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

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1 Dose-dependent genomic DNA hypermethylation and  
2 mitochondrial DNA damage in Japanese tree frogs  
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4

5 Abstract

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

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

18

19 Keywords

20 Japanese tree frog- *Dryophytes japonicus* ; Fukushima ; DNA methylation ; mitochondrial  
21 DNA damage ; ionizing radiation  
22  
23

24 BACKGROUND

25 The nuclear disaster at the Fukushima Daiichi Nuclear Power Plant (FDNPP) which occurred  
26 on March 11 2011 was rated at the highest level (7) on the international nuclear disaster scale.  
27 It is considered as the second major accident in the history after the Chernobyl NPP accident,  
28 which occurred on April 26, 1986, with vast amounts of artificial radionuclides released into  
29 the environment. Ten to fifteen % (400-630 PBq) and 35 % (58 PBq) of the total iodine and  
30 cesium isotopes were indeed emitted by the Chernobyl NPP accident, respectively<sup>1,2</sup>.  
31 Subsequent dose rates absorbed by non-human biota have been estimated to be high shortly  
32 after the accident (e.g. 20 mGy h<sup>-1</sup> for macroalgae for the northern drainage channel near the  
33 FDNPS site) but fell rapidly. However for the late phase after the accident a potential risk of  
34 effects on individuals of certain species, especially mammals, may exist in areas of relatively  
35 high deposition<sup>2</sup> with some estimated values being above the generic benchmark value of 10  
36  $\mu\text{Gy h}^{-1}$  recommended for the protection of ecosystems<sup>3</sup>.  
37 Long term consequences of this ionizing radiation exposure on wild animals have been scarcely  
38 studied to date. In birds, a decrease of abundance was linked with the increase of ambient<sup>4,5</sup> or  
39 absorbed radiological dose rate<sup>6</sup>. In wild monkeys from the forests of Fukushima City, the  
40 number of white blood cells was negatively correlated with muscle radiocesium concentration,  
41 suggesting an impairment of the immune system<sup>7</sup>. The same authors found also smaller head  
42 size for monkey foetuses after the FDNPP accident<sup>8</sup>. Several studies on the endemic pale grass

43 blue butterfly (*Zizeeria maha*)<sup>9-14</sup> 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<sup>9</sup>. 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<sup>15-19</sup>.

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<sup>20</sup>. 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)<sup>21-26</sup> 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<sup>26</sup>, 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<sup>27</sup>, birds<sup>22</sup> and small mammals<sup>28</sup> 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<sup>26, 29</sup>.

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’<sup>30</sup>. 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<sup>31</sup>. DNA methylation is  
73 involved in several biological functions such as development, regulation of gene expression<sup>32,33</sup>,  
74 chromosomal stability<sup>34</sup> and organisms capacity to cope with environmental stress<sup>35</sup>. 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<sup>36</sup>. DNA methylation is also involved in  
78 DNA repair mechanisms following radiation-induced damage by exposure to high levels of  
79 IR<sup>37</sup> (absorbed doses from 5 to 60 Gy).

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

95

96 MATERIALS AND METHODS

97

98 ***In situ* sampling.** The *in situ* sampling campaign was carried out in 2013 during the Japanese  
99 tree frog breeding season (June and July). Four sites were sampled (Fig. 1), one with a low  
100 ambient level (mean±standard deviation) of radioactivity (Nihonmatsu town, control site:  
101  $0.38\pm 0.16 \mu\text{Gy h}^{-1}$ ) and three contaminated sites near Iitate town R1 ( $2.76\pm 0.86 \mu\text{Gy h}^{-1}$ ), R2  
102 ( $2.67\pm 0.89 \mu\text{Gy h}^{-1}$ ) and R3 ( $2.63\pm 0.76 \mu\text{Gy h}^{-1}$ ). Ten calling male frogs were sampled in each  
103 site and dissected to collect the tibia muscle. This tissue was chosen because it is composed by  
104 similar cell types (supposed to have rather homogenous DNA methylation pattern) and provides  
105 a sufficient amount of biological material. Collected tissue was quick frozen and stored at -80  
106 °C until further analysis. Whole frogs and samples of the surrounding media (water, sediment,  
107 soil) were taken to determine activity concentrations and calculate internal and external  
108 radiological doses.

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

112  
113 **Estimation of the total dose rate (TDR).** The total dose rate is the sum of internal dose rate  
114 (absorbed from food, soil ingestion etc) and external dose rate. These components of the total  
115 dose rates are calculated using Dose Coefficients (DCs), which enable to convert activity  
116 concentrations ( $\text{Bq kg}^{-1}$ ) into dose rate (Gy per unit of time). DCs were calculated both for

Commenté [A1]: Jean-Marc, peux-tu me confirmer STP que c'est correct ? ou sinon mettre plutôt le nom des districts...

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 ( $^{134}\text{Cs}$ ,  $^{137}\text{Cs}$ )  
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

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

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

150

151

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

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

164

165 **Assessment of mitochondrial DNA damage.** The method chosen to assess DNA damages  
166 is focused on mitochondrial DNA damages. It enables to measure DNA damages in frozen  
167 samples, in opposition to other methods such as the classical COMET assay which is better  
168 suited to analyse genotoxicity in fresh samples. It is a PCR-based technic which provides the  
169 average number of lesions in the Mt DNA<sup>42,43</sup>. Mt DNA is used instead of nuclear DNA as the  
170 tree frog genome is not fully sequenced and Mt DNA is the best known DNA sequence in this  
171 species. However, it must be kept in mind that mitochondrial DNA does not have the same level  
172 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 ( $^{134}\text{Cs}$  and  $^{137}\text{Cs}$ ) 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<sup>-1</sup> w.w. respectively for  
202  $^{137}\text{Cs}$  and  $^{134}\text{Cs}$  (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 ( $\pm$ SD) TDR  
206 at the contaminated sites was higher than the TDR at control site (control site:  $0.41\pm 0.04$   $\mu\text{Gy}$   
207  $\text{h}^{-1}$ ; site R1:  $2.4\pm 0.54$   $\mu\text{Gy h}^{-1}$ ; site R2:  $4.9\pm 0.38$   $\mu\text{Gy h}^{-1}$ ; site R3:  $4.3\pm 2.40$   $\mu\text{Gy h}^{-1}$  ([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](#)).

211

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

219

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

224

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

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

234

## 235 DISCUSSION

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

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

249 biological effects<sup>4,5,9-14,24,46</sup>, which may under-estimate the total dose rate absorbed by wildlife.

250 As already underlined in other works, such comprehensive calculation of total absorbed dose

251 rates is important to assess thresholds of toxicity in wildlife and compare them to toxicity

252 thresholds or benchmarks obtained under laboratory control conditions<sup>3,6</sup>.

253

254 This study focusing on Japanese tree frogs exposed *in situ* following the FDNPP accident

255 shows significant positive correlations between TDR, mitochondrial DNA damages and DNA

256 methylation levels in frogs. These molecular responses may be more sensitive than responses

257 observed at higher biological organisation levels. Indeed, in a previous study on five frog

258 species (*Rana japonica*, *R. ornativentris*, *R. tagoi tagoi*, *Pelophylax porosus porosus*, and

259 *Dryophytes japonicus*) sampled in the radiocontaminated area around Fukushima, the

260 histological examination of ovaries and testes using conventional microscopy, failed to detect

261 any overt aberrations in the morphology of germ cells in the testes and ovaries of frogs<sup>44</sup>. To

262 the contrary, chromosomal aberrations and malformed cells in bone marrow were observed in

263 *R. temporaria* L. in radiocontaminated areas in Belarus in a period of 3-7 years after the

264 Chernobyl accident<sup>26</sup>. In laboratory experiments, chromosomal aberrations, a decreased

265 reproductive capacity and an increase in male number were also observed in 4 generations of

266 frogs produced from gametes exposed to 1.5-3.5 Gy of X-rays<sup>47</sup>. These different results could

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

269

270 Our study highlights a dose-dependent correlation between TDR and 5-hmC levels, and a  
271 strong but not statistically significant trend to cytosine hypermethylation in radiocontaminated  
272 area (Table 1). 5hmC is a product of 5mC oxidation during the process of DNA demethylation,  
273 but it also plays an important role in gene expression, pluripotency of stem cells, stress response,  
274 disease progression and aging<sup>48</sup>. As such, it is interesting to note that there is also a trend for a  
275 positive correlation between 5-hmC and age (Table 1,  $p=0.0503$ ), underlining the reliability of  
276 5hmC as biomarker of aging<sup>48</sup>. However, as aged frogs may also have been exposed to higher  
277 total doses just after the FDNPP accident, it cannot be ruled out that this increase of 5hmC  
278 could also reflect the past exposure of frogs. Nonetheless, changes in global DNA methylation  
279 have already been observed in several organisms exposed to IR, such as plants<sup>29, 48-52</sup> or fish<sup>53</sup>.  
280 Hence, we hypothesize that the changes in methylation levels observed in our data could be  
281 implicated in a physiological response or a phenotypic plasticity leading to adaptation of frogs  
282 exposed chronically to IR.

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

286 irradiated microcrustaceans<sup>55</sup>. 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<sup>56</sup>. 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)<sup>56</sup>: 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<sup>57</sup>. 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.

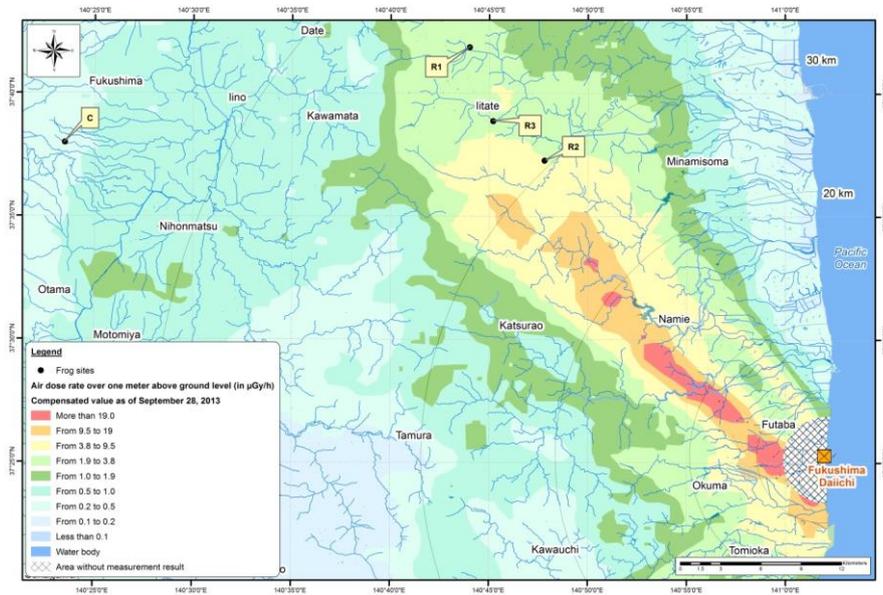
298 Our results show a significant positive correlation between the frogs' TDR, 5-hmdC but also  
299 the average number of lesions to the Mt DNA ([Table 1](#)). Epigenetic mechanisms and genome  
300 stability are closely linked<sup>58</sup>. Epigenetic marks such as DNA methylation are involved in 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<sup>59</sup> has been proposed as a potential mechanism for radiation

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

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

316

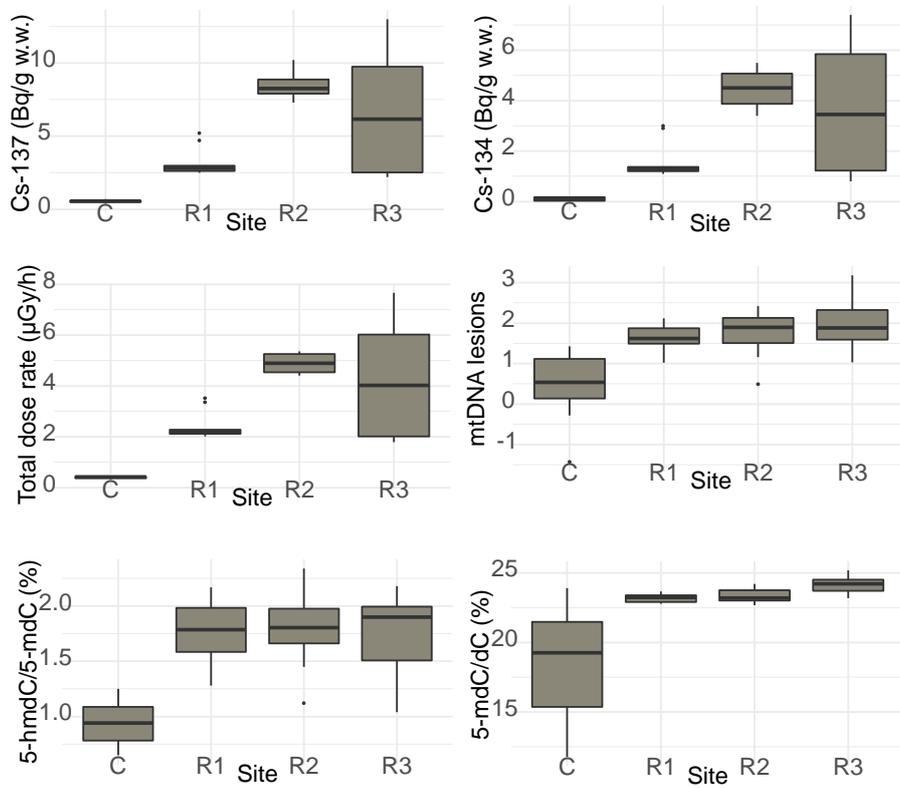
317



318  
 319 **Figure 1.** Map of ambient dose rate ( $\mu\text{Gy h}^{-1}$ ) 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.

322

323



324  
 325 Figure 2. Boxplots of Cs-137 and Cs-134 activity concentrations, total dose rates, mitochondrial  
 326 DNA lesions and DNA methylation in frogs from the four sites (control site C, contaminated  
 327 sites R1, R2 and R3, n=10).

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

<b>Model</b>	<b>Factor</b>	<b>Estimate</b>	<b>Standard Error</b>	<b>df</b>	<b>t-value</b>	<b>P value</b>
Methylated cytosine (5-mdC)	<b>Fixed effects</b>					
	(Intercept)	23.023434	0.4621987	26	49.81285	<b>&lt;0.0001</b>
	Age	-0.038076	0.1067110	26	-0.35682	0.9932
	Body Condition Index	0.369256	0.1275974	26	2.89391	<b>0.0149</b>
	Total Dose Rate	0.715193	0.3085177	26	2.31816	0.0773
Hydroxymethylated cytosine (5-hmdC)	<b>Fixed effects</b>					
	(Intercept)	1.5273975	0.06559786	26	23.284257	<b>&lt;0.001</b>
	Age	0.1079019	0.04391403	26	2.457117	<b>0.0513</b>
	Body Condition Index	0.0819409	0.06029821	26	1.358928	0.4924
	Total Dose Rate	0.2967815	0.05422058	26	5.473595	<b>&lt;0.001</b>
MtDNA lesions	<b>Fixed effects</b>					
	(Intercept)	1.4754382	0.1214342	26	12.150109	<b>&lt;0.0001</b>
	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	<b>&lt;0.001</b>

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## 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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