5-azacytidine experiment, a large number of aborted broods was observed at the three highest concentrations.

3.3 Multigeneration experiment

As observed also in the chronic experiment, the 5-azacytidine concentration decreased between medium renewals, with no detectable concentration after three days and on average 29% of the initial concentration after two days. The genistein concentration of 4.7 ± 0.7 mg/L was very similar to the highest concentration in the chronic experiment and remained stable throughout the multigeneration experiment. Vinclozolin concentrations measured in the fresh test media of the multigeneration experiment decreased with time from 0.54 ± 0.19 mg/L in F_0 to 0.45 ± 0.16 mg/L in F_1 and 0.18 ± 0.15 mg/L in F_2. The vinclozolin concentration decreased between two renewals. After three days, approximately 0.1% to 1.1% of the initial vinclozolin concentration in freshly prepared medium was detected.
The highest 5-azacytidine concentration of the chronic test, for which a reduction in overall DNA methylation was observed, yielded a reproduction of only 1.5 juveniles per surviving female. A reproduction as low as this is not suitable for a multigeneration experiment. Therefore the second highest concentration was chosen for the A^+ exposures. The highest genistein concentration of the chronic experiment was selected for the G^+ exposures, to confirm the absence of an effect on overall DNA methylation. For vinclozolin, the highest concentration of the chronic test, in which a reduction in overall DNA methylation was observed, was chosen as exposure concentration in the multigeneration experiment.
Reproduction was affected in the F_0 daphnids exposed to 5-azacytidine and genistein (Fig. 2). This effect was not passed on to the F_1G^- offspring. The F_1A^- treatment was accidentally stopped at day 14, at which time no third brood was present yet. However, reproduction at day 14 was significantly lower in F_1A^- compared to F_1C (Mann-Whitney U test, $p = 0.029$). A clear effect on reproduction was also observed in F_1A^+, in which no reproduction was

observed up to the end of the test (day 21). In the F_2 generation, none of the treatments exhibited a significantly lower reproduction than the control. No mortality was observed in the controls of the three generations.

On day 7, reductions in the length of the daphnids was noted in all exposed treatments except in F_1V^+ (Fig. 3). Of the non-exposed F_1 and F_2 treatments, only A^- exhibited a reduction in length.

Overall DNA methylation expressed as %[mdC]/[dG] ranged from 0.11% to 0.40%. The two quality controls for DNA methylation showed that the RSDs were 6.9% and 0.7% for mdC and 2.7% and 0.001% for dG. REs were 2.8% and -6.7% for mdC and -0.4% and 5.4% for dG. The relative proportion of 5mdC in DNA was reduced in F_0A^+ and F_0V^+, but not reduced nor increased in F_0G^+ (Fig. 4). The reduction in F_0A^+ was also observed in its F_1A^- and F_2A^- offspring. In the F_1A^+ treatment, only one replicate could be measured due to high mortality and low biomass of the organisms. The overall DNA cytosine methylation level was only 57% of that in the control, but no statistical significance could be attributed to this. The reduction in methylation observed in F_0V^+ was also present in the F_1V^+ offspring (exposed), but not in the F_1V^- (non-exposed) offspring. In the subsequent generation however, the F_2V^- organisms exhibited a smaller amount of global DNA methylation than the F_2C.

4 Discussion

4.1 Acute tests

From the acute test results, it is clear that 2'-deoxy-5-azacytidine (21 mg/L) and vinclozolin (1.685 mg/L) had no effect on the immobility of the daphnids at the tested concentrations. This is somewhat unexpected because the material safety data sheet of vinclozolin (Sigma) reports a (nominal) 48 h EC_{50} for *D. magna* of 3.65 mg/L. However, our results are in agreement with those of Haeba et al. (2008), who found no acute effect of vinclozolin up to its water solubility. The noted decrease in concentration of the (deoxy)nucleoside analogues and vinclozolin during the exposure, which was also observed in the subsequent chronic and multigeneration experiments, was not unexpected. Indeed, 5-azacytidine, 2'-deoxy-5-azacytidine and vinclozolin are not stable in aqueous environments and hydrolyze to

several by-products (Lin et al. 1981; Szeto et al. 1989; Zhao et al. 2004). However, it was not the purpose of this study to determine exact effective concentrations of these substances. Instead the main goal of this study was to investigate whether the substances or their degradation products could elicit possible transgenerational epigenetic effects (and this based on measured substance concentrations).

4.2 Chronic experiments

Based on the results of the acute tests, a range of concentrations was chosen for the chronic experiment aimed at establishing a sublethal concentration which has an effect on DNA methylation and reproduction or growth. This concentration could subsequently be used in the multigeneration experiment. For all five compounds, an effect on length or reproduction was noted in at least one of the tested concentrations.

No effect on overall DNA methylation was observed in the chronic experiments with biochanin A, genistein and 2'-deoxy-5-azacytidine. The potential inhibition of DNMT activity by biochanin A and 2'-deoxy-5-azacytidine, as described by Fang et al.(2005) and Piekarz et al. (2009) respectively, did not result in an overall decrease in DNA methylation in exposed *Daphnia*. Genistein has been shown to inhibit DNMT activity, resulting in reduced methylation in the methylated promoter regions of three genes in a human esophagous carcinoma cell line (Fang et al. 2005). On the other hand, genistein induced hypermethylation in CpG islands and restored hypomethylated loci in mice (Day et al. 2002; Dolinoy 2007). Our results suggest that in *D. magna*, genistein either did not affect DNA methylation mechanisms at all, or induced hypomethylation and hypermethylation at different loci, resulting in an unchanged overall DNA methylation compared to the control. In the highest genistein concentration, which was selected for the multigeneration experiment, reproduction was reduced. A negative effect on reproduction has also been described in mice, where administration of genistein via drinking water resulted in decreased oocyte maturation and *in vitro* fertilization, as well as early embryonic developmental injury (Chan 2009).

Exposure to the nucleoside analog 5-azacytidine caused a concentration dependent effect on reproduction, with a high number of aborted broods in the highest treatment. This compound is known to cause preimplantation loss and reduced fertility when administered

459 to male rats before mating (Doerksen et al. 1996). The demethylating effect, which was
460 detected in *D. magna* exposed to the highest concentration, was expected based on the
461 known interaction of 5-azacytidine with DNMTs (Ghoshal et al. 2002).
462 The absence of an effect on reproduction of vinclozolin at the highest tested concentration
463 of 0.43 mg/L corroborates the results of Haeba et al. (2008) who reported no effects on
464 reproduction at a nominal concentration of 1 mg/L. However, whereas a small but
465 significant decrease in body length was observed in daphnids exposed to 0.43 mg/L
466 vinclozolin in the current study, no such effect was noted by those authors. Vinclozolin
467 exposure induced both hypermethylation and hypomethylation events at 25 regions in the
468 rat genome (Anway et al. 2005). Inawaka et al. (2009), however, could not confirm the
469 vinclozolin-induced DNA methylation changes in one of those regions within the
470 lysophospholipase gene. In *D. magna*, we observed a reduction in overall DNA methylation
471 upon exposure to 0.43 mg/L vinclozolin, indicating that vinclozolin or its degradation
472 products do interact with DNA methylation.
473 Global DNA hypomethylation, as observed here in vinclozolin and 5-azacytidine exposed
474 daphnids, has been associated with cell proliferation and with hypomethylation of
475 transposable elements which can alter gene expression (Schulz 2006; Huang et al. 2008).
476 This observation has also been reported in rat, mouse and human cells or tissues after
477 exposure to various environmental chemicals (Baccarelli et al. 2009)
478
479 *4.3 Multigeneration experiment*
480 First, the results of the F_0 generation are compared with the results of the chronic
481 experiment. Effects on length, reproduction and DNA methylation in exposed F_0 daphnids
482 generally corroborate the effects observed in the chronic experiment. In F_0A^+, however, the
483 reduced length at day 7 and the reduction in DNA methylation were not observed at the
484 corresponding nominal concentration in the chronic test. It may be noted that the control
485 length of 2.61 ± 0.08 mm at day 7 in the multigeneration experiment is lower than that of
486 2.73 ± 0.12 in the chronic 5-azacytidine experiment. The batch of smaller daphnids with
487 which the multigeneration experiment was initiated appears to be more sensitive to 5-
488 azacytidine exposure.
489 The following paragraph discusses the effects in daphnids exposed during consecutive

generations. Increased effects on reproduction (reduced number of produced eggs) in different generations under continuous exposure to environmental stress have been reported for *D. magna* (Alonzo et al. 2008). Similar continuing phenotypic effects were observed in our study for reproduction during azacytidine exposure and for length during azacytidine and genistein exposure. Global DNA hypomethylation in both F_0A^+ and F_1A^+ indicates a possible link with the reduced length and reproduction. In porcine fetal fibroblasts, growth reduction combined with lower DNA methylation was observed after treatment with 5-azacytidine (Mohana Kumar et al. 2006). No connection between overall DNA methylation status, which was not altered, and length reduction in genistein exposed daphnids can be made. The length reduction in the F_0V^+ daphnids was not observed in the F_1V^+ daphnids, but returned in the F_2V^+ daphnids. The overall DNA-methylation in F_1V^+ on the contrary remained smaller than that of the control organisms, while it was not significantly different from the control in F_2V^+, suggesting that the length reduction in the V^+ treatments is not directly linked to the reduced DNA methylation.

When evaluating the effects in non-exposed offspring produced by exposed F_0 daphnids, a reduction in length and reproduction was noted in the F_1A^- daphnids, who were only exposed to 5-azacytidine during the first hours of their life cycle. This coincided with a similar decrease in DNA methylation compared to the control as in F_0. The reduced DNA methylation in the non-exposed F_2A^- daphnids demonstrates, for the first time in *Daphnia*, a transgenerational alteration in an epigenetic system. The reproduction in F_2A^- returned to a level not significantly differing from the control. However, body length at day 7 remained reduced. Although we cannot demonstrate any direct relationship with the epigenome, these observations suggest the possibility of an epigenetic transgenerational effect on juvenile growth in *D. magna*. Transgenerational transfer of 5-azacytidine to F_2A^- is highly unlikely because of its short half-lives of 1.82 ± 1.51 h in plasma and approximately 4 h in neutral to alkaline solutions. It can be demonstrated that metabolites of 5-azacytidine do not inhibit DNMTs (Zhao et al. 2004; Chabner et al. 2006; Esteller 2008a).

The absence of any effect on body length, reproduction/mortality or overall DNA methylation in F_1G^- and F_1V^- reveals that the observed effects in the genistein and vinclozolin exposed F_0 treatments are not transgenerationally heritable to non-exposed offspring. There is no obvious explanation for the reduction in overall DNA methylation in

F_2V^-. If this would be an epigenetic effect induced by the F_0V^+ vinclozolin exposure, a similar reduction in DNA methylation should have been observed in F_2V^+.

It should be noted that with the methylation assessment method used in this study, no information could be obtained on the location and hence the possible function of the methylated cytosines in *D. magna* DNA from different treatments. The *D. magna* genome is currently being sequenced at Indiana University's Center for Genomics and Bioinformatics and next-generation sequencing also opens new possibilities with regard to genome wide DNA methylation analysis. Future research should therefore focus on the specificity of the epigenetic effects on DNA methylation caused by exposure to environmental chemicals and the molecular pathways involved. This may elucidate the possible epigenetic mechanism behind the juvenile growth reduction in the offspring of 5-azacytidine exposed daphnids.

5 Conclusions

For the first time, direct effects of exposure to chemicals on overall DNA methylation in *Daphnia* have been described. Exposure to elevated concentrations of the fungicide vinclozolin and the nucleoside analog 5-azacytidine (in combination with their degradation products in aqueous media) resulted in a decrease in overall DNA-methylation. This effect on DNA methylation was not observed after exposure to lower concentrations of these substances. The isoflavones genistein and biochanin A and the deoxynucleoside analog 2'-deoxy-5-azacytidine did not induce an effect on overall *D. magna* DNA methylation at exposure concentrations for which effects on reproduction were observed. 5-azacytidine was the only compound for which the effect of reduced DNA methylation was stably transferred to two subsequent non-exposed generations. The demonstration of a transgenerational alteration in an epigenetic system in *D. magna* indicates the possibility of transgenerational inheritance of environment-induced epigenetic changes in non-exposed subsequent generations.

Acknowledgements

The authors want to thank Emmy Pequeur 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).

References

Agrawal, A.A., Laforsch, C., Tollrian, R., 1999. Transgenerational induction of defences in animals and plants. Nature 401, 60-63.

Alonzo, F., Gilbin, R., Zeman, F.A., Garnier-Laplace, J., 2008. Increased effects of internal alpha irradiation in Daphnia magna after chronic exposure over three successive generations. Aquat. Toxicol. 87, 146-156.

Anway, M.D., Cupp, A.S., Uzumcu, M., Skinner, M.K., 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308, 1466-1469.

Baccarelli, A., Bollati, V., 2009. Epigenetics and environmental chemicals. Curr. Opin. Pediatr. 21, 243-251.

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6-21.

Bird, A.P., 1995. Gene number, noise reduction and biological complexity. Trends Genet. 11, 94-100.

Bongiorni, S., Cintio, O., Prantera, G., 1999. The relationship between DNA methylation and chromosome imprinting in the coccid Planococcus citri. Genetics 151, 1471-1478.

Bongiorni, S., Pugnali, M., Volpi, S., Bizzaro, D., Singh, P.B., Prantera, G., 2009. Epigenetic marks for chromosome imprinting during spermatogenesis in coccids. Chromosoma 118, 501-512.

Cavalli, G., Paro, R., 1998. The Drosophila Fab-7 chromosomal element conveys epigenetic inheritance during mitosis and meiosis. Cell 93, 505-518.

Chabner, B.A., Longo, D.L., 2006. Cancer chemotherapy and biotherapy: principles and practice. Lippincott Williams & Wilkins, Philadelphia, PA.

Chan, W.-H., 2009. Impact of genistein on maturation of mouse oocytes, fertilization, and fetal development. Reprod. Toxicol. 28, 52-58.

Chuang, J.C., Jones, P.A., 2007. Epigenetics and microRNAs. Pediatr. Res. 61, 24R-29R.

Clark, S.J., Harrison, J., Paul, C.L., Frommer, M., 1994. High-sensitivity mapping of methylated cytosines. Nucleic Acids Res. 22, 2990-2997.

Crain, P., 1990. Preparation and enzymatic hydrolysis of DNA and RNA for mass spectrometry. Meth. Enzymol. 193, 782-790.

Cropley, J.E., Suter, C.M., Beckman, K.B., Martin, D.I.K., 2006. Germ-line epigenetic modification of the murine A(vy) allele by nutritional supplementation. Proc. Natl. Acad. Sci. USA 103, 17308-17312.

Day, J.K., Bauer, A.M., desBordes, C., Zhuang, Y., Kim, B.E., Newton, L.G., Nehra, V., Forsee, K.M., MacDonald, R.S., Besch-Williford, C., Huang, T.H.M., Lubahn, D.B., 2002. Genistein alters methylation patterns in mice. J. Nutr. 132, 2419S-2423S.

Doerksen, T., Trasler, J.M., 1996. Developmental exposure of male germ cells to 5-azacytidine

592 results in abnormal preimplantation development in rats. Biol. Reprod. 55, 1155-1162.
593 Dolinoy, D., 2007. Maternal nutrient supplementation counteracts bisphenol A-induced DNA
594 hypomethylation in early development. Proc. Natl. Acad. Sci. USA 104, 13056-13061.
595 Dolinoy, D.C., Weidman, Jennifer R., Waterland, Robert A., Jirtle, R.L., 2006. Maternal genistein
596 alters coat color and protects avy mouse offspring from obesty by modifying the fetal
597 epigenome. Environ. Health Perspect. 114, 567-572.
598 Elendt, B.-P., Bias, W.-R., 1990. Trace nutrient deficiency in Daphnia magna cultured in standard
599 medium for toxicity testing. Effects of the optimization of culture conditions on life history
600 parameters of D. magna. Water Res. 24, 1157-1167.
601 Esteller, M., 2008a. Epigenetics in Biology and Medicine. CRC Press, Boca Raton, FL.
602 Esteller, M., 2008b. Epigenetics in evolution and disease. Lancet 372, S90-S96.
603 Fang, M.Z., Chen, D., Sun, Y., Jin, Z., Christman, J.K., Yang, C.S., 2005. Reversal of
604 hypermethylation and reactivation of p16INK4a, RARβ, and MGMT genes by genistein and
605 other isoflavones from soy. Clin Cancer Res 11, 7033-7041.
606 Feil, R., 2008. Epigenetics, an emerging discipline with broad implications. C. R. Biol. 311, 837-
607 843.
608 Ghoshal, K., Datta, J., Majumder, S., Bai, S., Dong, X., Parthun, M., Jacob, S.T., 2002. Inhibitors of
609 histone deacetylase and DNA methyltransferase synergistically activate the methylated
610 metallothionein I promoter by activating the transcription factor MTF-1 and forming an open
611 chromatin structure. Mol. Cell. Biol. 22, 8302-8319.
612 Guil, S., Esteller, M., 2009. DNA methylomes, histone codes, miRNAs: Tying it all together. Int. J.
613 Biochem. Cell Biol. 451, 87-95.
614 Haeba, M., Hilscherová, K., Mazurová, E., Bláha, L., 2008. Selected endocrine disrupting
615 compounds (Vinclozolin, Flutamide, Ketoconazole and Dicofol): Effects on survival,
616 occurrence of males, growth, molting and reproduction of Daphnia magna. Environ Sci Pollut
617 Res Int 15, 222-227.
618 Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman-Karber method for
619 estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714-
620 719.
621 Hebert, P.D.N., 1987. Genetics of *Daphnia*. In: Peters, R.H., de Bernardi, R. (Eds.), *Daphnia*, vol.
622 45. Memorie dell'Istituto Italiano di Idrobiologia, Pallanza, pp. 245-284
623 Huang, D., Zhang, Y., Qi, Y., Chen, C., Ji, W., 2008. Global DNA hypomethylation, rather than
624 reactive oxygen species (ROS), a potential facilitator of cadmium-stimulated K562 cell
625 proliferation. Toxicol. Lett. 179, 43-47.
626 Inawaka, K., Kawabe, M., Takahashi, S., Doi, Y., Tomigahara, Y., Tarui, H., Abe, J., Kawamura,
627 S., Shirai, T., 2009. Maternal exposure to anti-androgenic compounds, vinclozolin, flutamide
628 and procymidone, has no effects on spermatogenesis and DNA methylation in male rats of
629 subsequent generations. Toxicol. Appl. Pharmacol. 237, 178-187.
630 Lin, K.-T., Momparler, R.L., Rivard, G.E., 1981. High-performance liquid chromtographic analysis
631 of chemical stability of 5-aza-2'-deoxycytidine. J. Pharm. Sci. 70, 1228-1232.
632 Mandrioli, M., 2007. A new synthesis in epigenetics: towards a unified function of DNA

633 methylation from invertebrates to vertebrates. Cell. Mol. Life Sci. 64, 2522-2524.
634 Mohana Kumar, B., Jin, H.F., Kim, J.G., Song, H.J., Hong, Y., Balasubramanian, S., Choe, S.Y.,
635 Rho, G.J., 2006. DNA methylation levels in porcine fetal fibroblasts induced by an inhibitor
636 of methylation, 5-azacytidine. Cell Tissue Res. 325, 445-454.
637 Muyssen, B.T.A., De Schamphelaere, K.A.C., Janssen, C.R., 2006. Mechanisms of chronic
638 waterborne Zn toxicity in Daphnia magna. Aquat. Toxicol. 77, 393-401.
639 Oakeley, E.J., 1999. DNA methylation analysis: a review of current methodologies. Pharmacol.
640 Ther. 84, 389-400.
641 OECD, 1994. Guidelines for testing of chemicals, Daphnia sp., Acute Immobilisation Test and
642 Reproduction Test. OECD, Paris.
643 OECD, 1998. Guidelines for testing of chemicals, Daphnia magna reproduction test. OECD, Paris.
644 Piekarz, R.L., Bates, S.E., 2009. Epigenetic modifiers: Basic understanding and clinical
645 development. Clin. Cancer Res. 15, 3918-3926.
646 Reamon-Buettner, S.M., Mutschler, V., Borlak, J., 2008. The next innovation cycle in
647 toxicogenomics: Environmental epigenetics. Mutat. Res./Rev. Mutat. Res. 659, 158-165.
648 Schulz, W.A., 2006. L1 retrotransposons in human cancers. J Biomed Biotechnol 2006, 83672
649 Song, L.G., James, S.R., Kazim, L., Karpf, A.R., 2005. Specific method for the determination of
650 genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass
651 spectrometry. Anal. Chem. 77, 504-510.
652 Stephan, C.E., 1977. Methods for calculating an LC50. In: Mayer, F.L., Hamelink, J.L. (Eds.),
653 Aquatic Toxicology and Hazard Evaluation, vol. ASTM STP 634. American Society for
654 Testing and Materials, Philadelphia, PA, pp. 65-84
655 Suzuki, M.M., Kerr, A.R.W., De Sousa, D., Bird, A., 2007. CpG methylation is targeted to
656 transcription units in an invertebrate genome. Genome Res. 17, 625-631.
657 Szeto, S.Y., Burlinson, N.E., Rahe, J.E., Oloffs, P.C., 1989. Kinetics of hydrolysis of the
658 dicarboximide fungicide Vinclozolin. J. Agric. Food Chem. 37, 523-529.
659 Vallero, D.A., Pierce, J.J., 2003. Engineering the risks of hazardous wastes. Butterworth-
660 Heinemann, Oxford, UK.
661 Vandegehuchte, M.B., Kyndt, T., Vanholme, B., Haegeman, A., Gheysen, G., Janssen, C.R., 2009a.
662 Occurrence of DNA methylation in Daphnia magna and influence of multigeneration Cd
663 exposure. Environ. Int. 35, 700-706.
664 Vandegehuchte, M.B., Lemière, F., Janssen, C.R., 2009b. Quantitative DNA-methylation in
665 *Daphnia magna* and effects of multigeneration Zn exposure. Comp Biochem Physiol C:
666 Toxicol Pharmacol 150, 343-348.
667 Wang, Y., Wang, C., Zhang, J., Chen, Y., Zuo, Z., 2009. DNA Hypomethylation induced by
668 tributyltin, triphenyltin, and a mixture of these in Sebastiscus marmoratus liver. Aquat.
669 Toxicol. 95, 93-98.
670 Watson, R.E., Goodman, J.I., 2002. Epigenetics and DNA methylation come of age in toxicology.
671 Toxicol. Sci. 67, 11-16.
672 Youngson, N., Whitelaw, E., 2008. Transgenerational epigenetic effects. Annu. Rev. Genomics
673 Hum. Genet. 9, 233-257.

674 Zaffagnini, F., 1987. Reproduction in *Daphnia*. In: Peters, R.H., de Bernardi, R. (Eds.), *Daphnia*,
675 vol. 45. Memorie dell'Istituto Italiano di Idrobiologia, Pallanza, pp. 245-284
676 Zhao, M., Rudek, M.A., He, P., Hartke, C., Gore, S., Carducci, M.A., Baker, S.D., 2004.
677 Quantification of 5-azacytidine in plasma by electrospray tandem mass spectrometry coupled
678 with high-performance liquid chromatography. J. Chromatogr. B 813, 81-88.
679
680 **Figure captions**

681 Fig. 1 Overview of the experimental culture setup for the multigeneration
682 experiment. F_0, F_1, F_2: generations. White rectangles represent control medium.
683 Grey rectangles represent medium with Vinclozolin (0.54 ± 0.19 mg/L in F_0, 0.45 ±
684 0.16 mg/L in F_1, 0.18 ± 0.15 mg/L in F_2), Genistein (4.7 ± 0.7 mg/L in F_0-F_2) or 5-
685 azacytidine (2.9 ± 0.4 mg/L in F_0, 2.3 ± 0.3 mg/L in F_1). Arrows represent offspring.
686
687 Fig. 2 Mean reproduction in the multigeneration experiment depicted as the
688 number of living juvenile offspring per surviving female at the day of a third brood
689 in the control treatment: day 16 for F_0, day 15 for F_1 and F_2. Error bars indicate
690 standard deviations. : significantly different from the control in the same
691 generation (Mann-Whitney U test or Dunnett test, $p = 0.0004$ and 0.022 for F_0A^+ and
692 F_0G^+, respectively); : reproduction at day 14, significantly different from control
693 reproduction at day 14 (Mann-Whitney U test, $p = 0.029$, see text).
694
695 Fig. 3 Mean length (mm) at day 7 and 15 for the different treatments of the
696 multigeneration experiment. Error bars indicate standard deviations. :
697 significantly different from the control in the same generation (Mann-Whitney U test
698 or Dunnett test, $p < 0.05$).
699
700 Fig. 4 Mean overall DNA cytosine methylation expressed as % [mdC]/[dG] at the
701 day of the third brood in the different treatments of the multigeneration
702 experiment. Error bars indicate standard deviations. : significantly different from
703 the control in the same generation (Dunnett test or Mann-Whitney U test, $p < 0.05$).
704 : Only one replicate could be measured due to high mortality; no reproduction
705 took place and DNA samples were taken at day 21.

Table 1 – EC50 (concentration causing 50% immobility) in the acute tests with *D. magna* exposed to 5-azacytidine, 5-aza-2'-deoxycytidine, biochanin A, genistein and vinclozolin, based on measured concentrations at the beginning of the test.

	EC_{50} ± standard deviation(mg/L)	remarks
5-azacytidine	310 ± 11[1]	95 % confidence interval: 180-534 mg/L
5-aza-2'-deoxycytidine	> 20.8 ± 0.5	0 % immobility at this concentration
biochanin A	8.50 ± 0.89[2]	>95 % confidence interval: 6.59 – 14.67 mg/L
genistein	> 6.93[3]	33 % immobility at this concentration
vinclozolin	> 1.7 ± 1.0	6.7 % immobility at this concentration

[1] Estimated with the trimmed Spearman-Karber method (Hamilton et al. 1977)

[2] Estimated with the binomial method (Stephan 1977)

[3] This value is an underestimation of the real concentration found by extrapolating a polynomial standard curve

Table 2 – Lowest observed effect concentrations (LOECs) for reproduction, length and overall DNA methylation (based one one-tailed Dunnett test or Kruskal-Wallis test with Mann-Whitney U test, $p < 0.05$) as well as relative reproduction, length and DNA methylation at the LOEC as a percentage of the control (ctrl) for the chronic tests with *D. magna* exposed to 5-azacytidine, 5-aza-2'-deoxycytidine, biochanin A, genistein and vinclozolin. LOECs are given as average measured concentrations (± standard deviation) in freshly prepared medium.

	Reproduction (nr of juveniles per surviving female)			Length (mm)			DNA cytosine methylation (% [mdC]/[dG])		
	LOEC (mg/L)	% of ctrl	Ctrl reproduc-tion	LOEC (mg/L)	% of ctrl	Ctrl length	LOEC (mg/L)	% of ctrl	Ctrl methy-lation
5-azacytidine	16 ± 2	46	81	27.8 ± 3.4[a]	91	2.73[a]	27.8 ± 3.4	30	0.26
5-aza-2'-deoxycytidine	4.8 ± 0.5	45	81	> 12.8 ± 0.5[a,b]	-	2.73[a], 3.61[b]	> 12.8 ± 0.5	-	0.26
biochanin A	4.9 ± 0.9	73	76	0.11 ± 0.04[b]	73	3.70[b]	> 4.9 ± 0.9	-	0.20
genistein	3.4 ± 1.5	56	76	1.8 ± 0.4[a,b]	93[a,b]	2.84[a], 3.70[b]	> 3.4 ± 1.5	129	0.20
vinclozolin	> 0.43 ± 0.09	-	76	0.43 ± 0.09[b]	91[b]	3.70[b]	0.43 ± 0.09	69	0.20

[a] Length at day 21

[b] Length at day 7

Figure 1

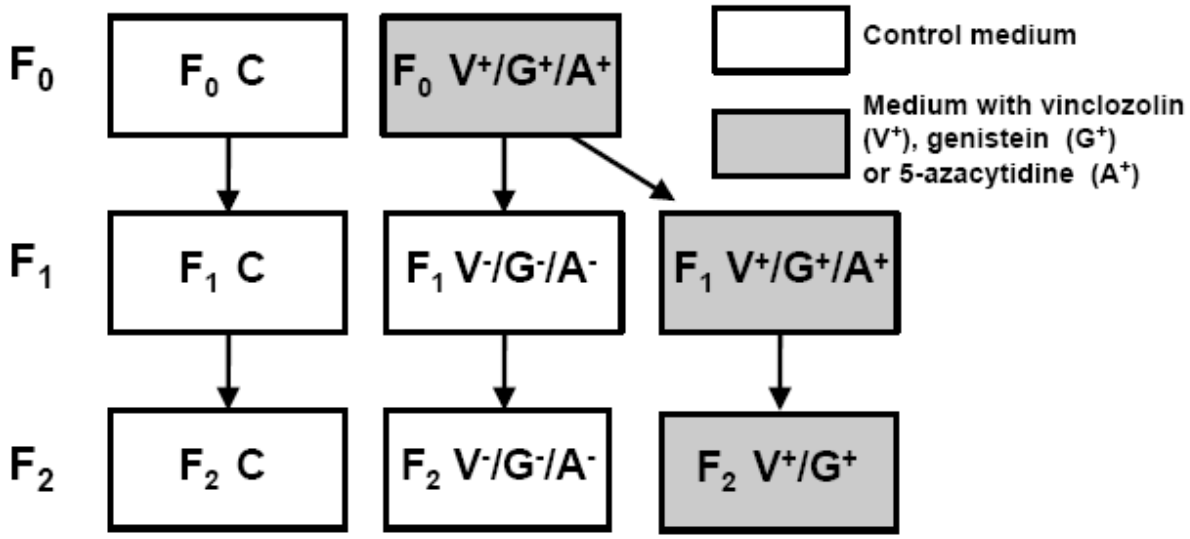

Figure 2

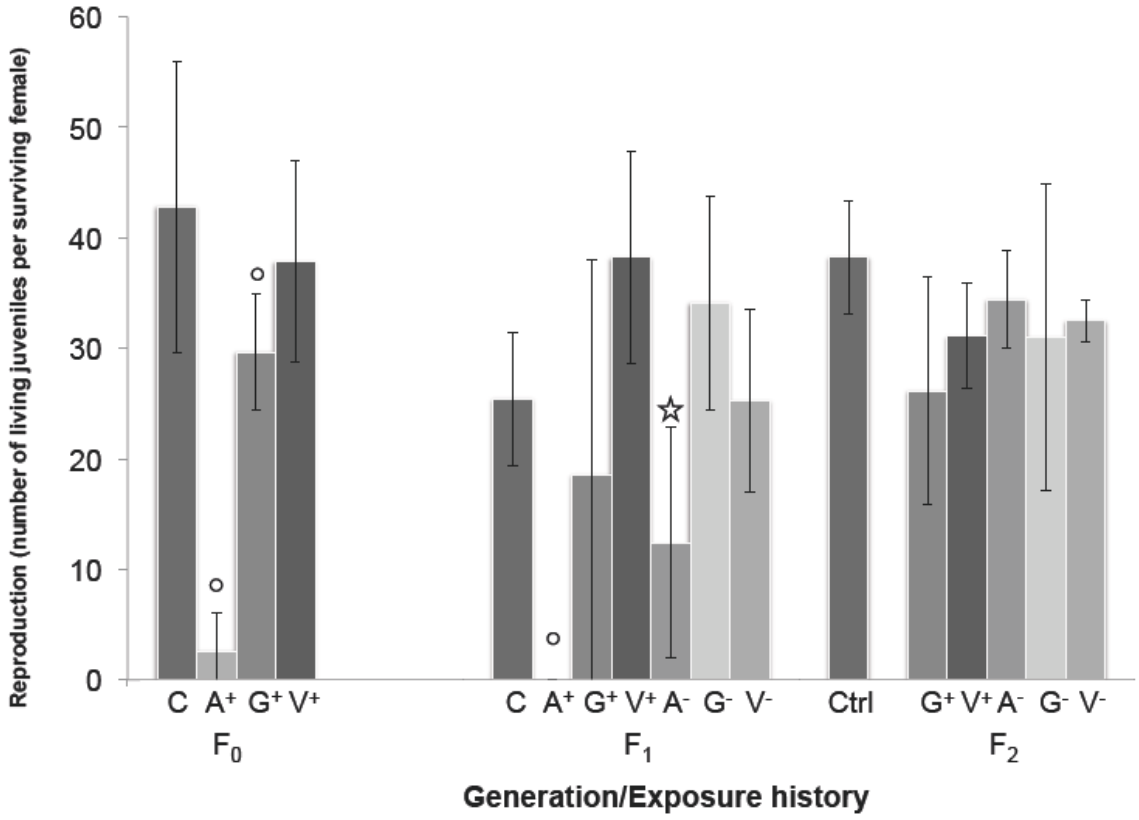

Figure 3

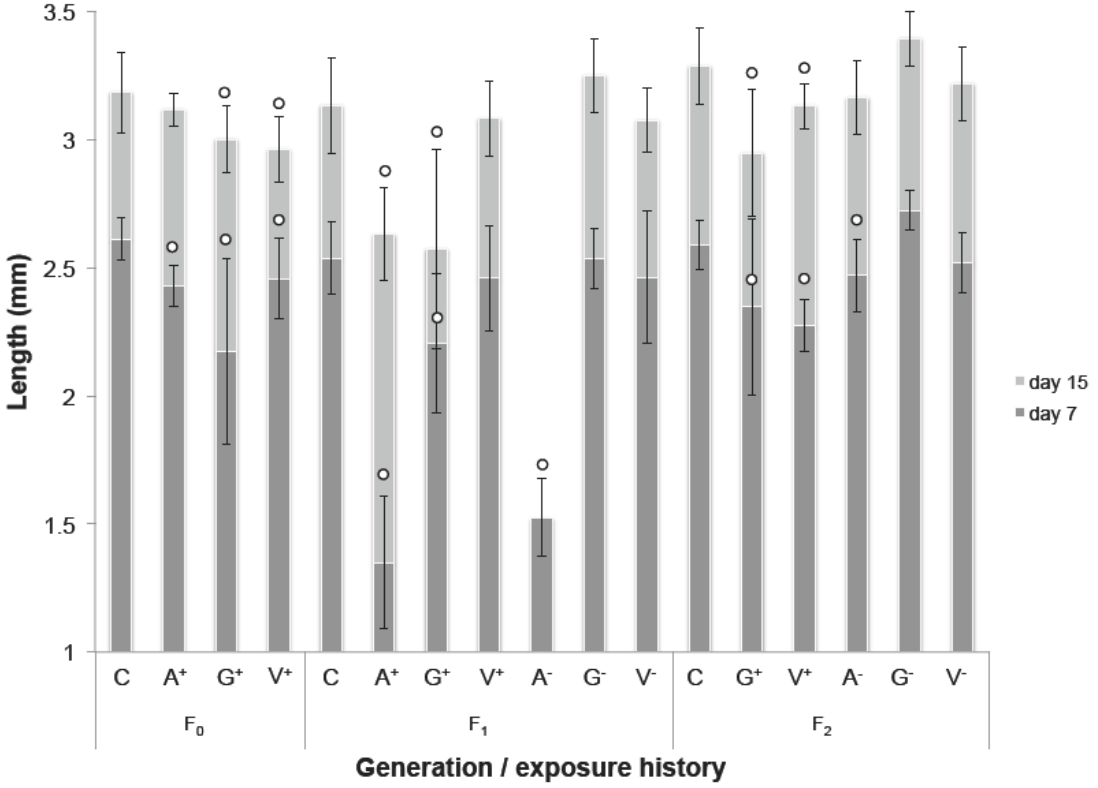

Figure 4