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

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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 was injected (split flow 60 mL min^{-1}, splitless time 1 min). Chromatographic separation of the analytes and internal standard was performed on a BPX5 capillary column (25 m x 0.22 mm ID) with a 5% phenyl-polysilphenylene-siloxane phase (0.25 µm film) (SGE Analytical Science Pty. Ltd., Victoria, Australia). The temperature program started at an initial temperature of 80°C. Temperature was increased to 140°C applying a ramp of 50°C min^{-1}. Subsequently, an increase to 260°C was assessed using a ramp of 5°C min^{-1}, holding this temperature for 3 minutes. Spectra were obtained in positive electron impact ionisation (EI) mode MS-MS scan. Mass range depended on the selected precursor ion, and the collision energy ranged from 1.15 to 1.30 V.

For all analyses data processing was performed using Xcalibur® 2.0 software (Thermo Electron).

2.3 DNA methylation analysis

DNA was extracted from 21 day-old daphnids at the end of the chronic test and from daphnids on the first day the third brood was observed (day 14 to day 16) in the multigeneration experiment. This was not possible in the F_1A^+ treatment, in which no reproduction was observed up to day 21. Here DNA extraction of the 21 day-old daphnids was performed. The MasterPure™ kit (Epicentre, Madison, WI, USA) was used following the protocol for DNA extraction from tissue as provided by the manufacturer. Four to six adult organisms per replicate were rinsed with deionized water, blotted dry and shock frozen in liquid nitrogen prior to extraction. Hydrolysis of DNA was performed following Crain (Crain 1990). A sample of 1.3 to 4.25 µg DNA was adjusted to 16.8 µL with Tris-HCl (1 mM, pH 7.4). The DNA was denatured by heating at 100 °C for 3 min in a warm water bath. The denatured DNA was hydrolyzed by adding 0.75 µL (1.5 units) nuclease P1 (Sigma-Aldrich, Bornem, Belgium) and 1/10 volume of 0.1 M NH_4OAc (pH 5.3). This was incubated at 45°C for 2 h. Subsequently, 0.002 units phosphodiesterase I (Sigma-Aldrich, Bornem, Belgium) and 1/10 volume of 1 M NH_4HCO_3 at pH 7.8 were added to the sample. This was incubated at 37 °C for 2 h. Phosphates were removed by adding 0.5 units alkaline phosphatase (Fermentas, St. Leon-Rot, Germany) and 1/10 volume phosphatase buffer and this mixture was incubated at 37 °C for 1 h, after which it was stored at -20°C prior to 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 after 48 h exposure to the highest concentration. The EC_{50}s are based on concentrations measured at the beginning of the test. The concentrations generally decreased during the 48h tests. At the end of the test, the concentrations of 5-azacytidine, 5-aza-2'-deoxycytidine and vinclozolin were reduced to the following fractions of the initial concentrations: 6 to 77 %, 29% and 1 to 15%, respectively (see electronic supplementary material). The isoflavone concentrations remained rather constant throughout the test. From nominal concentration of ≥ 3.6 mg/L (measured concentration 0.182 ± 0.099 mg/L) vinclozolin, small non-dissolved particles could be observed in the test medium. This is in accordance with the water solubility of 3.5 mg/L vinclozolin at 20 °C (Vallero et al. 2003).

3.2 Chronic experiments

Differences between treatments will only be discussed when they are statistically significant ($p < 0.05$).

LOECs are expressed as average measured concentrations in freshly prepared medium (Table 2). The concentration of some compounds decreased considerably between two medium renewals. 5-azacytidine and 5-aza-2'-deoxycytidine were not detectable after three days, while after two days on average 22% and 49% (respectively) of the original concentration was present in the medium. For biochanin A and genistein, there was no consistent trend. Vinclozolin concentrations decreased to approximately 0.4 to 1.5% of the initial concentration after three days.

Two quality controls (QCs) for DNA methylation were measured in triplicate, resulting in relative standard deviations (RSDs) of 2.5% and 7.4% for mdC and 1.3% and 1.6% for dG. Relative Errors (REs) were -0.1% for mdC for both QCs and 5.2 and 0.3% for dG.

No difference was detected in reproduction, length or DNA methylation between controls with 0, 0.0176 and 0.05% DMSO that were started with the same batch of daphnids. These controls were pooled for the calculation of the pooled residual standard deviation with ANOVA for the experiments with biochanin A, genistein and vinclozolin.

For all test substances, an effect on at least one of the endpoints (reproduction, length and overall DNA methylation) could be observed. Vinclozolin did not elicit an effect on reproduction at the tested concentrations. 5-aza-2'-deoxycytidine did not induce an effect 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 to male rats before mating (Doerksen et al. 1996). The demethylating effect, which was detected in *D. magna* exposed to the highest concentration, was expected based on the known interaction of 5-azacytidine with DNMTs (Ghoshal et al. 2002).

The absence of an effect on reproduction of vinclozolin at the highest tested concentration of 0.43 mg/L corroborates the results of Haeba et al. (2008) who reported no effects on reproduction at a nominal concentration of 1 mg/L. However, whereas a small but significant decrease in body length was observed in daphnids exposed to 0.43 mg/L vinclozolin in the current study, no such effect was noted by those authors. Vinclozolin exposure induced both hypermethylation and hypomethylation events at 25 regions in the rat genome (Anway et al. 2005). Inawaka et al. (2009), however, could not confirm the vinclozolin-induced DNA methylation changes in one of those regions within the lysophospholipase gene. In *D. magna*, we observed a reduction in overall DNA methylation upon exposure to 0.43 mg/L vinclozolin, indicating that vinclozolin or its degradation products do interact with DNA methylation.

Global DNA hypomethylation, as observed here in vinclozolin and 5-azacytidine exposed daphnids, has been associated with cell proliferation and with hypomethylation of transposable elements which can alter gene expression (Schulz 2006; Huang et al. 2008). This observation has also been reported in rat, mouse and human cells or tissues after exposure to various environmental chemicals (Baccarelli et al. 2009)

4.3 Multigeneration experiment

First, the results of the F_0 generation are compared with the results of the chronic experiment. Effects on length, reproduction and DNA methylation in exposed F_0 daphnids generally corroborate the effects observed in the chronic experiment. In F_0A^+, however, the reduced length at day 7 and the reduction in DNA methylation were not observed at the corresponding nominal concentration in the chronic test. It may be noted that the control length of 2.61 ± 0.08 mm at day 7 in the multigeneration experiment is lower than that of 2.73 ± 0.12 in the chronic 5-azacytidine experiment. The batch of smaller daphnids with which the multigeneration experiment was initiated appears to be more sensitive to 5-azacytidine exposure.

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.

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Figure captions

Fig. 1 Overview of the experimental culture setup for the multigeneration experiment. F_0, F_1, F_2: generations. White rectangles represent control medium. Grey rectangles represent medium with Vinclozolin (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), Genistein (4.7 ± 0.7 mg/L in F_0-F_2) or 5-azacytidine (2.9 ± 0.4 mg/L in F_0, 2.3 ± 0.3 mg/L in F_1). Arrows represent offspring.

Fig. 2 Mean reproduction in the multigeneration experiment depicted as the number of living juvenile offspring per surviving female at the day of a third brood in the control treatment: day 16 for F_0, day 15 for F_1 and F_2. Error bars indicate standard deviations. : significantly different from the control in the same generation (Mann-Whitney U test or Dunnett test, $p = 0.0004$ and 0.022 for F_0A^+ and F_0G^+, respectively); : reproduction at day 14, significantly different from control reproduction at day 14 (Mann-Whitney U test, $p = 0.029$, see text).

Fig. 3 Mean length (mm) at day 7 and 15 for the different treatments of the multigeneration experiment. Error bars indicate standard deviations. : significantly different from the control in the same generation (Mann-Whitney U test or Dunnett test, $p < 0.05$).

Fig. 4 Mean overall DNA cytosine methylation expressed as % [mdC]/[dG] at the day of the third brood in the different treatments of the multigeneration experiment. Error bars indicate standard deviations. : significantly different from the control in the same generation (Dunnett test or Mann-Whitney U test, $p < 0.05$). : Only one replicate could be measured due to high mortality; no reproduction 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 reproduction	LOEC (mg/L)	% of ctrl	Ctrl length	LOEC (mg/L)	% of ctrl	Ctrl methylation
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

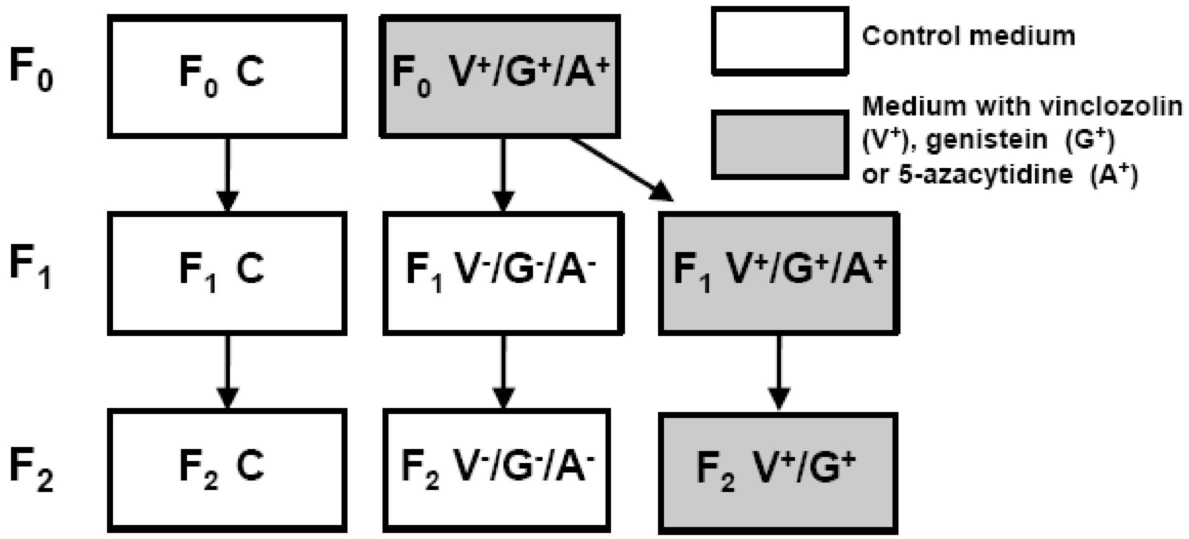

F_0
F_0 C
F_0 $V^+/G^+/A^+$
Control medium
Medium with vinclozolin (V^+), genistein (G^+) or 5-azacytidine (A^+)
F_1
F_1 C
F_1 $V^-/G^-/A^-$
F_1 $V^+/G^+/A^+$
F_2
F_2 C
F_2 $V^-/G^-/A^-$
F_2 V^+/G^+

Figure 2

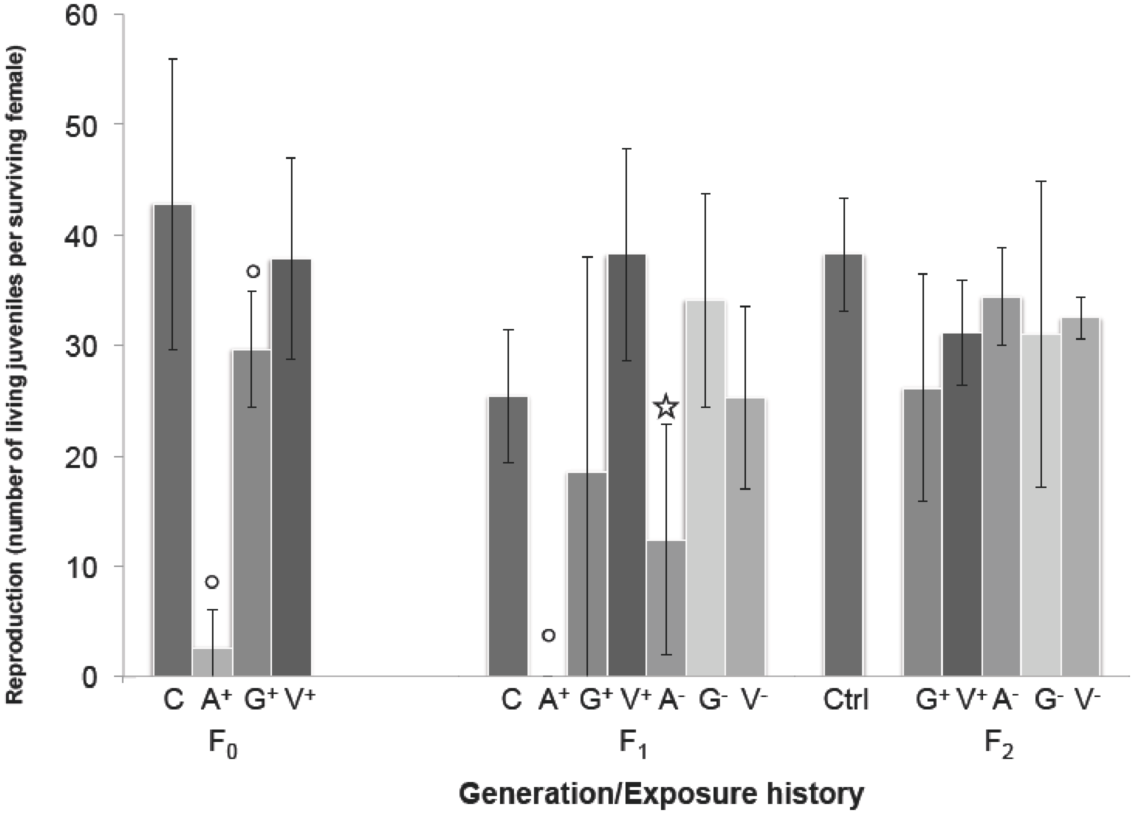

Reproduction (number of living juveniles per surviving female)
0
10
20
30
40
50
60
C A+ G+ V+
F0
C A+ G+ V+ A- G- V-
F1
Ctrl G+ V+ A- G- V-
F2
Generation/Exposure history
○
○
○
☆

Figure 3

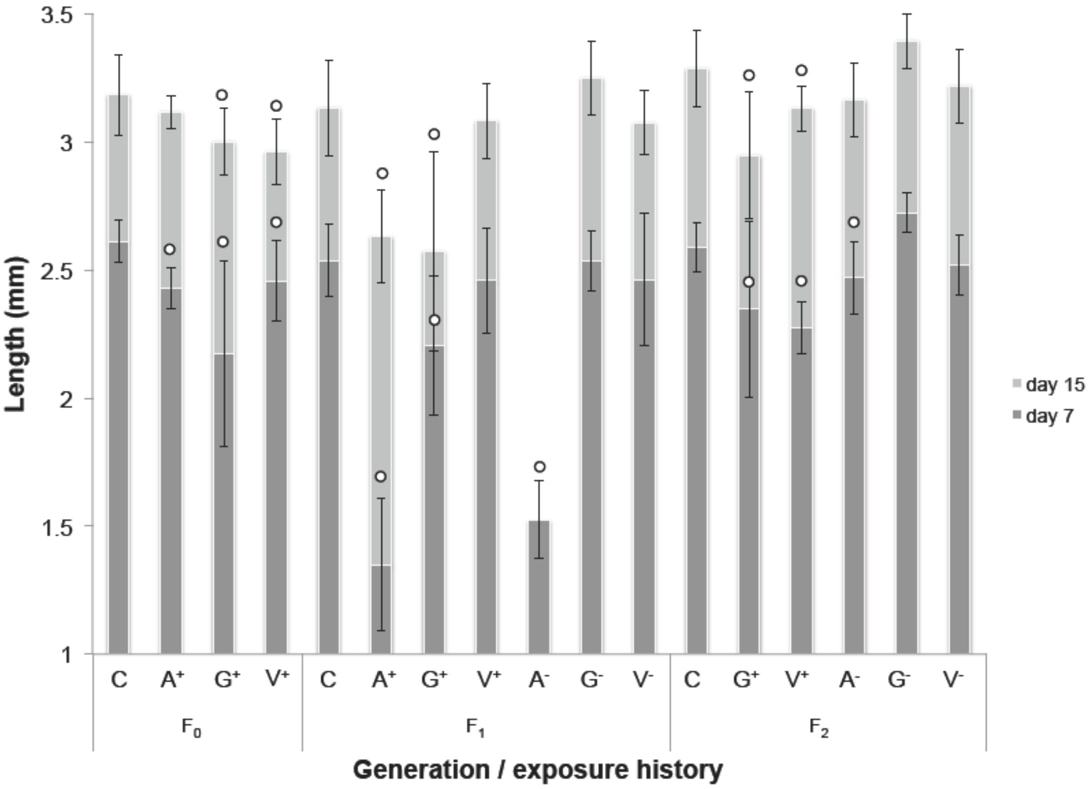
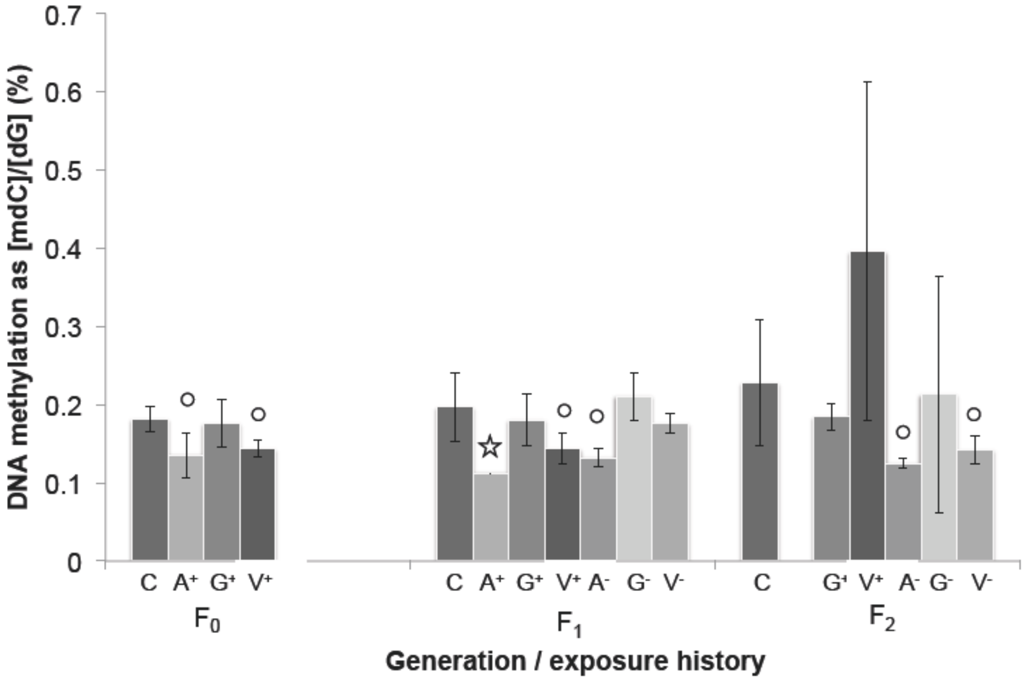

3.5
3
2.5
2
1.5
1
Length (mm)
day 15
day 7
C
A+
G+
V+
C
A+
G+
V+
A-
G-
V-
C
G+
V+
A-
G-
V-
F0
F1
F2
Generation / exposure history

Figure 4


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DNA methylation as [mdC]/[dG] (%)
C A⁺ G⁺ V⁺
F0
C A⁺ G⁺ V⁺ A⁻ G⁻ V⁻
F1
C G⁺ V⁺ A⁻ G⁻ V⁻
F2
Generation / exposure history