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Epigenetic, transcriptional and phenotypic responses in *Daphnia magna* exposed to low-level ionizing radiation

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ABSTRACT

Ionizing radiation is known to induce oxidative stress and DNA damage as well as epigenetic effects in aquatic organisms. Epigenetic changes can be part of the adaptive responses to protect organisms from radiation-induced damage, or act as drivers of toxicity pathways leading to adverse effects. To investigate the potential roles of epigenetic mechanisms in low-dose ionizing radiation-induced stress responses, an ecologically relevant crustacean, adult *Daphnia magna* were chronically exposed to low and medium level external ^{60}Co gamma radiation ranging from 0.4, 1, 4, 10, and 40 mGy/h for seven days. Biological effects at the molecular (global DNA methylation, histone modification, gene expression), cellular (reactive oxygen species formation), tissue/organ (ovary, gut and epidermal histology) and organismal (fecundity) levels were investigated using a suite of effect assessment tools. The results showed an increase in global DNA methylation associated with loci-specific alterations of histone H3K9 methylation and acetylation, and downregulation of genes involved in DNA methylation, one-carbon metabolism, antioxidant defense, DNA repair, apoptosis, calcium signaling and endocrine regulation of development and reproduction. Temporal changes of reactive oxygen species (ROS) formation were also observed with an apparent transition from ROS suppression to induction from 2 to 7 days after gamma exposure. The cumulative fecundity, however, was not significantly changed by the gamma exposure. On the basis of the new experimental evidence and existing knowledge, a hypothetical model was proposed to provide in-depth mechanistic understanding of the roles of epigenetic mechanisms in low dose ionizing radiation induced stress responses in *D. magna*.

1. Introduction

Organisms are constantly exposed to gamma radiation, a high energy penetrating electromagnetic radiation, from naturally occurring radionuclides such as uranium and thorium and their progenies, or from artificially produced radionuclides such as nuclear fission (e.g., ^{137}Cs) or activation products (e.g., ^{60}Co) being released to the environments following nuclear weapons tests or nuclear accidents. In contaminated areas such as the exclusion zones of Chernobyl and Fukushima, the external dose to living organisms is attributed to gamma radiation from man-made radionuclides. The dose-rates within contaminated areas vary significantly, reaching 633 μGy/h in Chernobyl (Kryshev, 1998), 100–633 μGy/h in Fukushima (Battle et al., 2014) and 450 μGy/h at Mayak Production Association (PA) in the Urals (Kryshev et al., 1997). In extreme cases such as Lake Karachay at Mayak, as high as 1 Gy/h has been reported (reviewed in Standring et al. (2009)). At all these sites, lake waters and rivers have been contaminated due to run-off from contaminates soils or from direct release from nuclear installations. Due to the rather long half-lives of ^{60}Co (5.27 years) and ^{137}Cs (30.17 years) environmental exposure is in most cases long-term and thereby of great concern to both terrestrial and aquatic organisms.

By ionization and excitation of water molecules, gamma radiation

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can generate highly reactive free radicals, such as reactive oxygen species (ROS) which can cause oxidative damage to macromolecules such as DNA, proteins and lipids (Reisz et al., 2014). Direct ionization of DNA molecules can also lead to double-strand breaks (Vignard et al., 2013), which in combination with other types of DNA damage is considered a main cause of mutation, genomic instability and tumorigenesis in organisms (Mavragani et al., 2017). Oxidative stress and DNA damage are known to cause diverse types of adverse effects in directly irradiated organisms, such as mortality, growth retardation, developmental arrest and reproductive decline (Real et al., 2004; Dallas et al., 2012; Fuller et al., 2015; Hurem et al., 2017). Interestingly, accumulating evidence from the past two decades suggests that besides posing direct hazards to exposed organisms, ionizing radiation can also cause heritable effects on unexposed progenies of exposed organisms (Schofield and Kondratowicz, 2017; Adam-Guillermin et al., 2018; Kamstra et al., 2018; Horemans et al., 2019). These transgenerational effects have been proposed to be produced through epigenetic mechanisms (reviewed in Horemans et al. (2019)), such as DNA methylation, histone modifications and non-coding RNA interference, which are found to be the master regulators of gene expression (Allis and Jenuwein, 2016).

One of the most widely studied epigenetic mechanisms is DNA methylation. In general, cytosine residues in DNA are methylated to 5-methylcytosine (5 mC) by DNA methyltransferases (DNMTs) through addition of a methyl group donated by S-adenosylmethionine (SAM). In vertebrates, DNA methylation is maintained by DNMT1 during the S phase of mitosis and *de novo* by DNMT3a and DNMT3b to establish new methylation patterns to unmodified DNA (Okano et al., 1998; Cheng and Blumenthal, 2008). Demethylation of 5 mC is achieved either by direct or indirect (through modification of cytosine bases by ten-eleven translocation (TET) methylcytosine dioxygenases (Rasmussen and Helin, 2016) removal of 5 mC via processes such as base excision repair (BER) and mismatch repair (MMR) (Grin and Ishchenko, 2016). Another key epigenetic mechanism to regulate gene expression is via histone post-translational modifications (PTM). Different types of histone PTM, such as methylation, acetylation, phosphorylation and ubiquitination allow the control of accessibility for RNA polymerase to the DNA helix in the promoter region of a gene, thereby inducing or suppressing gene transcription (Bannister and Kouzarides, 2011). For instance, tri-methylation of histone H3 lysine residue 9 (H3K9) has been found to repress transcription (Greer and Shi, 2012), whereas acetylation of H3K9 has been reported to upregulate gene expression in vertebrates (Meyer et al., 2016). The interactions between DNA methylation and histone modifications play active roles in regulation of gene expression (Razin, 1998).

While a significant number of epigenetic studies have been conducted to investigate the effects of ionizing radiation on vertebrates, relatively little is known for invertebrates, especially aquatic crustaceans that are of great ecological relevance and are found in areas contaminated by nuclear accidents (Wada et al., 2016; Fuller et al., 2017, 2019bib_Fuller_et_al_2017bib_Fuller_et_al_2019; Goodman et al., 2019). In recent years, the water flea *Daphnia* have been increasingly used as aquatic crustacean models in environmental epigenetic research to understand the transgenerational effects of metals (Vandegehuchte et al., 2009a, 2009b, 2010a, 2010b, 2010cbib_Vandegehuchte_et_al_2009abib_Vandegehuchte_et_al_2009bbib_Vandegehuchte_et_al_2010abib_Vandegehuchte_et_al_2010bbib_Vandegehuchte_et_al_2010c), pharmaceuticals (Athanasio et al., 2018; Lindeman et al., 2019b), nutrition (Asselman et al., 2015; Hearn et al., 2019), cyanobacteria (Asselman et al., 2015, 2017bib_Asselman_et_al_2015bib_Asselman_et_al_2017), salinity (Jeremias et al., 2018) and ionizing radiation (Trijau et al., 2018). *Daphnia* possess many advantages of being an invertebrate epigenetic model, such as parthenogenetic reproduction which produces genetically-identical progenies, rapid reproduction cycles for investigating transgenerational effects, sequenced genome for gene identification and functional characterization, small sizes and transparent bodies for easy phenotyping, and high

susceptibility for detecting effects of environmental stressors (Harris et al., 2012; Wojewodzic and Beaton, 2017). The orthologs of all three mammalian DNMTs have been identified in *Daphnia*, demonstrating the presence of CpG-based methylation processes (Vandegehuchte et al., 2009a, 2010bbib_Vandegehuchte_et_al_2009abib_Vandegehuchte_et_al_2010b). Dissimilar to mammals which have around 70% of methylated CpGs, *Daphnia* are hypomethylated under normal conditions, with *Daphnia magna* and *D. pulex* having 0.52% and 0.70% global CpG methylation, respectively (Asselman et al., 2016; Lindeman et al., 2019b). Knowledge of ionizing radiation-mediated epigenetic effects on *Daphnia* is, however, limited. The only relevant study to date was published recently and used whole-genome bisulfite sequencing to understand the transgenerational effects of chronic gamma radiation exposure on *D. magna* (Trijau et al., 2018). Although a number of differentially methylated regions (DMR) were identified after gamma exposure, the causal relationships between DNA methylation, gene expression and transgenerational reproductive effects were not investigated and still remain to be established.

The present study was conducted to investigate the involvement of epigenetic mechanisms in the stress responses of *Daphnia* exposed to low levels of gamma radiation. Adult female *D. magna* were chronically exposed to external cobalt-60 gamma radiation for seven days to investigate the reproductive effects of gamma radiation on at least two molt and reproductive cycles. A suite of bioassays was employed to identify effects occurring at multiple levels of biological organization. A general hypothesis was that epigenetic mechanisms were involved in the stress responses in D. magna after chronic exposure to gamma radiation. The objectives of the present study were to: 1) investigate temporal changes of ROS formation in *D. magna* after exposure; 2) identify epigenetic, transcriptional and phenotypic effects in *D. magna* after chronic exposure; 3) decipher the potential relationships between radiation exposure, ROS formation, DNA methylation, histone modification, gene expression and phenotypic changes for mechanistic understanding of ionizing radiation effects in this key freshwater crustacean. The present study was among the first to investigate the epigenetic regulation of stress responses at multiple levels of biological organization in adult *D. magna* after chronic exposure to gamma radiation.

2. Materials and methods

2.1. Daphnia culture

Daphnia magna DHI strain (DHI Water & Environment, Hørsholm, Denmark) were cultured in the M7 medium (20 daphnids/L) in glass beakers under stable temperatures (20 ± 1 °C) and a constant photoperiod (16 h light:8 h dark). The culture medium was renewed twice per week. After renewal, *D. magna* were fed with concentrated green algae *Raphidocelis subcapitata* (0.1 mg carbon daphnid^{-1} day^{-1}) (OECD, 2012). For exposure studies, synchronized neonatal (<24 h old) *D. magna* were cultured under the same conditions as described above until 14-15 d old.

2.2. Exposure and sampling

The exposure studies were conducted at the FIGARO ^{60}Co gamma irradiation facility (Lind et al., 2018), Norwegian University of Life Sciences (NMBU, Norderås, Ås, Norway). The applied non-lethal dose-rates (0-control, 0.4, 1, 4, 10, and 40 mGy/h) were proven to be sublethal to *D. magna* and achieved by varying the distance to the radiation source, as previously described (Gomes et al., 2018). These dose-rates were representative of the low exposure levels immediately after nuclear contaminations or accidents. Exact dosimetry (front and back of the beakers) for the studies has been previously described in Song et al. (2020). Two exposure studies were run sequentially to assess reproductive effects using synchronized (14-15 d old) adult female

D. magna from the same parental animals, but different broods, according to the principles of a short-term screening (STS) protocol (Abe et al., 2015). The exposure conditions were identical to the culturing conditions. The test medium was renewed every two days and each daphnid was daily fed with concentrated *R. subcapitata* (2.1×10^7 cells corresponding to 0.1 mg carbon/daphnid/day). The first exposure mainly focused on the apical effects (i.e. survival, fecundity, molting and histopathology) and molecular endpoints (i.e. methylation, histone modifications, gene expression and ROS), whereas the second exposure focused on temporal ROS formation. *Exposure I*: individual daphnid per beaker was exposed to all dose-rates of external gamma radiation for 7 days in a plastic beaker containing 50 mL of culture medium (n = 10). Survival, molting, ovulation and reproduction (number of offspring) were recorded every day. The offspring were removed immediately after daily observation to avoid high population density and food shortage. After 7 days, the test animals (n = 1) were fixed in 1 mL Bouin's fluid (Sigma-Aldrich, St. Louis, USA) and stored at 4 °C until histological analysis. In parallel, 18 daphnids per beaker were exposed to 0 (control), 0.4, 1 and 10 mGy/h gamma radiation in a 1 L plastic beaker containing 900 mL culture medium for 7 days (n = 5) to obtain required amount of materials for molecular analyses. Only three dose-rates were tested due to the use of larger beakers and limitations in space at the irradiation facility. After 7 days, samples for global methylation (5 individuals pooled per replicate) were snap-frozen in liquid nitrogen and stored at −80 °C. Samples for histone analysis (8 individuals pooled per replicate) were immediately cross-linked as previously described (Lindeman et al., 2019b), snap-frozen in liquid nitrogen and stored at −80 °C. For gene expression analysis, samples (4 individuals pooled per replicate) were collected in RNALater (Qiagen, Hilden, Germany) and stored at −80 °C. Only one time point was included in the analyses due to limited materials. *Exposure II*: individual daphnid per beaker was exposed to all dose-rates of external gamma radiation for 7 days in a plastic beaker containing 50 mL of culture medium (n = 18). Samples for determining mitochondrial ROS (n = 3) and lipid peroxidation associated ROS (n = 3) were collected and immediately analyzed after 2, 4, and 7 d exposure to gamma radiation to investigate temporal change of ROS formation, as ROS formation is widely known as a direct biochemical response to gamma radiation exposure. The water quality (pH and dissolved oxygen) and temperature were recorded throughout the exposures.

2.3. Global methylation analysis

Global cytosine methylation analysis (n = 5) was conducted as previously described (Kamstra et al., 2017). In brief, genomic DNA was extracted from pooled (5 individuals) *D. magna* using the Gentra Puregene Tissue Kit (Qiagen), according to the manufacturer's instructions. For each sample, 200 ng of total DNA was used for global methylation analysis. DNA was digested at 37 °C for 6 h with a mixture of venom phosphodiesterase (60 mU/mL), alkaline phosphatase (40 U/mL) and benzonase (50 U/mL) in digestion buffer (20 mM TRIS, 100 mM NaCl, 20 mM MgCl2, pH 7.9) (Sigma-Aldrich) in a final volume of 20 μL. After incubation, internal standards 2′-deoxyguanosine-$^{13}C_{10}$,$^{15}N_5$ ($^{13}C^{15}$N-G) (TRC, Canada) and (5-methyl-2′-deoxycytidine-d3 (DmC) (TRC, Canada) were added at final concentrations of 345 nM $^{13}C_{10}$−$^{15}N_5$-G and 20 nM DmC and diluted to 200 μL in water. Liquid chromatography–mass spectrometry (LC-MS) analysis was performed exactly as described by Kamstra et al. (2017). After correction with internal standards, the ratio between 5 mC and guanine was determined as a measure for global cytosine methylation.

2.4. Histone analysis

The chromatin immunoprecipitation coupled with quantitative polymerase chain reaction (ChIP-PCR) was used to determine histone modifications in *D. magna* after exposure to gamma radiation. The ChIP PCR assay (n = 3, 8 pooled daphnids/replicate) was performed as

previously described (Lindeman et al., 2019b). Briefly, the daphnids were cross-linked with 1% formaldehyde for 8 min and chromatin fragmented on a Bioruptor pico (Diagenode, Liege, Belgium) in 100 μL lysis buffer. Antibodies were coupled to magnetic beads and incubated with 100 μL chromatin with a concentration of 3 ng/μL overnight with rotation. Empty beads worked as no antibody control as a measure of unspecific binding. After incubation, the chromatin-antibody-bead complexes were washed three times in TRIS-EDTA (TE) buffer before cross-linking reversal and elution of the beads. Input samples contained the same volume of chromatin as the ChIP samples. Nucleic acids were isolated, real time PCR was performed and the percentage of ChIPed DNA relative to the input samples were calculated. All samples were normalized to the histone H3 enrichment. Antibodies used were H3K9me3 (Diagenode, prod. no. C15410056), H3K9ac (Diagenode, prod. no. C1541009) and histone H3 (Diagenode, prod. No C15310135). Previously published primers were used for ChIP-PCR analysis (Lindeman et al., 2019b). *Actin* was used as a reference gene for quality assurance, whereas *Dnmt1*, *Dnmt3a1*, *Dnmt3a2*, glycine N-methyltransferase (*Gnmt*), S-adenosylmethionine synthase (*MetK*), adenosylhomocysteinase (*Sahh*), methylenetetrahydrofolate reductase (*Mthfr*) were test genes.

2.5. Transcriptional analysis

Quantitative real-time reverse-transcription PCR (qPCR) was used to determine the transcriptional changes of selected biomarker genes in *D. magna* after exposure to gamma radiation. The qPCR assay (n = 5) was conducted as previously described (Song et al., 2016a). Briefly, total RNA was isolated from pooled (4 individuals) *D. magna* using the ZR Tissue & Insect RNA MicroPrep™ kit (Zymo Research Corp., Irvine, CA, USA). A Nanodrop® ND-1000 spectrometer (Nanodrop Technologies, Wilmington, Delaware, USA) was used to determine the purity of RNA (260/280 > 1.8), and the RNA integrity was assessed using an Agilent Bioanalyzer (Agilent technologies, Santa Clara, California, USA), as previously described (Song et al., 2016b). Total RNA with clear 28s/18s peaks and flat baselines was qualified for qPCR analysis.

The qPCR assay was performed using the Bio-Rad CFX96 platform (Bio-Rad Laboratories, Hercules, CA, USA). Previously published primers were used for qPCR analysis (Gomes et al., 2018; Lindeman et al., 2019b; Song et al., 2020). Complementary DNA (cDNA) was reversely transcribed from 200 ng total RNA using qScript™ cDNA SuperMix (Quanta BioSciences™, Gaithersburg, MD, USA), and amplified in a 20 μL reaction using PerfeCTa® SYBR® Green FastMix® (Quanta BioSciences™). For quality control, a non-template control (NTC) and a no-reverse-transcriptase control (NRT) were included in the analysis. The qPCR assay was run in technical duplicates. A standard curve was generated from a dilution series of pooled cDNA from all test samples for calculation of amplification efficiency (E) and correlation coefficient. The relative expression was calculated based on a combination of threshold cycle (Cq) and efficiency using the Pfaffl method (Pfaffl, 2001) and normalized to the geometric mean expression of two reference genes, *Actin* and glyceraldehyde 3-phosphate dehydrogenase (*Gadph*) using the ΔΔCq method (Vandesompele et al., 2002). Genes that are representative of different biological pathways, such as 1) DNA methylation: *Dnmt1*, *Dnmt3a1*, *Dnmt3a2*, Tet methylcytosine dioxygenase 2 (*Tet2*), 2) one carbon metabolism: *Gnmt*, *MetK*, *Sahh*, *Mthfr*, 3) calcium signaling: calmodulin (*Calm*), 4) DNA damage response: DNA repair protein RAD50 (*Rad50*), 5) apoptosis: TP53 regulated inhibitor of apoptosis (*Triap*), 6) antioxidant defense: glutathione S-transferase (*Gst*), Cu–Zn superoxide dismutase (*Sod*), 7) development: vitellogenin 1 (*Vtg1*), *Vtg2*, methoprene-tolerant (*Met*) were included as target genes for qPCR analysis.

2.6. Reactive oxygen species measurements

The ROS production in *D. magna* (n = 3) was assessed using two

fluorescent probes, dihydrorhodamine 123/DHR123 and 4,4-difluoro-3a, 4adiaza-s-indacene/BODIPY (Thermo Fisher Scientific, Waltham, USA). The former was used to measure the formation of mitochondrial ROS (mROS), whereas the latter was used to indicate the production of ROS associated with lipid peroxidation (lpoROS), such as peroxyl radicals. The ROS assays were conducted as previously described (Song et al., 2020). Briefly, individual daphnids were incubated in the culture media containing 5 mM of DHR123 or 1.5 mM of BODIPY for 1 h in a 96-well black microplate (Corning Costar, Cambridge, MA, USA). The fluorescence intensity at excitation/emission wavelength of 485/538 nm was measured using a VICTOR 3 microplate reader (PerkinElmer, Waltham, USA).

2.7. Histopathological analysis

Histopathological analysis was performed as previously described (Song et al., 2020). The Bouin's fluid fixed daphnids were washed with phosphate buffered saline (PBS, pH 7) and calcified. The samples (n = 1) were post fixed in OsO_4 (Sigma-Aldrich) for 30 min. After post fixation, the samples were briefly washed with PBS and distilled water. Dehydration were performed in graded ethanol series (30, 50, 70, 90 and 100% ethanol 1 h for each step). The samples were infiltrated in LR and embedded in the LR white. Embedded samples were sectioned into 1 μm thickness using an Ultracut microtome (Leica EM UC6, Germany) and stained with toluidine blue O (Sigma-Aldrich) and examined using a light microscope DM6B (Leica, Germany).

2.8. Statistical analyses

The raw data was checked for normality and equal variance prior to statistical analyses. Differences (p < 0.05) between treatment groups were determined using one-way analysis of variance (one-way ANOVA) followed by Tukey post-hoc test, or Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test (no normality or equal variance) in Graphpad Prism v8.0.2 (Graphpad Software Inc., San Diego, CA, USA). Outliers (q < 0.05) were checked and eliminated using the ROUT test (Motulsky and Brown, 2006). A principal component analysis (PCA) was performed based on the mean dose-response data of different endpoints to characterize and visualize major patterns and correlations. A scree plot was made first to determine the number of PCs. A biplot of dose-rates and variables (endpoints) was then used to visualize the clustering, correlation and contribution of the variables in different PCs. All steps for PCA was performed using XLSTAT v2019.3.2 (Addinsoft Inc., New York, USA), as previously described (Xie et al., 2019).
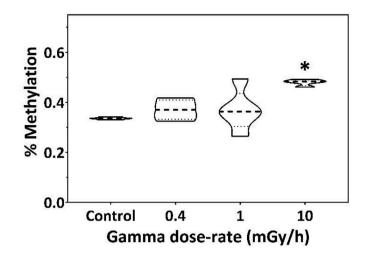
3. Results

3.1. Exposure quality

The measured gamma dose-rates were on average 0.4, 0.9, 3.8, 11.6, and 42.9 mGy/h (Song et al., 2020), which were similar to the nominal ones. It needs to be noted that the lead-shielded controls also received an average background dose-rate of 0.005 mGy/h due to scattered radiation. This value was in the range of the reported natural background radiation level of 0.17–90 μGy/h (UNSCEAR, 2000). The temperature, pH and dissolved oxygen in the exposure media was in the range of 8.0 ± 0.2, 20 ± 1 °C and above 8 mg/L, respectively throughout the studies, according to that required by the OECD test guideline (OECD, 2012).

3.2. Global methylation

Global DNA methylation analysis showed that exposure to 0.4, 1 and 10 mGy/h gamma radiation led to dose-rate dependent increase in global cytosine methylation in *D. magna* after 7 days, with 10 mGy/h causing a significant increase in global methylation (Fig. 1).

Fig. 1. Violin plots displaying global DNA methylation (n = 4–5, 5 pooled individuals/replicate) in adult female *Daphnia magna* after 7-day exposure to gamma radiation. The shape of the plot shows distribution of the data, with the top and bottom edges representing max and min, respectively. The upper, middle, and bottom dashed lines represent 75% quantile, median and 25% quantile of the data. * denotes significant difference from control.

3.3. Histone modification

As a quality control for the analysis, the H3K9ac of the housekeeping gene *Actin* was found to be highly enriched compared to H3K9me3, whereas no significant differences were found between the treatment groups. Dose-rate dependent increases in histone H3K9me3 enrichment were observed for *Dnmt3a1*, *Dnmt3a2*, *Gnmt*, and *Mthfr*, whereas dose-rate dependent increases in histone H3K9ac enrichment were found for *Dnmt3a1*, *Dnmt3a2* and *Gnmt* (Fig. 2). Significant enrichment of H3K9ac was identified for *Dnmt3a1* after exposure to 10 mGy/h and *Metk* after exposure to 1 mGy/h (Fig. 2).

3.4. Transcriptional responses

The two reference genes *Actin* and *Gadph* showed stable expression across treatment groups (Fig. 3) and were used for normalization. Seven out of sixteen genes were downregulated in a dose-rate dependent manner (Fig. 3), where exposure to 10 mGy/h significantly suppressed the expression of *Sahh*, *Dnmt3a2*, *Calm*, *Rad50*, *Triap*, and *Gst*. In addition, exposure to 0.4–10 mGy/h gamma radiation significantly downregulated the expression of *Dnmt3a2*. On the contrary, dose-rate dependent upregulation (Fig. 3) was identified for *Vtg1 and Vtg2*, where 10 mGy/h significantly induced the expression of the former three and 1 mGy/h significantly upregulated *Met*. An apparent, albeit non-significant trend of up-regulation of Tet2 was also observed.

3.5. ROS formation

Results from the ROS assays showed that exposure to gamma radiation caused time-and dose-rate dependent ROS production in *D. magna* during the 7-day exposure (Fig. 4). After 2 days, exposure to as low as 1 mGy/h gamma radiation led to significant reduction in mROS formation, whereas the lpoROS was only reduced by exposure to 10 and 40 mGy/h gamma radiation. After 4 days, no significant difference in mROS or lpoROS was found between the treatment groups. After 7 days, a tendency of increased ROS formation in irradiated *D. magna* was observed for both mROS and lpoROS (except for 0.4 mGy/h), albeit the differences were not statistically significant.
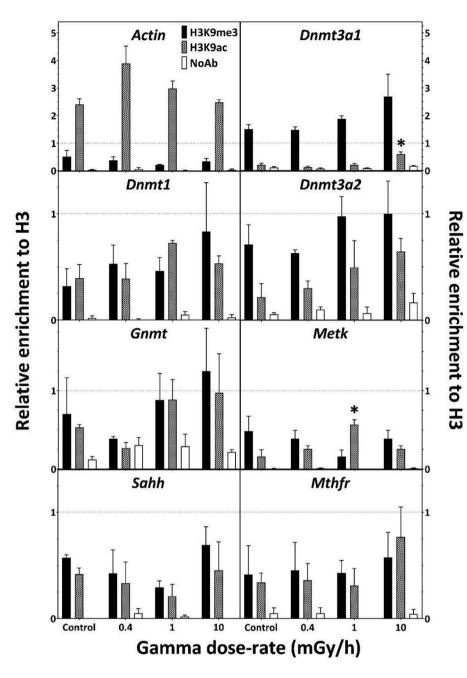
Fig. 2. Relative difference in promotor enrichment in relation to H3 (dashed line) in adult female *Daphnia magna* (mean ± SEM, n = 3, 8 pooled individual/replicate) after 7-day exposure to gamma radiation. *Dnmt1*: DNA (cytosine-5)-methyltransferase 1; *Dnmt3a1*: DNA (cytosine-5)-methyltransferase 3A1; *Dnmt3a2*: DNA (cytosine-5)-methyltransferase 3A2; *Gnmt*: glycine N-methyltransferase; *Metk*: s-adenosylmethionine synthase; *Sahh*: adenosylhomocysteinase; *Mthfr*: methylenetetrahydrofolate reductase.

3.6. Histological changes

The histopathological analysis showed that increased numbers of empty follicles were observed in *D. magna* after exposure to as low as 0.4 mGy/h gamma radiation (Fig. 5A). Abnormal morphology of oocytes was observed at gamma dose-rates as low as 4 mGy/h (Fig. 5A). Gut cells and gut hair were affected by exposure to gamma radiation and displayed irregular morphology and arrangement compared to the control (Fig. 5B). In addition, the irradiated *D. magna* apparently displayed thinner epidermis at gamma dose-rates as low as 4 mGy/h compared to control daphnids (Fig. 5C).

3.7. Reproductive effects

The fecundity of *D. magna* was not significantly affected by exposure to gamma radiation, albeit an apparent positive trend observed as an increase in the number of offspring in brood 1, brood 2 and total

fecundity were observed in *D. magna* after exposure to 4–40 mGy/h gamma radiation (Fig. 6).

3.8. Principal component analysis

The PCA showed that PC1 and PC2 explained 85.41% of the variance (Fig. 7). The majority of the molecular endpoints, such as global methylation, histone modifications and gene expression, in combination with fecundity were well represented by PC1, with global DNA methylation negatively correlated with the transcription of most genes tested, whereas positively correlated with *Dnmt3a1*, *Tet2*, *Gnmt*, *Vtg1*, *Vtg2* and *Met* (Fig. 7). Histone H3K9 methylation and acetylation were in most cases positively correlated. These histone modifications were also positively correlated with global DNA methylation and *Dnmt3a1*, *Tet2*, *Gnmt*, *Vtg1*, *Vtg2* and *Met* transcription, whereas negatively correlated with the transcription of other genes tested. These variations of these endpoints were well represented by the first principal component
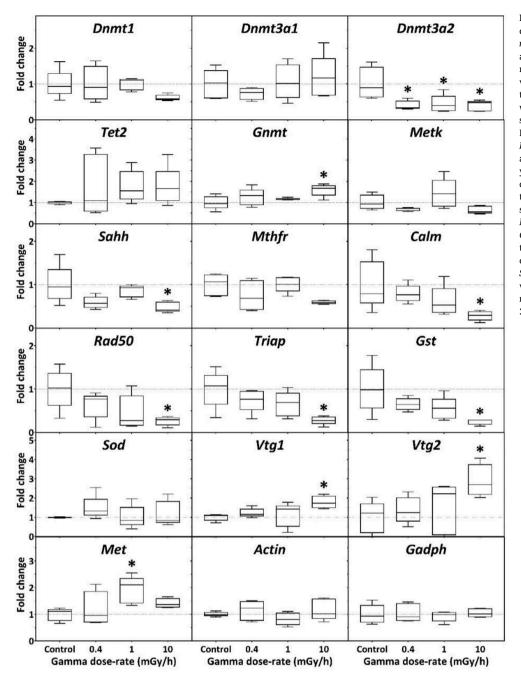
Fig. 3. Boxplots displaying transcriptional changes (n = 4–5) in adult female *Daphnia magna* after 7-day exposure to gamma radiation. The lower and upper edge of the box represents 25% quantile and 75% quantile values, respectively, the line in the middle of the box represents the median, and the whiskers represent min and max. * denotes significant difference from control. *Dnmt1*: DNA (cytosine-5)-methyltransferase 1; *Dnmt3a1*: DNA (cytosine-5)-methyltransferase 3A1; *Dnmt3a2*: DNA (cytosine-5)-methyltransferase 3A2; *Tet2*: Tet methylcytosine dioxygenase 2; *Gnmt*: glycine N-methyltransferase; *Metk*: s-adenosylmethionine synthase; *Sahh*: adenosylhomocysteinase; *Mthfr*: methylenetetrahydrofolate reductase; *Calm*: calmodulin; *Rad50*: DNA repair protein RAD50; *Triap*: TP53 regulated inhibitor of apoptosis; *Gst*: glutathione S-transferase; *Sod*: Cu–Zn superoxide dismutase; *Vtg1*: vitellogenin 1; *Vtg2*: vitellogenin 2; *Met*: methoprene-tolerant; *Gadph*: glyceraldehyde 3-phosphate dehydrogenase.

(Fig. 7). The contribution of different endpoints to the total variance of PC1 was similar, with the exceptions of *Sahh* and *Sod* gene expression (Fig. 7). Most endpoints were well correlated at the gamma dose-rate of 10 mGy/h (Fig. 7). For PC2, the levels of ROS (at 7 days exposure) were negatively correlated with transcription of genes involved in the anti-oxidant defense (*Gst*), DNA repair (*Rad*), apoptosis (*Triap*) and calcium signaling (*Calm*), whereas positively correlated with transcription of genes involved in reproductive processes (e.g., *Met* and *Vtg*) and DNA methylation processes (Fig. 7). Two and four-day mROS and expression of different DNMTs contributed less to the total variance compared to other endpoints.

4. Discussion

Ionizing radiation such as gamma radiation has been reported to cause heritable transgenerational adverse effects on organisms through alterations to the epigenome (reviewed in Horemans et al. (2019)). However, how exposure to ionizing radiation induces epigenetic changes, and how the epigenetic mechanisms is involved in stress responses following low-level radiation exposure have not been fully elucidated for aquatic organisms. The present study employed a suite of bioassays to assess biological effects of gamma radiation occurring at multiple levels of biological organization in an aquatic model crustacean, *D. magna*, with special focus on radiation-mediated changes in ROS formation, global DNA methylation, histone PTM, gene transcription and apical effects. The gamma dose-rates (0–40 mGy/h) used in the present study have been selected in order to cover dose-rates from low-level situations (Lerebours et al., 2018; Fuller et al., 2019; Goodman et al., 2019) to dose-rates expected immediately after nuclear accidents (Kryshev, 1998; Battle et al., 2014). Potential relationships between different endpoints will be characterized and discussed in detail in the following sections.
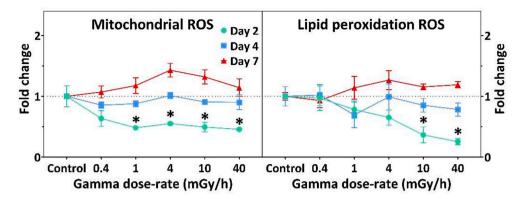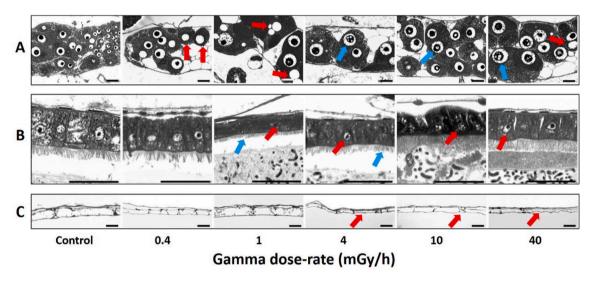
Fig. 4. Temporal changes in mitochondrial (left) and lipid peroxidation-associated (right) reactive oxygen species (ROS) formation (mean ± SEM, n = 3) in adult female *D. magna* after exposure to gamma radiation. * denotes significant difference from control.

Fig. 5. Histological changes (n = 1) in adult female *D. magna* after 7-day exposure to gamma radiation. A) Follicles and oocytes, red arrows indicate empty follicles and blue arrows indicate abnormal oocytes compared to the control; B) Gut cells and hair, red arrows indicate abnormal gut cells and blue arrows indicate not well-arranged gut hair compared to control; C) Epidermis, red arrows indicate thinner layers of epidermis compared to the control. Scales: 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
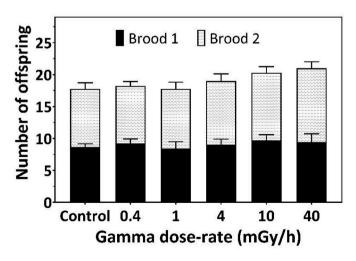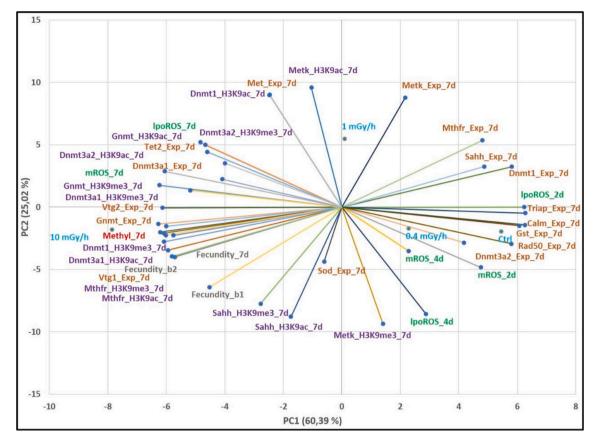

Fig. 6. Fecundity (mean ± SEM, n = 10) in adult female *Daphnia magna* during a 7-day exposure to gamma radiation.

4.1. DNA methylation

The average level of global methylation (approx. 0.34%) in the controls was in the range of that previously reported (0.25–0.53%) for this species (Asselman et al., 2015, 2016bib_Asselman_et_al_2015bi-b_Asselman_et_al_2016; Lindeman et al., 2019b). A dose-rate dependent increase in global DNA methylation was identified in irradiated daphnids. Low-dose ionizing radiation induced DNA hypermethylation has been documented for a wide range of organisms (reviewed in Miousse et al. (2017) and Horemans et al. (2019)), albeit a recent study identified more hypomethylated than hypermethylated gene regions in *D. magna* after chronic (25 d) exposure to 6.5 μGy/h and 41.3 mGy/h gamma radiation by whole-genome bisulfite sequencing analysis (Trijau et al., 2018). Interestingly, the discrepancy in findings between these two apparently comparable studies might be due to the use of different methodologies for quantification of global methylation, as well as different dose-rates and exposure duration. The whole-genome bisulfite approach by Trijau et al. (2018) has only reported the significantly altered loci, whereas in the present study, the entire pool of 5 mC was measured. Although more hypomethylated sites were found by (Trijau et al., 2018), the total pool of mC might lead to a different conclusion. In addition, the discrepancy may also be due to differences in radiation sensitivity of the life-stages exposed (14-15 d adult versus <24 h

Fig. 7. Principal component analysis (PCA) of the potential relationships between different endpoints. blue: gamma radiation dose-rates; green: reactive oxygen species (ROS); purple: histone modification; orange: gene expression; grey: fecundity. Methyl: global DNA methylation; *Dnmt1*: DNA (cytosine-5)-methyltransferase 1; *Dnmt3a1*: DNA (cytosine-5)-methyltransferase 3A1; *Dnmt3a2*: DNA (cytosine-5)-methyltransferase 3A2; *Tet2*: Tet methylcytosine dioxygenase 2; *Gnmt*: glycine N-methyltransferase; *Metk*: S-adenosylmethionine synthase; *Sahh*: adenosylhomocysteinase; *Mthfr*: methylenetetrahydrofolate reductase; *Calm*: calmodulin; *Rad50*: DNA repair protein RAD50; *Triap*: TP53 regulated inhibitor of apoptosis; *Gst*: glutathione S-transferase; *Sod*: Cu–Zn superoxide dismutase; *Vtg1*: vitellogenin 1; *Vtg2*: vitellogenin 2; *Met*: methoprene-tolerant. H3K9me3: tri-methylation of histone H3 lysine residue 9; H3K9ac: acetylation of H3K9; 2 d: after 2 days exposure; 4 d: after 4 days exposure; 7 d: after 7 days exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

juvenile) in the two studies. It has been suggested that juveniles are more susceptible to ionizing radiation than adults (Styron, 1971; Sasaki, 1991) and it is widely known that dramatic epigenetic reprograming processes can occur at different life stages in both animals and plants (Feng et al., 2010). In addition, it has been shown that DNA hypermethylation may be a defense mechanism against radiation as higher DNA methylation and other epigenetic factors make the chromatin more compact (Takata et al., 2013). At the transcription level, genes involved in the one-carbon metabolism pathway (Gatta et al., 2017) to promote

DNA methylation, such as *Dnmt 1*, *Dnmt 3a2*, *Sahh*, *Mthfr*, and *Metk* were downregulated by exposure to 10 mGy/h gamma radiation, whereas the competitor of DNMTs for methyl groups (*Gnmt*), and the gene regulating DNA demethylation processes (*Tet2*) were upregulated by 10 mGy/h gamma radiation, possibly indicating reduced demand for DNA methylation and elevated needs for demethylation. In addition, dose-rate dependent downregulation of genes associated with antioxidant defense (*Gst*), DNA double-strand break repair (*Rad50*), apoptosis inhibition (*Triap*) and calcium signaling (*Calm*) were identified and
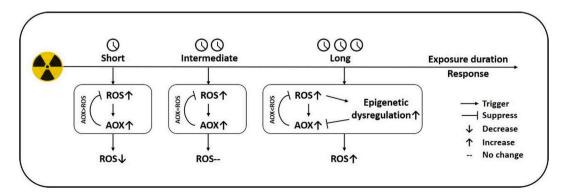

Fig. 8. Temporal changes of reactive oxygen species (ROS) formation and putative relationships between ROS, antioxidant defense (AOX) and epigenetic dysregulation.

found to be negatively correlated with global methylation. Although not determined directly in the present study, this may suggest a global DNA methylation induced suppression of target-specific gene expression after exposure to gamma radiation.

4.2. Histone modification

The present study also showed that exposure to gamma radiation caused changes to histone modifications in *D. magna*. The loci investigated in the putative upstream regulatory region of *Actin* displayed a histone PTM signature with low enrichment of transcriptionally repressive H3K9me3 and high enrichment of transcriptionally permissive H3K9ac, thus confirming the high level of transcription and stability of this reference gene across treatments. Marginal changes in H3K9me3 and H3K9ac enrichment were observed for most of the genes tested, with positive correlation between histone H3K9ac and gene expression for *Gnmt* and *Dnmt3a1* (10 mGy/h) and for Metk (1 mGy/h). Ionizing radiation-mediated changes to histone PTM have been reported in a number of studies, and have also been suggested as markers for ionizing radiation (Hall et al., 2017). In zebrafish embryos, short-term (3 h) exposure to 10 mGy/h gamma radiation induced hypermethylation of lysine 9 residues on histone H3 on a selection of genes such as hepatocyte nuclear factor 4 alpha (*hnf4a*), geminin DNA replication inhibitor (*gmnn*) and vascular endothelial growth factor Ab (*vegfab*) (Lindeman et al., 2019a). This was in line with a tendency of higher enrichment of H3K9me3 with increasing gamma dose-rates in the present study.

4.3. Temporal changes of ROS

Results from the present study showed that exposure to gamma radiation led to time- and dose-rate dependent changes in ROS formation in *D. magna* (Figs. 4 and 8). Interestingly, after short-term (2-day) exposure to gamma radiation, both mROS and lpoROS were suppressed in a dose-rate dependent manner. This is in contrast to an earlier study which showed significant induction of ROS after a 2-day exposure using the same experimental Co-60 source and similar nominal dose-rates (0.4, 1, 4, 10, 40, 100 mGy/h) (Gomes et al., 2018). However, Gomes and co-workers used juvenile *D. magna* and a simpler exposure medium without feeding, thus indicating that adult *D. magna* have better developed antioxidant defense system to regulate ROS formation compared to developing juveniles. The cellular ROS production was also found to be reduced in *D. magna* after 24 h exposure to 0.1–0.3 nM metal nanoparticles (MNP - quantum dots), whereas increased at higher (0.6–10 nM) MNP concentrations (Galdiero et al., 2017). Reduction in cellular ROS and lpoROS formation has also been observed in zebrafish (*Danio rerio*) larvae (72–120 hpf) after 2-day exposure to 9.4–37.4 J/cm^2 ultraviolet A radiation (Hurem et al., 2018). Inhibition of ROS formation was likely an adaptive response to low levels of oxidative stress. Such adaptive response can be exerted through a sudden enhancement of antioxidant defense leading to reduced ROS (Birben et al., 2012), and/or via reduction of ROS-generating physiological processes, such as oxidative phosphorylation (OXPHOS) in the mitochondria (Munro and Treberg, 2017). It is known that adaptive responses to ionizing radiation, such as activation of different antioxidants, can rapidly take place (e.g., a few hours) in mammals after irradiation (Miura, 2004). It has also been suggested that organisms can moderately reduce the mitochondrial OXPHOS activities as a compensatory mechanism to restrict the endogenous ROS formation without significant effects on ATP synthesis (Zorov et al., 2014).

After an intermediate exposure duration (4-day), no significant difference in ROS formation was observed in gamma-irradiated daphnids compared to the control, suggesting a temporary balance between ROS formation and antioxidant defense (Fig. 8).

After 7-day exposure, a tendency of increased ROS formation was observed for both mROS and lpoROS, albeit the changes were not statistically significant. Induction of ROS has been reported in juvenile

D. magna after acute (24–48 h) (Gomes et al., 2018) and chronic (8 d) (Song et al., 2020) exposure to 1–100 mGy/h of gamma radiation. Interestingly, *Gst*, a gene encoding for an antioxidant against lipid hydroperoxides (Singhal et al., 2015) and a widely used marker for oxidative stress, was downregulated by exposure to gamma radiation in a dose-rate dependent manner, indicating potential suppression of glutathione-associated antioxidant processes. In comparison, Cu/Zn-SOD activity was increased at mild stress but decreased after severe stress leading to excess ROS accumulation (Liu et al., 2015; Phull et al., 2018). Suppression of antioxidant genes/enzymes have been reported for aquatic organisms after exposure to environmental stressors such as metals (Lee et al., 2017), organics (Lee et al., 2006) and nanoparticles (Galdiero et al., 2017; Nunes et al., 2018). In addition, it is widely known that a number of antioxidant genes are regulated by the Kelch-like ECH-associated protein 1-nuclear factor erythroid 2 (Keap1-Nrf2) pathway (Kansanen et al., 2013). DNA hypermethylation may suppress the Keap1-Nrf2 pathway thus affect the transcription of downstream antioxidant genes (Figs. 3 and 8), such as *Gst* and NAD(P)H quinone dehydrogenase 1 (Guo et al., 2015).

On the basis of the current findings, it is hypothesized that if gamma exposure endures, ROS may accumulate, and the organisms may subsequently suffer from increased oxidative damage.

4.4. Apical effects

At the tissue/organ level, increased empty follicles in combination with abnormally developed oocytes were identified in *D. magna* exposed to as low as 0.4 mGy/h gamma radiation, suggesting an adverse effect of radiation on oogenesis. Similar effects on oocytes have been observed in *D. magna* after 8- and 14-day exposure to 0.4–100 mGy/h gamma radiation (Song et al., 2020). Impairment of oogenesis was likely caused by radiation-triggered apoptosis (Song et al., 2020), as also evidenced by downregulation of the apoptosis inhibitor *Triap* (Adams et al., 2015) in the present study. The morphologies of gut cells and hair were also affected by exposure to as low as 1 mGy/h gamma radiation. Similar effects have also been reported in *D. magna* after exposure to the same levels of gamma radiation in a previous study (Song et al., 2020) to support the relevance of radiation-induced cell death as one of the main causes for disruption of normal tissue development and functions (Munoz et al., 2017). In addition, the epidermis was affected by as low as 4 mGy/h gamma. The epidermis and gut of *D. magna* consist of cuticle-secreting cells which contain chitin (Halcrow, 1976; Lu et al., 2018). Modification of chitin synthesis can affect epidermal cuticle and midgut matrix in insects such as *Tribolium castaneum* (Arakane et al., 2005). The mechanisms of epidermal and gut modification in *Daphnia*, however, is still unclear and warrants more investigations.

Although effects were found on oocytes at the histological level, surprisingly no effects were found on fecundity at the individual level. On the contrary, a marginal (albeit non-significant) trend towards increases in fecundity were found in *D. magna* after 7-day exposure to 4–40 mGy/h gamma radiation. It has been proposed that exposure to low-dose ionizing radiation can induce hormesis (beneficial effects) in organisms (Luckey, 2006; Vaiserman, 2010). Reactive oxygen species at low levels are also considered to be important secondary messengers for diverse types of physiological functions (Martin and Barrett, 2002), including oocyte development (Kala et al., 2016) and female reproduction (Agarwal et al., 2005). It is therefore plausible that a marginal increase in ROS can promote reproduction (Agarwal et al., 2005). Additional evidence such as the upregulation of genes involved in the endocrine regulation of reproduction (*Met*) and embryogenesis (*Vtg1* and *Vtg2*) also supported the speculation that exposure to low-dose gamma radiation may positively regulate reproduction. Nevertheless, observations from the histological analysis further indicate that if irradiation proceeds, adverse effects on fecundity may be observed, possibly due to accumulation of ROS and associated oxidative damage to the oocytes. This hypothesis is further supported by two previous studies
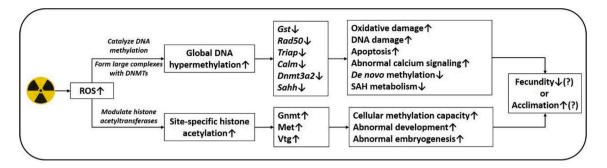
Fig. 9. A hypothetical mechanistic model describing the involvement of epigenetic mechanisms in the stress responses of *Daphnia magna* after a 7-day exposure to low level of gamma radiation.

where significant reduction in fecundity was observed in *D. magna* after exposure to gamma radiation for longer (>14-day) durations (Trijau et al., 2018; Song et al., 2020).

4.5. Mechanistic insights and pathway assembly

The mechanisms of how gamma radiation induces heritable changes in the epigenome in organisms have not been completely understood. However, several potential mechanisms have been proposed to link radiation-induced ROS formation with DNA methylation and histone modification (Fig. 9): a) Superoxide can act as a nucleophile to promote the transferring of methyl from SAM to cytosine, thus inducing DNA methylation without the participation of DNMTs (Afanas'ev, 2014); b) Hydrogen peroxide can form large complexes with DNMTs and facilitate the binding of DNMTs to the promoter of a gene (Wu and Ni, 2015); c) Superoxide anions can induce the expression of DNMTs through the RAS (mitogen-activated protein kinases/extracellular signal-regulated kinase) pathway (MacLeod et al., 1995); d) Hydrogen peroxide can affect the histone acetylation either in a positive or negative manner, depending on the sites and exposure conditions (Rahman et al., 2002; Kang et al., 2003). It is more likely that mechanism a) and b) were involved in ROS-induced DNA methylation in this study, whereas c) was not likely involved as both *Dnmt1* and *Dnmt3a2* showed dose-rate dependent downregulation. As a result of global methylation, genes involved in key defense mechanisms, such as antioxidant defense, DNA repair and calcium signaling were suppressed, potentially leading to enhanced oxidative stress, DNA damage and calcium efflux from the cells (Fig. 9). On the contrary, inhibition of the apoptosis inhibitor promotes apoptosis thus leading to cell death. Increased histone H3K9 acetylation at specific loci can also promote the expression genes which may play important roles in reproduction and embryogenesis (Fig. 9). It is also expected that if the radiation exposure prolongs, adverse effects may be induced due to accumulation of oxidative damage and lack of sufficient defense mechanisms as a result of global methylation of repair genes. However, this hypothetical outcome and associated toxicity pathways still remain to be better understood.

5. Conclusions

The present study has generated new experimental evidence for understanding the involvement of epigenetic mechanisms in the stress responses of adult female *D. magna* after exposure to gamma radiation. The main findings suggest that radiation induced ROS formation and associated oxidative damage may trigger the epigenetic machinery in a dose-rate dependent manner. Exposure to mGy/h level of gamma radiation can cause increased global DNA methylation, histone modifications and subsequent suppression of gene transcription associated with major defense mechanisms, and abnormal induction of genes involved in developmental and reproductive processes. These collectively led to damage at the apical levels such as impairments of the ovary, gut and epidermis, albeit no significant effect was observed for cumulative

fecundity. A hypothetical model was assembled to provide in-depth knowledge on the roles of epigenetic mechanisms in the stress responses of *D. magna*, and may shed light on future investigations for understanding the sensitivity and adaptive strategies of aquatic organisms to ionizing radiation. Future investigations may focus more on the temporal changes of epigenetic markers as well as on linking high-content epigenomic data with phenotypic effects at multiple levels of biological organization to decipher how transition from adaptive responses to damage (toxicity) pathways occurs.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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