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25-day chronic gamma irradiation

Exposed F1 as embryo
Exposed F2 as germline cell
Unexposed F3

43.1 mGy h\(^{-1}\)
or
6.5 µGy h\(^{-1}\)

CH\(_3\)CH\(_3\)CH\(_3\)

Inheritance of radio-induced DNA methylation patterns?

TOC/Abstract Graphic
(for table of contents only)
Transgenerational DNA methylation changes in *Daphnia magna* exposed to chronic gamma irradiation

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KEYWORDS. DNA methylation, Epigenetic inheritance, Ionising radiation, *Daphnia magna*
1 Abstract

Our aim was to investigate epigenetic changes in *Daphnia magna* after a 25-day chronic external gamma irradiation (generation F0 exposed to 6.5 µGy h\(^{-1}\) or 41.3 mGy h\(^{-1}\)) and their potential inheritance by subsequent recovering generations, namely F2 (exposed as germline cells in F1 embryos) and F3 (the first truly unexposed generation). Effects on survival, growth and reproduction were observed and DNA was extracted for whole genome bisulfite sequencing in all generations. Results showed effects on reproduction in F0 but no effect in the subsequent generations F1, F2 and F3. In contrast, we observed significant methylation changes at specific CpG positions in every generation independent of dose rate, with a majority of hypomethylation. Some of these changes were shared between dose rates and between generations. Associated gene functions included gene families and genes which were previously shown to play roles during exposure to ionising radiation. Common methylation changes detected between generations F2 and F3 clearly showed that epigenetic modifications can be transmitted to unexposed generations, most likely through the germline, with potential implications for environmental risk.

2 Introduction

Ecosystems can be chronically exposed to ionising radiation, due to releases of radionuclides during the normal functioning of nuclear facilities or after major accidents such as in Chernobyl in 1986 and Fukushima in 2011. Three decades after the accident in Chernobyl, wildlife organisms can absorb dose rates ranging from 0.1 µGy h\(^{-1}\) to 10 mGy h\(^{-1}\) in the Exclusion Zone, due to the persistence of long-lived isotopes, such as \(^{137}\)Cs, \(^{90}\)Sr, \(^{240}\)Pu and \(^{239}\)Pu.\(^1\) In this context, ecologically relevant predictions of long term biological effects induced by chronic low doses of ionising radiation in non-human organisms are necessary. These predictions can gain in robustness by understanding radiotoxicity mechanisms over
several generations and underlying processes involved at the molecular scale. While effects of ionizing radiation on biomolecules, especially DNA, have been extensively described and are now well-understood, the contribution of other radiation-induced molecular alterations that might be transmitted from irradiated parents to their progeny remains unclear. Studies of radiation effects in fish and rodents on several generations reported a situation of genomic instability in the unexposed progeny, characterized by an increased frequency of mutations and other DNA aberrations compared to parents. The observation that inheritance of genomic instability did not follow a classical Mendelian pattern suggested that an epigenetic mechanism might be involved.

Epigenetic processes, which include all mitotically and/or meiotically heritable modifications that occur without changes in the DNA sequence are mechanisms of growing interest in ecotoxicology and environmental risk assessment. A large body of work suggested that epigenetic changes (DNA methylation, histone modifications, non-coding RNA) could be transmitted via the germline and cause adverse effects or adaptive responses in subsequent generations in absence of exposure. Field studies on frogs and plants in Fukushima and Chernobyl contaminated areas and laboratory-based tests on rodents showed that ionising radiation could affect DNA methylation, a well-studied epigenetic mechanism known to play key roles in diverse cellular mechanisms in different taxonomic groups. In particular, DNA methylation of cytosines followed by a guanine (commonly referred to as CpG) is involved in the regulation of gene expression in various species. The transgenerational inheritance of DNA methylation remains discussed and requires specific experimental design to be demonstrated. In fact, exposure designs in most standard toxicity tests imply that at least one following generation (F1 as embryos, and in some cases F2 as germline cells) is possibly exposed at the same time as generation F0, especially in gestating mammals,
ovoviviparous fish or cladoceran crustaceans. This needs to be taken into consideration in order to correctly assess true transgenerational epigenetic effects.

To our knowledge, a true transgenerational inheritance of radio-induced DNA methylation changes has never been demonstrated yet. The freshwater cladoceran *Daphnia magna*, is a particularly adequate model organism to address this issue. With its short life cycle and parthenogenetic reproduction, *D. magna* has been successfully used to study epigenetic patterns and DNA methylation under limited genetic variation, during exposure to different environmental stressors. In addition, effects of gamma radiation (\(^{137}\text{Cs}\)) on *D. magna* survival, growth and reproduction was previously shown to increase in severity, together with genotoxicity, across three continuously exposed generations. The present study aimed to investigate the possible involvement of epigenetic mechanisms in this increase. To do so, *D. magna* were exposed to external gamma irradiation for one generation (F0) until hatching of the following generation (F1) and DNA methylation modifications were examined up to the unexposed generation F3, using whole genome bisulfite sequencing. Our objectives were to test: 1) whether DNA methylation varied with the absorbed radiation dose; 2) whether radio-induced epigenetic changes were transmitted from exposed generations to subsequent unexposed generations; and 3) whether epigenetic modifications were associated with effects on survival, growth and reproduction, with potential direct implications for ecological risk assessment.

3 Material and methods

3.1 Exposure conditions

*Daphnia magna* (Strain A) were obtained from INERIS (Verneuil-en-Halatte, France) and were kept for many generations (> 30) in optimal laboratory conditions: 1 daphnid per 50 mL renewed every day; in M4 medium at pH8 (composition as Supporting Information, SI);
temperature of 20 ±1 °C; 16h:8h light-dark cycle; light intensity of 20 µE m⁻² s⁻¹; daily ration of 100 µg C per daphnid using axenic cultures of *Chlamydomonas reinhardtii*. Daphnids were chronically exposed to external gamma radiation at IRSN irradiation facility MIRE (Mini Irradiator for RadioEcology, Cadarache, France). Irradiation was performed in individual experimental units of 50 mL, placed in circles around ¹³⁷Cs sources of 1.64 MBq and 1.93 GBq from CERCA-LEA (Framatome ANP, Pierrelatte, France), in separate ventilated thermostatic chambers protected by 10-cm thick lead walls in order to avoid cross-irradiation among treatments. Exposure included a 25-day irradiation phase of a first generation (F0) followed with a recovery phase in subsequent generations F1, F2 and F3 (Figure 1). Exposure of generation F0 was started with freshly laid eggs (<24h) contained in the brood pouch of their mother. After hatching, irradiation was maintained until F0 daphnids released neonates from their 5th brood. Generations F1 and F2 were not irradiated any further and were therefore exposed to gamma radiation as embryos and as germ cells respectively. Generation F3 was the first truly unexposed generation. An unexposed control treatment (24 daphnids per generation) was monitored at all times throughout the experiment. Each treatment was composed of 22-24 daphnids per generation. Average dose rates absorbed by daphnids were estimated at experimental unit centers by Monte Carlo N-particle calculations and confirmed by radio photo luminescent dosimetry (SI and Table S1). Two dose rates were selected, based on previous results with *D. magna*: 41.3 mGy h⁻¹ as a high exposure level at which genotoxicity and reprotoxicity were detected early in F0 adults; 6.5 µGy h⁻¹ as a low exposure level at which genotoxicity was detected late in F0 adults and early in F2 adults, with no observed effect on reproduction.

### 3.2 DNA extraction and bisulfite sequencing

Daphnids were collected upon release of 5th (generation F0) or 4th brood (generations F2 and F3) and immediately flash frozen in liquid nitrogen for methylation analysis. Eggs were
removed from the brood pouch, prior to DNA extraction (DNeasy Blood and Tissue kit, Qiagen). Two to eight whole daphnids were pooled to form a genomic DNA sample per treatment. Pooling individuals was possible because variability is considered limited among individual daphnids with a clonal reproduction, compared to variability among tissues. Library preparation, bisulfite conversion and whole genome bisulfite sequencing (see SI for details) were performed by the BGI sequencing facility (Hong Kong, China). More than 80M of high quality (Q 30> 98%) 100bp paired-end reads were generated per sample (SI Table S2). The data were made accessible in Geo under accession number GSE108426.

3.3 Quality assessment and mapping

Methylation analysis (see SI for details) followed the protocol described for *D. magna*. Genetic variability at CpG sites might act as a confounding factor because the detection of non-methylated cytosines was based on C to T transitions after bisulfite treatment. In order to avoid this source of confusion, SNPs (single nucleotide polymorphisms) were detected by comparing a control genome, sequenced without bisulfite treatment (blank) with the reference genome using the function mpileup in SAMtools. All C to T transitions that were not due to the bisulfite treatment (but were due to genetic variation between the reference genome and the blank) were removed.

3.4 CpG methylation level

For every read, cytosine methylation status (methylated or not methylated) at each CpG site was extracted with Bismark. A total of ~5.4 × 10^6 CpGs were selected for the analysis, including those which were present in all DNA samples with a minimum coverage of 5 reads. False positives were detected using a binomial testing procedure at each CpG, based on the bisulfite conversion efficiency (from 99.4 to 99.6%) calculated on an unmethylated DNA (lambda phage) spiked in all samples. For each truly methylated cytosine, a methylation
level was calculated by dividing the number of methylated reads by the total number of reads at the CpG site.

### 3.5 Differential methylation analysis

Following recommendations in,⁴⁵ all CpGs which showed no variation in methylation levels in any sample were eliminated and differential methylation analysis was conducted on 74020 CpGs (out of the $5.4 \times 10^6$ total). Differentially methylated cytosines (DMC) and differentially methylated regions (DMR) were detected using Dispersion Shrinkage for Sequencing data package (DSS) in R.⁴⁶ DSS can estimate the biological variance in absence of replicate, based on the spatial correlation in methylation levels from nearby CpGs, used as pseudo-replicates.⁴⁷ A Wald test was performed on smoothed methylation levels in order to calculate a p-value and a false discovery rate (FDR) at each CpG site. This was achieved using the *DMLtest* function based on a simple moving average procedure (window size of 80 base pairs) and an empirical Bayesian procedure to estimate the dispersion among all CpGs within the smoothing window. DMCs were considered significant at FDR below 5%. DMRs were defined as a region of minimum 50 base pairs length, with a least 3 CpGs and 50 % significant DMCs. Differential methylation in each generation (F0, F2 or F3 respectively) was determined using the control sample from the same generation (i.e. F0 control, F2 control or F3 control respectively) for reference. An additional pairwise analysis among control samples was conducted in order to test whether some of the detected methylation differences might reflect a change in the control across generations rather than a response to radiation exposure.

### 3.6 Functional analysis

The annotation information (daphmagna_2011pubfc8.gff3) available for *Daphnia magna* through wFleabase.org⁴⁸ was used to identify the genomic feature to which each DMC belongs. Intergenic regions were defined as all regions that did not correspond to gene body
An enrichment analysis was performed for each treatment, using the Fisher exact test. Resulting P-values were corrected for multiple testing using the FDR Benjamini-Hochberg method, in order to identify in which genomic features DMCs were over-represented, compared to the distribution of CpGs among the different genomic features. Genome annotations are poor in *D. magna* and this limits the GO analysis in this organism. In this situation, we used the Eukaryotic orthologous groups system (KOG) which provides a gene classification per functional categories based on orthologous relationships between genes. We classified genes in 23 defined KOG categories representative of different cellular functions, based on the reference *Daphnia pulex* orthologous genes, available through the Joint Genomic Institute website (http://genome.jgi.doe.gov/cgi-bin/kogBrowser?db=Dappu1).

### 3.7 Effects on survival, growth and reproduction

Survival, age at brood release and brood size were measured every day in 10 units per treatment. Neonates were removed on the day of their release. Body size was measured in neonates and in adults upon deposition of brood 1, 3 and 5 (F0) or 6 (F1, F2 and F3). Body size was measured from apex of the helmet to the base of the apical spine under a binocular microscope with a micrometer. All statistical analyses were performed with R (version 3.3.2) as previously described (see SI for details), with statistical significance level of 0.05.

### 4 Results and discussion

#### 4.1 Radiation effects on DNA methylation at the whole genome scale

Effects of ionising radiation on DNA methylation levels were assessed in the whole genome and the genomic features (exons, introns, intergenic regions). A global methylation level of ~0.85% was found at the whole genome scale (SI Figure S1). The value was slightly higher, but in the same order of magnitude, than the values previously reported for *D. magna*, ranging
values from 0.25 to 0.52%. A variability in global methylation among D. magna genotypes could explain this difference. This global methylation level was consistent with the low methylation levels in invertebrate genomes in general, in contrast with most vertebrate genomes, such as mammals in which methylation range from 60 to 90%.

Average methylation levels at the whole genome scale and in the genomic features did not differ between the control and exposed samples across the three generations (SI Figure S1). This result contrasted with previous findings showing a significant global hypermethylation measured in plants and frogs from the contaminated areas in Chernobyl and Fukushima.

Other in vivo studies with rodents exposed to acute or chronic ionizing radiation reported a global DNA hypomethylation in specific tissues, like liver and thymus. In our study, analyses of DNA methylation were conducted on whole body samples including 3 to 5 individuals, thereby averaging inter-individual variability. Thus, our approach detected methylation changes which occurred most frequently independent of the type of organ, but could not distinguish tissue-specific methylation changes.

Our results showed that CpG methylation level varied among genomic features (SI Figure S1), with the highest levels observed in exons (~1.38% independent of generation and dose rate, compared to introns and intergenic regions with ~0.49 and ~0.69% respectively). This observation was in accordance with who reported similar methylation patterns in gene bodies, supporting the hypothesis that gene body might be the main target of DNA methylation in invertebrates.

### 4.2 Distribution of DMCs and DMRs among genomic features

Significant changes in methylation (FDR<0.05 compared to the controls) were detected in all treatments, including generations F0 exposed to both dose rates, and their subsequent generations F2 and F3 (Figure 2). In generation F0, DMCs represented proportions of 0.39 ×
$10^{-4}$ and $0.46 \times 10^{-4}$ (209 and 247 DMCs detected out of $5.4 \times 10^6$ CpGs) at 6.5 µGy h$^{-1}$ and 41.3 mGy h$^{-1}$ respectively. This proportion was greater in generation F2 with values of $0.73 \times 10^{-4}$ and $0.76 \times 10^{-4}$ (393 and 413 DMCs detected) at 6.5 µGy h$^{-1}$ and 41.3 mGy h$^{-1}$ respectively. Finally, in generation F3 we detected a DMC proportion of $0.62 \times 10^{-4}$ and 0.61 $\times 10^{-4}$ (334 and 330 DMCs) at 6.5 µGy h$^{-1}$ and 41.3 mGy h$^{-1}$ respectively. Differences in DMC distribution among genomic features (exon, intron and intergenic regions) were tested against that of all CpGs (Figure 2). In all treatments, DMC numbers in intergenic regions were significantly lower (p<0.001) than expected from a random distribution of methylation changes over the whole genome. Conversely, significantly higher than expected numbers of DMC (p<0.01) were found in exon and intron regions. In every treatment and genomic feature, both hypomethylated and hypermethylated DMCs (i.e with methylation levels significantly lower or higher than in the control respectively) were observed (Figure 2).

The total number of detected DMR ranged from 4 to 9 among treatments and generations (SI Table S3). These small numbers of DMR reflected a low clustering of DMC in the analyzed samples. Two hypotheses might explain the difference. First, our whole body approach possibly smoothed differences in cytosine methylation levels among tissues, thereby reducing the detection rate of DMCs. Second, the scarcity of DMR might be a specificity of the methylation pattern in the genome of daphnids or invertebrates in general. In fact, methylation in invertebrates was previously shown to form mosaic patterns, occurring primarily in exonic regions, whereas intergenic regions were sparsely methylated. Hence, such methylation patterns might make difficult a detection of long methylated regions in the genome.19

Our observations suggested that methylation changes in introns and exons might play important functional roles in the response to ionising radiation. Results in D. magna exposed to cyanobacteria similarly suggested that methylation changes in exons might be part of an
adaptive response to environmental stress, through the expression of different proteins isoforms. Although the role of methylation is not clear in invertebrates, it might be linked to the regulation of alternative gene splicing. In honeybees, methylation levels were shown to vary among exons depending on whether they were transcribed or not.

4.3 Methylation changes as a function of dose rate

The dependence of methylation changes on absorbed radiation dose was an important issue. The comparison between the two treatments suggested that a similar proportion of DMCs (to total 74020 CpGs) was observed between dose rates, with values of 0.3%, 0.5% and 0.4% in generations F0, F2 and F3 respectively. Common DMCs, responding to the two dose rates, were in a large majority (>75%) represented by hypomethylated DMCs. Numbers of common DMCs between dose rates (50, 86 and 74 in generations F0, F2 and F3 respectively) largely exceeded what might be expected from chance. In fact, observed proportions of DMCs (to total 74020 CpGs) of 0.5% maximum suggested a much smaller probability to share DMCs between dose rates (0.0025% maximum), with expected numbers ranging from 0 to 2 DMCs out of 74020 CpGs. In other words, DMCs commonly detected at both dose rates were largely over-represented in our samples. This observation suggested that methylation changes did not follow a random pattern and occurred on specific CpGs at the two dose rates, which might reflect the response of specific molecular mechanisms induced in the somatic cells of generation F0 and in the germ cells of generation F1.

The observed situation in our study contrasted with previous in vivo and in vitro results suggesting that the number of induced methylation changes might depend on the received radiation dose in mice organs and in rodents and humans cells. However, a strict comparison among studies is not straightforward, considering that differences in exposure conditions (between acute and chronic exposures, between high and low doses), analyzed tissue, sex and biological species might have a strong influence on DNA methylation.
4.4 Biological functions of genes with DMCs and DMRs

Previous studies showed that gene body methylation could play a major role in gene expression in bees. In order to examine this hypothesis in D. magna, an identification of genes associated with observed DMCs or DMRs and their corresponding biological functions (KOG categories) was carried out when possible (Figure 3, Tables S2–5). The most represented biological functions across all dose rates and generations were associated with signal transduction (KOG category T), protein modification and turnover (KOG category O) and transcription (KOG category K), RNA processing and modification (KOG category A), cytoskeleton (KOG category Z) and translation (KOG category J). These observations were consistent with previous findings that these gene families were involved in the cellular response to ionising radiation.

A few genes with DMCs identified in generation F0 (irradiated across lifecycle) were previously involved in radiological stress in in vitro and in vivo studies in humans or rodents. Two of these genes, with common DMCs to both dose rates, coded respectively for a X-box-binding protein (Dapma7bEVm007629t1) which was associated with endoplasmic reticulum (ER) stress following an irradiation and cell death, and for an Anaphase-promoting complex subunit (Dapma7bEVm003591t1) which was downregulated after irradiation to delay mitosis of damaged cells. One of the genes with DMCs detected at 6.5 µGy h coded for a Clip-domain serine protease (Dapma7bEVm005724t1). Serine proteases were involved in radiation-induced apoptosis in human cells, although Clip-domain serine proteases might have a different function in arthropods (immune responses). In addition, a gene homologous to methyl-CpG-binding domain protein 3 (Dapma7bEVm006374t1), which binds differentially depending on methylation status, was hypomethylated at 41.3 mGy h in generation F0. Complementary studies (with mRNAseq analyses and DNMT activity assays for example) are needed in D. magna in order to understand the molecular mechanisms.
leading to the hypo or hyper-methylation and determine the consequences of gene body
methylation changes for gene expression.

4.5 Methylation changes across generations

The hypothesis that CpG methylation status was transmitted across generations was
investigated by comparing DMC identities and methylation levels among generations. In
order to remain cautious with interpretation of DMCs, it is important to note that some of the
detected methylation changes were also detected as DMCs in the control across generations
(representing 30%, 25% and 24% of total DMCs detected in generations F0, F2 and F3
respectively). These DMCs might result from changes in unexposed daphnids and might not
reflect a response to radiation exposure.

The comparison of DMC identities (Figure 4) showed that the total number of common
DMCs was smallest (1 independent of dose rate) between generations F0 and F3. This number
was greater (18 independent of dose rate) between generations F0 and F2. A large proportion
of methylation changes observed in generation F0 after gamma irradiation were induced in
somatic cells. These changes could not be transferred through the germline and were thus
absent from subsequent generations. This could explain why generation F0 shared
comparatively fewer similarities with other generations. Beside this, few methylation changes
(2 hypermethylated DMCs) were common to generations F0, F2 and F3 (Figure 4), showing
that changes induced in the germline were possibly transferred across generations.

The total number of common DMCs was greatest between generations F2 and F3, ranging
from 26 to 34, depending on dose rate (Figure 4). A large proportion (23 out of 26 or 34) of
these DMCs in common between generations F2 and F3 were hypomethylated. A majority of
these hypomethylated DMCs (16 out of 26) were also shared between dose rates. Finally, the
comparison of DMC methylation levels of DMCs detected in gene bodies (Figure 5) showed
that common DMCs most often had similar methylation levels between generations F2 and F3. These observations strongly suggested that methylation changes (induced in the gametes of F1 during irradiation) could potentially be transmitted to generations F2 and F3, particularly in the case of hypomethylation. One could hypothesize that hypomethylated CpGs might contribute to the genomic instability observed in *D. magna*. Hypomethylation is often associated with genomic instability in vertebrates although a similar mechanism was not confirmed in invertebrates. One can also wonder whether the transmission of radiation-induced methylation changes is limited to parthenogenetic reproduction and whether a similar observation would be possible across generations of sexually reproducing *D. magna*. The generalization of the phenomenon to other species is an important question which needs further investigations. Inheritance of epigenetic marks is expected to be limited in rodents where most DNA methylation marks are erased after fertilization in contrast to other vertebrates like Zebrafish where no epigenetic erasure occurs during development.

Interestingly, genes among those with common DMCs between generations F2 and F3 (SI Table S5) included proteins were previously associated with radiation exposure. A 60S ribosomal proteins L28 (Dapma7bEVm004770t1, Figure 5p) involved in translation regulation was involved in a radio-induced dysregulation of gene expression observed in developing Murine heart. A 70 kDa HEAT shock protein (Hsp70) (Dapma7bEVm636207t1, Figure 5l and Figure 5m), involved in cellular responses to various stresses, protein stability and preventing cell death, was upregulated after exposure to ionizing radiation and was linked to an adaptive response. The association of this gene with common DMCs between generations F2 and F3 might suggest that *D. magna* can develop a resistance to radiation. This resistance ability was supported by fecundity results in daphnids during a gamma irradiation across generations F0 and F1.
4.6 Effects on survival, growth and reproduction

Our study finally tested whether methylation changes were associated with biological effects at the organism level. In generation F0, a significant reduction in fecundity (SI Figure S2) was observed at the highest tested dose rate only (41.3 mGy h\(^{-1}\)). This reduction occurred as early as brood 2 and reached 17% over the 5 broods (p≤0.05). No radiation effect was observed on survival and growth in body size at any dose rate in this generation (data not shown). These results were in agreement with previous results obtained with \(D. \ magn\)^{29,69} and with other studies in the marine polychaete \(Ophryotrocha \ diadema\) and the terrestrial oligochaete \(Eisenia \ fetida\), showing that gamma radiation induced reprotoxicity, at dose rates ranging from 0.19 to 43 mGy h\(^{-1}\) in the first exposed generation.\(^{70-72}\) A slight effect on growth was previously reported in generation F0 at 35.4 mGy h\(^{-1}\).\(^{29}\) This was not observed in our study, possibly due to a relatively greater individual variability.

In our study, no radiation effect was observed on survival, growth or reproduction at any dose rate in generations F1, F2 and F3 (data not shown). These results were in agreement with those in \(C. \ elegans\)^{73} showing that fecundity in unexposed generation F1 after a parental irradiation (F0) did not differ from the control up to 42.7 mGy.h\(^{-1}\). In our study, the transmitted methylation changes that we observed from generation F2 to generation F3 could not easily be linked to any specific phenotype at the organismal level. Despite the absence of significant macroscopic effects in recovering offspring generations, methylation changes remained good candidates as biomarkers of a parental exposure to gamma radiation. One can hypothesize that the gamma irradiation that we conducted in our experiment, was not sufficient (in dose rate or duration) to accumulate an amount of methylation changes that would lead to detectable effects in the unexposed generation F3. This cumulative mechanism was well described\(^{58}\) and was also suggested in studies showing that radiation effects at the organism level increased in intensity over successive exposed generations.\(^{29,70,72,73}\) In the
future, extending irradiation over a larger number of generations (N=2 or more) and investigating epigenetic changes and their inheritance and potential phenotypic consequences in unexposed generations N+2 and N+3 would be interesting.74

5 Associated content
Supporting Information includes additional Material and methods paragraphs (1.1. Composition of M4 medium; 1.2. Sequencing of the DNA samples; 1.3. DNA quality assessment and mapping; 1.4. Effects on survival, growth and reproduction). Tables report dose rates (mGy h⁻¹) delivered to *D. magna* (Table S1), summary statistics of the 100bp paired-end reads generated by WGBS (Table S2), list of detected DMRs (Table S3), lists of genes with DMCs in generation F2 and not in generation F3 (Table S4), in generation F3 and not in generation F2 (Table S5) and in generations F2 and F3 (Table S6). Figures report average CpG methylation levels in whole genome and in different genomic features (Figure S1) and cumulated production of neonates per daphnid as a function of age (Figure S2).
6 References


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7 Figure captions

Figure 1. Experimental design. “GC” = Germline cell. “B4” and “B5” = fourth and fifth brood used to start the next generation before DNA was extracted from daphnids.

Figure 2. Proportion of hypomethylated and hypermethylated DMCs (to total CpGs) in generations F0, F2 and F3 exposed or recovering from exposure to 6.5 µGy h⁻¹ and 41.3 mGy h⁻¹ per genomic features.

Figure 3. Number of genes containing at least one DMC in generations F0, F2 and F3, exposed or recovering from exposure to 6.5 µGy h⁻¹ and 41.3 mGy h⁻¹ per KOG category. Only the 10 most represented KOG categories are represented (including ex aequo counts). Some genes, representing proportions of 38 and 12 % in generation F0, 26 and 23 % in generation F2 and, 28 and 27 % in generation F3 at 6.5 µGy.h⁻¹ and 41.3 mGy.h⁻¹ respectively, could not be linked to any KOG.

Figure 4. Venn diagrams for hypomethylated, hypermethylated and total DMCs, showing numbers of common DMCs among generations F0, F2 and F3 in the 6.5 µGy h⁻¹ and 41.3 mGy h⁻¹ treatments.

Figure 5. Changes in methylation level (%) across generations F0, F2 and F3, for CpGs located in gene bodies and corresponding to common DMCs between generation F2 and F3, at 6.5 µGy h⁻¹ (from a to e), at 41.3 mGy h⁻¹ (from f to k) or at both 6.5 µGy h⁻¹ and 41.3 mGy h⁻¹ (from l to s). DMCs are marked with a star. Letters with grey shading indicate that CpGs are also detected as DMCs across generations in the control. Corresponding gene codes: a) Dapma7bEVm643574t1; b) Dapma7bEVm006642t1; c) Dapma7bEVm005020t1; d,e) Dapma7bEVm000594t1; f) Dapma7bEVm007421t1; g,h) Dapma7bEVm008419t1; i) Dapma7bEVm004647t1; j) Dapma7bEVm637841t1; k) Dapma7bEVm005882t1; l,m)
Dapma7bEVm636207t1; n,o) Dapma7bEVm001664t1; p) Dapma7bEVm004770t1; q,r)

Dapma7bEVm004226t1; s). Dapma7bEVm001898t1
Figure 1

ACS Paragon Plus Environment
Figure 2

ACS Paragon Plus Environment
Table:<br>KOG categories<br>Number of genes<br><br>Graphs showing the number of genes in different KOG categories for three different radiation levels:
- **F0**<br>- **F2**<br>- **F3**

**KOG categories**<br>Cellular processes and signaling<br>O Post-translational modification, protein turnover, chaperone functions<br>T Signal transduction<br>U Intracellular trafficking and secretion<br>Z Cytoskeleton<br><br>Information storage and processing<br>A RNA processing and modification<br>J Translation<br>K Transcription<br><br>Metabolism<br>G Carbohydrate metabolism and transport<br><br>Poorly characterized<br>R General function prediction only<br>S Function unknown

Figure 3
ACS Paragon Plus Environment
Figure 4

ACS Paragon Plus Environment
Figure 5

ACS Paragon Plus Environment