Dose-dependent genomic DNA hypermethylation and mitochondrial DNA damage in Japanese tree frogs sampled in the Fukushima Daiichi area

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Abstract

The long-term consequences of the nuclear disaster at the Fukushima Daiichi Nuclear Power Plant (FDNPP) that occurred on March 2011, have been scarcely studied on wildlife. We sampled Japanese tree frogs (*Dryophytes japonicus)*, in a 50 –km area around the FDNPP to test for an increase of DNA damages and variation of DNA methylation level. The ambient dose rate ranged between 0.4 and 2.8 µGy h^{-1} and the total estimated dose rate absorbed by frogs ranged between 0.4 and 4.9 µGy h^{-1}. Frogs from contaminated sites exhibited a dose dependent increase of global genomic DNA methylation level (5-mdC and 5-hmdC) and of mitochondrial DNA damages. Such DNA damages may indicate a genomic instability, which may induce physiological adaptations governed by DNA methylation changes.

This study stresses the need for biological data combining targeted molecular methods and classic ecotoxicology, in order to better understand the impacts on wildlife of long term exposure to low ionizing radiation levels.

Keywords

20 Japanese tree frog- *Dryophytes japonicus* ; Fukushima ; DNA methylation ; mitochondrial
21 DNA damage ; ionizing radiation
22

23

BACKGROUND

The nuclear disaster at the Fukushima Daiichi Nuclear Power Plant (FDNPP) which occurred on March 11 2011 was rated at the highest level (7) on the international nuclear disaster scale. It is considered as the second major accident in the history after the Chernobyl NPP accident, which occurred on April 26, 1986, with vast amounts of artificial radionuclides released into the environment. Ten to fifteen % (400-630 PBq) and 35 % (58 PBq) of the total iodine and cesium isotopes were indeed emitted by the Chernobyl NPP accident, respectively[1,2]. Subsequent dose rates absorbed by non-human biota have been estimated to be high shortly after the accident (e.g. 20 mGy h^{-1} for macroalgae for the northern drainage channel near the FDNPS site) but fell rapidly. However for the late phase after the accident a potential risk of effects on individuals of certain species, especially mammals, may exist in areas of relatively high deposition[2] with some estimated values being above the generic benchmark value of 10 µGy h^{-1} recommended for the protection of ecosystems[3].

Long term consequences of this ionizing radiation exposure on wild animals have been scarcely studied to date. In birds, a decrease of abundance was linked with the increase of ambient[4,5] or absorbed radiological dose rate[6]. In wild monkeys from the forests of Fukushima City, the number of white blood cells was negatively correlated with muscle radiocesium concentration, suggesting an impairment of the immune system[7]. The same authors found also smaller head size for monkey foetuses after the FDNPP accident[8]. Several studies on the endemic pale grass

43 blue butterfly (*Zizeeria maha*)[9-14] reported morphological abnormalities as soon as in May
44 2011, such as deformation of the eyes, wings, palps, and colouration anomalies on the wings,
45 which were not observed at control sites. Interestingly, more severe morphological
46 abnormalities were observed in the two successive generations raised in laboratory controlled
47 conditions[9]. The molecular processes involved in this increase of biological effects through
48 generations are unknown, but the authors hypothesized the implication of genetic or epigenetic
49 mechanisms. In parallel with these results, other studies have found no or weak effects of low
50 doses in radiation contaminated fields, leading to a debate or even a controversy, about effects
51 from low dose exposure in wildlife[15-19].
52 Among the biological impairments inducible by chronic exposure to low levels of ionizing
53 radiation (IR), those targeting DNA are the most susceptible to trigger deleterious effects over
54 generations, if the germline is affected. Such a link between increase of reprotoxicity and DNA
55 damages was demonstrated in a laboratory study where 3 generations of parthenogenetic
56 microcrustaceans have been continuously irradiated with gamma radiation[20]. Increase in
57 mutation rate and DNA damages have also been observed in several organisms (bacteria, plants
58 and animals) from the Chernobyl Exclusion Zone (CEZ)[21-26] but the long-term phenotypic
59 consequences of these mutations are difficult to predict. For example, strongly affected
60 populations of organisms such as pine trees or small mammals seemed to recover rapidly after
61 the Chernobyl NNP accident despite high DNA damages[26], which may indicate the involvement

62 of adaptation processes. The molecular mechanisms involved in these radio-adaptive processes
63 may involve specific genetic selection at loci leading to biochemical changes that underpin
64 adaptation, as already observed for fish[27], birds[22] and small mammals[28] exposed to
65 radionuclides. Although this selection phenomenon is largely observed, some data have shown
66 that rapid adaptation towards radionuclides in organisms cannot be explained only by increased
67 mutation rates, but could also be due to non-genetic changes in the activity of functional genes
68 that reveal the action of epigenetic mechanisms on gene structure and regulation[26, 29].
69 Epigenetic changes are defined as 'the study of mitotically and/or meiotically heritable
70 changes in gene function that cannot be explained by changes in gene sequence'[30]. The three
71 main identified epigenetic mechanisms are histone modification, non-coding RNA and DNA
72 methylation, this latter being the most studied epigenetic mechanism[31]. DNA methylation is
73 involved in several biological functions such as development, regulation of gene expression[32,33],
74 chromosomal stability[34] and organisms capacity to cope with environmental stress[35]. Studies
75 conducted on pine trees exposed in Chernobyl have shown that chronic exposure to low levels
76 of IR increase genomic DNA methylation and mutagen resistance, suggesting that DNA
77 methylation plays a role in DNA molecule stabilization[36]. DNA methylation is also involved in
78 DNA repair mechanisms following radiation-induced damage by exposure to high levels of
79 IR[37] (absorbed doses from 5 to 60 Gy).

In this general context, we hypothesize here that organisms exposed to IR in Fukushima may present an increase of DNA damages and a variation of DNA methylation levels. We therefore sampled an amphibian, the Japanese tree frog (*Dryophytes japonicus*), chosen because they may be exposed to high levels of radioactivity from various environmental sources (e.g. food, tree, water, sediment, soil). In addition, frogs are among the 12 Reference Animals and Plants of the International Commission on Radiological Protection. Tree frogs were sampled in contaminated area of the Fukushima Prefecture at three contaminated sites (with ambient dose rates up to 2.8 µGy h^{-1}) and at one control site (0.38 µGy.h^{-1}). We assessed the biological effect induced by the chronic exposure to FDNPP releases through the quantification of both mitochondrial DNA (Mt DNA) damage and global genomic DNA methylation level. In parallel, based on a quantification of activity concentrations in different living environment (water, sediment, soil) and in the organisms, we estimated the internal, external and total dose rate (TDR) for each frog, in order to evaluate the contribution of internal dose rate to the total dose rate. Using linear mixed effect model analyses, we tested for a relationship between TDR, age, body condition index (BCI) of each frog and the observed biological impairments.

MATERIALS AND METHODS

***In situ* sampling.** The *in situ* sampling campaign was carried out in 2013 during the Japanese tree frog breeding season (June and July). Four sites were sampled (Fig. 1), one with a low ambient level (mean±standard deviation) of radioactivity (Nihonmatsu town, control site: 0.38±0.16 µGy h^{-1}) and three contaminated sites near Iitate town R1 (2.76±0.86 µGy h^{-1}), R2 (2.67±0.89 µGy h^{-1}) and R3 (2.63±0.76 µGy h^{-1}). Ten calling male frogs were sampled in each site and dissected to collect the tibia muscle. This tissue was chosen because it is composed by similar cell types (supposed to have rather homogenous DNA methylation pattern) and provides a sufficient amount of biological material. Collected tissue was quick frozen and stored at -80 °C until further analysis. Whole frogs and samples of the surrounding media (water, sediment, soil) were taken to determine activity concentrations and calculate internal and external radiological doses.

The complete measures of radionuclide activity concentrations and dose rate measurement and calculation are provided in Table S1 and the procedures used to capture frogs in the Supporting information file available online.

Estimation of the total dose rate (TDR). The total dose rate is the sum of internal dose rate (absorbed from food, soil ingestion etc) and external dose rate. These components of the total dose rates are calculated using Dose Coefficients (DCs), which enable to convert activity concentrations (Bq kg^{-1}) into dose rate (Gy per unit of time). DCs were calculated both for

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117 internal and external exposure of frogs using the EDEN v3 software, considering shapes,
118 element composition of organisms (i.e. individual frog) and their environmental exposure
119 sources (i.e. water and soil) for the two radionuclides detected in frog samples (^{134}Cs, ^{137}Cs)
120 (Figure 2, Table S1). These DCs were then combined to radionuclide activity concentrations
121 measured in the collected samples (i.e. whole body, water, soil and sediment samples, expressed
122 in Bq per unit of mass or volume) to estimate the TDR absorbed by each frog. The individual
123 TDR estimated for each frog is provided in Figure 2 and Table S2. The whole process is detailed
124 in the Supporting information file available online.
125
126 **Estimation of the body condition index (BCI)**. Body condition is a scale of the energy
127 stores, and has important implications for fitness. It is frequently estimated as a body condition
128 index (BCI) using length and mass measurements, because it is itself difficult to measure
129 directly. The body size of each frog was measured from the snout to the vent using a digital
130 calliper, rounded to the nearest 0.1 mm. The body mass was measured with a digital scale and
131 rounded to the nearest 0.1 g. The BCI was estimated as the residuals of the log-log least-squares
132 linear regression of body mass against snout-vent length. Using this method, a positive value
133 of BCI indicates a fat animal and a negative value indicates a skinny animal.
134

Skeletochronology analysis. The skeletochronological analysis is used for age calculation using the tibiofibular, considered as one of the best long bones to measure age in hylid frogs. It was performed following previously published procedures[38]. Briefly, muscle and skin were removed and the bone decalcified in 4% (v/v) nitric acid for 1-4 h (depending on the size of the bone), and washed in running tap water for 12 h. Cross sections of the diaphyseal region of the bone were obtained using a freezing microtome (Microtom heidelberg HM330), stained with Ehrlich's haematoxylin, and analysed with a light microscope (Olympus CX40). Since annual periodicity in lines of arrested growth (LAGs) has been previously demonstrated[39], we used these marks to estimate the age of our individuals (in years). The results corresponding to BCI and age for each sampling site are presented in Table S2.

Genomic DNA extraction. Genomic DNA was extracted using the DNAeasy Blood and Tissue Kit (Qiagen, Les Ulis, France), according to the manufacturer's protocol, from the tibia muscle of frogs (n=10). Following the extraction, 1 µL of sample was used to determine DNA concentration and purity (ratio 260/280 ~ 1.8 and 260/230 between 1.8 - 2.2) using the NanoDrop 2000 (Thermo Scientific, Villebon sur Yvette, France).

Genome-wide DNA methylation measured with HPLC–MS/MS. The 2′-deoxycytosine (dC), 5-methyl-2′-deoxycytosine (5-mdC), and 5-hydroxymethyl-2′-deoxycytosine (5-hmdC)

nucleosides were detected and quantified with mass spectrometry in the positive ionization mode using the so-called multiple reaction monitoring mode (mrm) with transitions m/z = 228 → m/z = 112, m/z =242 → m/z = 126, and m/z = 258 → m/z = 142, respectively[40]. These measurements were made with a TSQ Quantum Ultra electrospray ionization tandem mass spectrometer (Thermo Fisher Scientific Inc., Illkirch, France). The conditions for DNA digestion were similar to those described previously[41] and provided in the Supporting information file available online. The levels of methylated cytosines (expressed as the percentage of 5-mdC relative to dC) and hydroxymethylated cytosines (expressed as the percentage of 5-hmdC relative to 5-mdC) measured in each sampling site are presented in Table S2 and Figure 2.

Assessment of mitochondrial DNA damage. The method chosen to assess DNA damages is focused on mitochondrial DNA damages. It enables to measure DNA damages in frozen samples, in opposition to other methods such as the classical COMET assay which is better suited to analyse genotoxicity in fresh samples. It is a PCR-based technic which provides the average number of lesions in the Mt DNA[42,43]. Mt DNA is used instead of nuclear DNA as the tree frog genome is not fully sequenced and Mt DNA is the best known DNA sequence in this species. However, it must be kept in mind that mitochondrial DNA does not have the same level of DNA damage repair as genomic DNA, hence DNA damage is more prevalent in this

173 organelle. To measure Mt DNA damage, the efficiency of synthesis of two PCR products was
174 analysed using : (i) a long fragment sizing 10.7 kb, called mitochondrial long fragment (MtLF);
175 (ii) a short fragment sizing 180 bp, called mitochondrial short fragment (MtSF, comprised in
176 the MtLF's sequence, see Fig. S2). Briefly, two sets of PCRs were run on the same DNA
177 sample, the first PCR targeting the MtLF and the second the MtSF. Following PCRs, samples
178 were diluted and incubated with Picogreen® (Life Technologies, Saint Aubin, France) to allow
179 the quantification of the PCRs efficiency by fluorescence. Then, for each biological sample, the
180 fluorescence data obtained during the three analytical replicates were used to calculate the
181 normalized average number of lesions over 10.7 kbp. The results obtained for each sampling
182 site are presented in Figure 2 and Table S2. The complete protocol used to perform the analysis
183 of the MtDNA damage is provided in the Supporting information file available online.
184
185 **Statistical analyses.** All statistical comparisons between sampling sites were performed
186 using 10 biological replicates per group and using the R software version 3.2.4. The statistical
187 differences presented in the Table 1, corresponding to the comparison of the results obtained
188 between each sampling sites, were obtained using the following tests. To evaluate the dose-
189 response relationship, we proceeded to statistical modelling. A linear mixed effects model was
190 used considering the TDR, the age or the BCI as fixed effects to evaluate their influence on the
191 biological impacts observed (i.e. mitochondrial DNA (Mt DNA) damage, and methylation

192 level). Additionally, to take into account the random variability across the sampling sites, these
193 sites were modelled as random effect on the intercept. The results of these analyses are
194 presented in Table 1. The complete protocol used to perform the statistical modelling is
195 provided in the Supporting information file available online.
196
197 RESULTS
198
199 Radiocesium isotopes (^{134}Cs and ^{137}Cs) were detected in frogs sampled in the FDNPP
200 accident impacted area (Figure 2). The highest concentrations were found in frogs of the R3
201 site, with values of activity concentrations going up to 13 and 7.4 Bq g^{-1} w.w. respectively for
202 ^{137}Cs and ^{134}Cs (wet weight, whole body) (Table S2). The average frog's age was of 3 years at
203 each site (range of 2-5 years) (Table S2).
204
205 **Internal DR is the main contributor to the TDR received by frogs.** The mean (±SD) TDR
206 at the contaminated sites was higher than the TDR at control site (control site: 0.41±0.04 µGy
207 h^{-1} ; site R1: 2.4±0.54 µGy h^{-1} ; site R2: 4.9±0.38 µGy h^{-1} ; site R3: 4.3±2.40 µGy h^{-1} (Figure
208 2, Table S2)). Additionally, within each sampling site the internal DR represented the highest
209 contribution to the TDR (58, 67, 90 and 83 % in frogs from the control site, contaminated sites
210 R1, R2 and R3, respectively) (Table S2).

Hypermethylation of genomic DNA. We quantified the average global levels of both the methylated (5-mdC) and the hydroxymethylated (5-hmdC) forms of cytosine in frogs' genomic DNA. An increase of the average level of 5-mdC (26 to 31 %) was observed in frogs from the three contaminated sites as compared to the control site (Figure 2). Similarly, a doubling of the level of 5-hmdC was observed in frogs from all three contaminated sites as compared to levels from the control site (1.77, 1.78 and 1.75 % in frogs from sites R1, R2 and R3, respectively, versus 0.94 % in frogs from the control site).

Increase of Mt DNA damage. In frogs from the control site, the average number of lesions to the Mt DNA was of 0.5 lesions/10.7 kbp (Figure 2). The number of lesions to DNA increased in frogs from the contaminated sites up to 4 times as compared to controls (3.5, 3.7 and 4.2 fold higher than in controls for sites R1, R2 and R3, respectively).

Correlation between TDR and biological factors on one hand, and global DNA methylation and Mt DNA damages. The relationships between the frog's TDR and the observed biological responses (DNA methylation and damage) were the most significant among fixed effects (Table 1). The results indicated a significant positive correlation between the frogs' TDR and both the average level of 5-hmdC ($p_{adj} < 0.001$) and the level of Mt DNA damages

(p_{adj} < 0.001). To the contrary, for 5-mdC, the relationship with the frogs' TDR was not significant (p_{adj} = 0.0748). We also observed a significant positive relationship between the average level of 5-hmdC and the frogs' age (p_{adj} < 0.001). Finally, a significant positive relationship was detected between the BCI and the level of 5-mdC (p_{adj} = 0.0149).

DISCUSSION

The radiocesium activity concentrations measured in frogs in this study are of a few Bq g^{-1} w.w. These values are in the range of activity concentrations measured in 5 frog species sampled in the Fukushima Prefecture in August and September 2012 at similar air dose rate[44]. In addition, the Concentration Ratio estimated between water and frogs ($CR_{frog\text{-}water}$) (Table S1) in our study, ranges from 400 to 1400 L kg^{-1} w.w., which is in adequacy with the mean $CR_{whole\ organism\text{-}water}$ value estimated at 580 L kg^{-1} w.w. for adult frogs from the Fukushima Prefecture from April 2012 to April 2016[45].

The mean TDRs absorbed by frogs in the different sampling sites were all lower than the 10 µGy h^{-1} generic screening value that below which 95% of all species should be protected from ionising radiation. The calculation of the TDR highlighted a major contribution of the internal exposure, representing from 58 % to 90% of TDR in contaminated sites. In addition, the calculated TDR is higher than the ambient dose rate. This is an important result since most of the studies carried out in Chernobyl and Fukushima area are based on the ambient DR to assess

biological effects[4,5,9-14,24,46], which may under-estimate the total dose rate absorbed by wildlife. As already underlined in other works, such comprehensive calculation of total absorbed dose rates is important to assess thresholds of toxicity in wildlife and compare them to toxicity thresholds or benchmarks obtained under laboratory control conditions[3,6].

This study focusing on Japanese tree frogs exposed *in situ* following the FDNPP accident shows significant positive correlations between TDR, mitochondrial DNA damages and DNA methylation levels in frogs. These molecular responses may be more sensitive than responses observed at higher biological organisation levels. Indeed, in a previous study on five frog species (*Rana japonica*, *R. ornativentris*, *R. tagoi tagoi*, *Pelophylax porosus porosus*, and *Dryophytes japonicus*) sampled in the radiocontaminated area around Fukushima, the histological examination of ovaries and testes using conventional microscopy, failed to detect any overt aberrations in the morphology of germ cells in the testes and ovaries of frogs[44]. To the contrary, chromosomal aberrations and malformed cells in bone marrow were observed in *R. temporaria* L. in radiocontaminated areas in Belarus in a period of 3-7 years after the Chernobyl accident[26]. In laboratory experiments, chromosomal aberrations, a decreased reproductive capacity and an increase in male number were also observed in 4 generations of frogs produced from gametes exposed to 1.5-3.5 Gy of X-rays[47]. These different results could

be explained by several factors, including the different levels (dose and dose rates) and types of radiocontaminants studied.

Our study highlights a dose-dependent correlation between TDR and 5-hmdC levels, and a strong but not statistically significant trend to cytosine hypermethylation in radiocontaminated area (Table 1). 5hmC is a product of 5mC oxidation during the process of DNA demethylation, but it also plays an important role in gene expression, pluripotency of stem cells, stress response, disease progression and aging[48]. As such, it is interesting to note that there is also a trend for a positive correlation between 5-hmC and age (Table 1, p=0.0503), underlining the reliability of 5hmC as biomarker of aging[48]. However, as aged frogs may also have been exposed to higher total doses just after the FDNPP accident, it cannot be ruled out that this increase of 5hmC could also reflect the past exposure of frogs. Nonetheless, changes in global DNA methylation have already been observed in several organisms exposed to IR, such as plants[29, 48-52] or fish[53]. Hence, we hypothesize that the changes in methylation levels observed in our data could be implicated in a physiological response or a phenotypic plasticity leading to adaptation of frogs exposed chronically to IR.

At the whole-genome scale, hypermethylation of cell cycle and detoxification gene promoters have been observed in offspring of irradiated humans long term after radiation exposure[54] while the hypomethylation of gene coding for stress protein *hsp70* was observed in offspring of

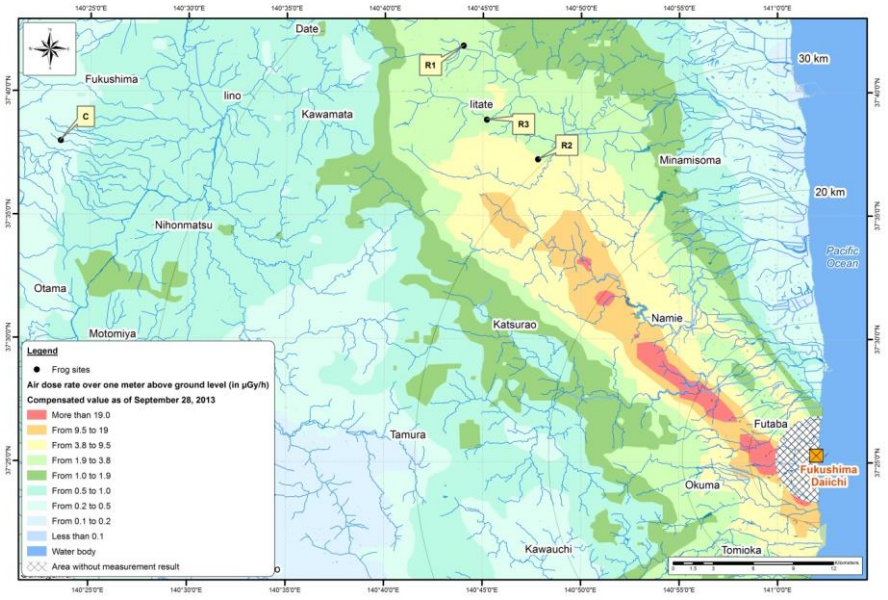
286 irradiated microcrustaceans[55]. DNA methylation is used to predict adverse effects in terms of
287 tumour recurrence as some gene-specific DNA methylations are predictors of response to
288 radiotherapy[56]. Such a whole-genome study of DNA methylation would be useful to better
289 understand the mechanisms involved in the response of Japanese tree frogs to IR.

290 The mechanisms of radiation-induced changes in DNA methylation remain largely unknown
291 but some hypotheses are proposed by Miousse et al. (2018)[56] : the most plausible scenario is an
292 effect of IR on DNA methyltransferases, as decreases of DNA methyltransferases' levels of
293 mRNA and proteins have been observed. Additionally, IR has been shown to affect several
294 microRNAs that specifically target DNA methyltransferases[57]. Further studies at higher
295 biological organisation levels are needed in order to demonstrate that these processes do occur
296 on wild animals and contribute to phenotypical changes or adaptive process in contaminated
297 area.

298 Our results show a significant positive correlation between the frogs' TDR, 5-hmdC but also
299 the average number of lesions to the Mt DNA (Table 1). Epigenetic mechanisms and genome
300 stability are closely linked[58]. Epigenetic marks such as DNA methylation are involved in several
301 aspects that can enhance genetic stability (e.g. transcriptional repression of repetitive elements
302 by DNA methylation preventing homologous recombination or chromatin condensation
303 induced by DNMTs (DNA methyltransferases)). This favourable role of DNA methylation in
304 increasing genomic DNA stability[59] has been proposed as a potential mechanism for radiation

adaptation in plants exposed at Chernobyl[36,60] or to environmental stress in general[61]. There is likely an "epigenetic advantage" to phenotypic switching by epigenetic inheritance, rather than by gene mutation, as an epigenetically-inherited trait can arise simultaneously in many individuals[62]. However, genomic destabilization can also occur through DNA hypermethylation of DNA repair genes, leading to reduced expression of genes required for genetic stability, which has been evidenced in cancer.

While the issue of inheritance of epigenetic characters in humans is still a matter of controversy, the transmission of acquired states can occur in plants and animals[62,63]. As such, it may contribute to evolution[61], underlining the importance of integrating the study of (epi)genetic molecular markers in ecological risk assessment, to assess for effects of long term chronic exposure to IR.



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319 **Figure 1**. Map of ambient dose rate (µGy h^{-1}) in the Fukushima area during the sampling period

320 (June and July 2013). The location of the four sites (control site C, contaminated sites R1, R2

321 and R3) are indicated on the map by the black dots.

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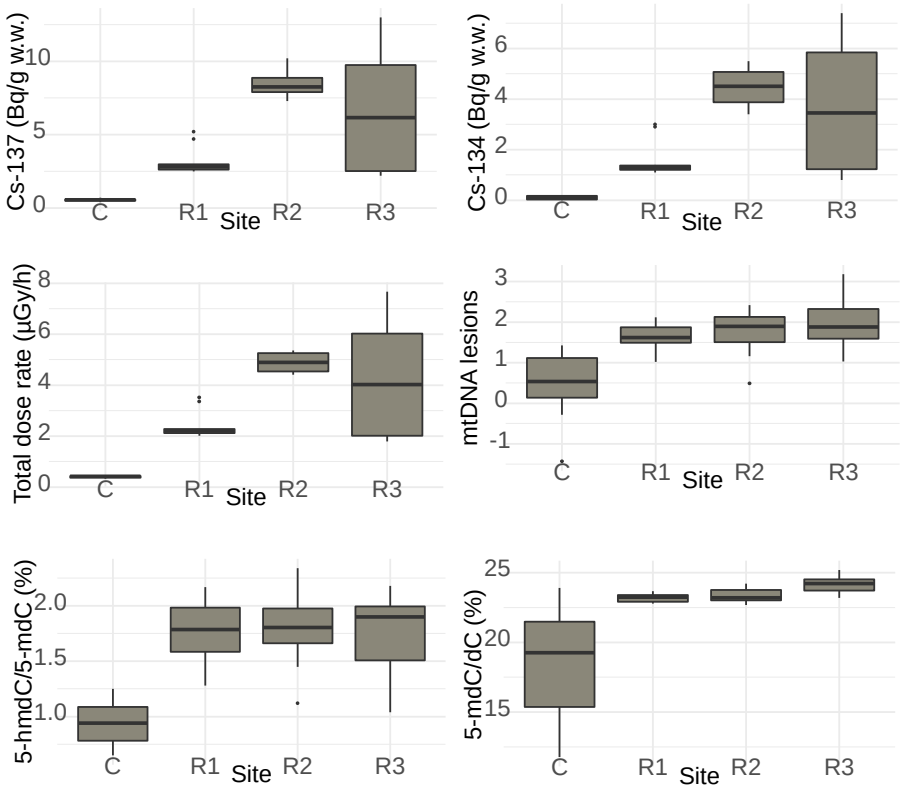

Figure 2. Boxplots of Cs-137 and Cs-134 activity concentrations, total dose rates, mitochondrial DNA lesions and DNA methylation in frogs from the four sites (control site C, contaminated sites R1, R2 and R3, n=10).

Table 1: linear mixed-effects models for methylated cytosine, hydroxymethylated cytosine and mitochondrial DNA (MtDNA) lesions.

Model	**Factor**	**Estimate**	**Standard Error**	**df**	***t*-value**	**P value**
Methylated cytosine (5-mdC)	**Fixed effects**					
	(Intercept)	23.023434	0.4621987	26	49.81285	**<0.0001**
	Age	-0.038076	0.1067110	26	-0.35682	0.9932
	Body Condition Index	0.369256	0.1275974	26	2.89391	**0.0149**
	Total Dose Rate	0.715193	0.3085177	26	2.31816	0.0773
Hydroxymethylated cytosine (5-hmdC)	**Fixed effects**					
	(Intercept)	1.5273975	0.06559786	26	23.284257	**<0.001**
	Age	0.1079019	0.04391403	26	2.457117	**0.0513**
	Body Condition Index	0.0819409	0.06029821	26	1.358928	0.4924
	Total Dose Rate	0.2967815	0.05422058	26	5.473595	**<0.001**
MtDNA lesions	**Fixed effects**					
	(Intercept)	1.4754382	0.1214342	26	12.150109	**<0.0001**
	Age	0.0323862	0.1246704	26	0.259774	0.998040
	Body Condition Index	0.2143823	0.1450854	26	1.477629	0.440104
	Total Dose Rate	0.5287266	0.1352902	26	3.908093	**<0.001**

ASSOCIATED CONTENT

Supporting information. The protocols used to capture frogs and determine radionuclides concentrations, to calculate the DCs and TDRs, the HPLC-MS/MS and Mt DNA damage methods are provided in supporting information. Raw data corresponding to individual internal, external and total DRs, HPLC-MS/MS and Mt DNA damage are also provided in supplementary information.

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