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Effects of gamma ionizing radiation exposure on *Danio rerio* embryo-larval stages - comparison with tritium exposure

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22

23 **Abstract**

24 The objective was to investigate the effects of ionizing radiation induced in zebrafish early
25 life stages by coupling responses obtained at the molecular (genotoxicity, ROS production,
26 gene expression) and phenotypic (tissue alteration, embryo-larval development) levels. Here
27 we present results obtained after exposure of 3 hpf larvae to 10 days of gamma irradiation at
28 3.3×10^1 , 1.3×10^2 and 1.2×10^3 $\mu\text{Gy/h}$, close to and higher than the benchmark for protection of
29 ecosystems towards ionizing radiations of 10^1 $\mu\text{Gy/h}$. Dose rates used in these studies were
30 chosen to be in the ‘derived consideration reference level’ (DCRL) for gamma irradiation
31 where deleterious effects can appear in freshwater fish. Also, these dose rates were similar to
32 the ones already tested on tritium (beta ionizing radiation) in our previous work, in order to
33 compare both types of ionizing radiation. Results showed that gamma irradiation did not
34 induce any effect on survival and hatching. No effect was observed on DNA damages, but
35 ROS production was increased. Muscle damages were observed for all tested dose rates,
36 similarly to previous results obtained with tritium (beta ionizing radiation) at similar dose
37 rates. Some molecular responses therefore appeared to be relevant for the study of gamma
38 ionizing radiation effects in zebrafish.

39

40 **Keywords**

41 Gamma irradiation; tritium; zebrafish, *Danio rerio*; embryo-larvae stages; molecular
42 responses.

43

44

45

46 1. Introduction

47 Recommendations from international organizations underline the need for a radiological
48 protection of the environment towards deleterious effects of ionizing radiation (IAEA 2014,
49 ICRP 2007). They aim to guarantee for the present and future human generations (i) a durable
50 use of natural ecosystems for agricultural needs, forest exploitation, fishing, tourism; (ii) to
51 prevent the effects of ionizing radiations on flora and fauna. In line with that, considering that
52 the status of ecosystem can impact human health at long-term, European Union supported a
53 politic protecting the environment from harmful effects of ionizing radiation (directive
54 2013/59/Euratom). Several approaches are proposed to evaluate the potential effects of
55 ionizing radiation on ecosystems. The system recommended by the ICRP is based on a range
56 of dose rates called the 'Derived Consideration Reference Levels' (DCRL) for 'Reference
57 Animals and Plants' (RAP), in which deleterious effects to specific wildlife groups (eg.
58 Pelagic fish, Crustacean...) are considered (ICRP 2008). The ERICA and PROTECT projects
59 enabled to define a benchmark value or reference no effect dose rate (10 $\mu\text{Gy/h}$) for
60 ecosystems (considered as generic) exposed chronically to ionizing radiation (Garnier-
61 Laplace et al. 2010).

62 In the environment, natural populations are exposed to different radionuclides, emitting
63 different kinds of radiations (α , β or γ). The effects of ionizing radiation depend on the type of
64 radiation, the absorbed dose, the duration of exposure but also on the sensitivity of the
65 exposed organism or organ. Therefore, it is necessary to take into account these variations in
66 biological efficiency in the environmental risk assessment of ionizing radiations. To this end,
67 the concept of Relative Biological Effectiveness (RBE) was developed. RBE corresponds to
68 the ratio between the dose of reference radiation (γ or X) needed to produce a level of effect
69 and the dose of the studied radiation (α or β) needed to produce the same kind of effect
70 (similar mode of action) and the same level of effect on a common endpoint (Dobson and
71 Kwan 1978, Hall et al. 1967). RBE depends on several factors, including the type of irradiated
72 cell, the effect considered and the distribution (homogenous or not) of radionuclides. In the
73 area of radioprotection of non-human species, there is no consensus on the choice of RBE
74 values, due to a lack of data, notably in the domain of low dose rates. Therefore, on the basis
75 of literature data, a RBE of 5 was proposed for alpha emitters, but with values ranging
76 between 1 and 377, showing the complexity to precisely determine the biological efficiency
77 of a radiation (Chambers et al. 2006). For tritium, the literature reports a controversy
78 concerning the RBE value (Higley et al. 2012). Therefore, work are needed to better

79 characterize tritium effects and mode of action on non-human species and to compare to a
80 reference radiation such as gamma ionizing radiation.

81 Fish represent a relevant vertebrate model to study effects of ionizing radiation, as
82 they are considered to be among the most radiosensitive animals in aquatic species (ICRP
83 2008). Several kinds of effects have been observed in fish exposed to gamma ionizing
84 radiation such as disruption of immune response (Strand et al. 1982), apoptosis (Zhao et al.
85 2019), genomic instability (Hurem et al. 2017a) and effects on hormonal levels (Erickson
86 1971). These alterations could have consequences on survival (especially for early life
87 stages), fecundity and development. In a previous work, we also exposed zebrafish larvae,
88 *Danio rerio*, a well-known model organism in toxicology and ecotoxicology (OECD 2004),
89 during 4 days at low and high dose rates ($3,3 \cdot 10^1$ and $2,4 \cdot 10^4$ $\mu\text{Gy/h}$) of external chronic
90 gamma irradiation and showed effect on hatching, gene expression and muscular tissue
91 integrity (Gagnaire et al. 2015b).

92 The effects of tritium were also investigated at the molecular and cellular levels in zebrafish
93 larvae early life stages using of a battery of molecular responses of immunotoxicity,
94 neurotoxicity and genotoxicity. Tritiated water (HTO) effects were studied at dose rates of
95 $3.4 \cdot 10^1$, $1.36 \cdot 10^2$ and $1.26 \cdot 10^3$ $\mu\text{Gy/h}$ until larvae reached 10 dpf (days post fertilization), in
96 order to observe a key phase of larval development until the total resorption of yolk bag, and
97 therefore to investigate potential effects on metabolism (Gagnaire et al. 2020a). The links
98 between responses observed at the molecular, tissular and individual levels were also
99 investigated using specific tools such as RNAseq (Arcanjo et al. 2018) and behavioral
100 analyses (Arcanjo et al. 2020). Tritiated thymidine was also studied at dose rates of $8.7 \cdot 10^1$ to
101 $2.4 \cdot 10^3$ $\mu\text{Gy/h}$ (Adam-Guillermin et al. 2013). This previous work allowed to better
102 understand the mode of action of tritium on zebrafish early life stages. However, results were
103 difficult to compare to those obtained for gamma ionizing radiation, as the exposure
104 conditions were not the same in duration and dose rates.

105 Therefore, the aim of the study presented in this paper was to complete the previous study
106 realised on gamma irradiation (Gagnaire et al. 2015b) by studying the same dose rates as we
107 used for HTO exposure (Gagnaire et al. 2020a), and to continue the exposure until 10 dpf, in
108 order to study the potential effects of gamma irradiation on metabolism and to compare the
109 mode of action of HTO and gamma irradiation. Dose rates tested were in the range of the
110 DCRLs for fish ($4 \cdot 10^1$ - $4 \cdot 10^2$ $\mu\text{Gy/h}$) for which deleterious effects are supposed to appear
111 (ICRP 2008). Biological effects were evaluated in terms of immunotoxicity and oxidative
112 stress (production of reactive oxygen species (ROS)) and genotoxicity (DNA strand breaks

113 measured by the comet assay). These markers were previously measured on HTO and gamma
114 irradiation, which justifies their choice. Moreover, they are classical molecular responses used
115 in a wide range of fish species in ecotoxicology (van der Oost et al. 2003). Moreover, the
116 expression of genes linked to the studied mechanisms was followed. Molecular responses
117 were related to individual responses by the analysis of muscle histopathology and embryo-
118 larval development. The whole responses were compared to those obtained on zebrafish
119 larvae contaminated to HTO at 1, 4, 7 and 10 dpf and also on zebrafish contaminated with
120 tritiated thymidine at 4 dpf in order to compare and better understand the mode of actions of
121 different types of ionizing radiations.

122 2. Material & Methods

123

124 2.1 Chemicals

125 The chemicals used in this study were purchased from Sigma-Aldrich (St Quentin-Fallavier,
126 France) and Molecular Probes (Invitrogen, Cergy-Pontoise, France), as mentioned before
127 (Gagnaire et al. 2015b).

128

129 2.2 Egg production

130 All experimental procedures of this study were approved by IRSN Animal Care Committee
131 and followed French regulations for animal experimentation (protocol P2017-05; registration
132 number of IRSN laboratory: C13-013-07).

133 The eggs were obtained using zebrafish aged of 6-12 months (AB strain, GIS Amagen, Gif
134 sur Yvette, France) as described before in Gagnaire et al. (2020a). Genitors were maintained
135 in a Tecniplast ® rearing system with a temperature of $28 \pm 1^\circ\text{C}$ and a photoperiod of
136 12h/12h. They were fed three times per day with GemmaWean fish feed (Planktovie, France).
137 Embryos were obtained from at least 40 animals by putting together 2 females and 2 males.
138 All of the eggs spawned by all couples of genitors were pooled; egg viability was confirmed
139 when the blastula stage was reached without visible abnormalities at 3 hpf (hours post
140 fertilization). Experiments were started when the viability rate at 3 hpf was at least 90%.

141

142 2.3 Egg exposure to gamma irradiation

143 Eggs used in these experiments were aged of 3 hpf. They were maintained in embryo medium
144 (Westerfield 2007). Irradiations took place in incubators at a temperature of $28 \pm 1^\circ\text{C}$ and a
145 photoperiod of 12h dark/12h light. Eggs were randomly distributed on a standard 25 well-
146 plate (5 eggs in 2 mL of embryo medium; VWR). Three dose rates were studied (one source
147 by incubator). Plates were placed circularly around the central ^{137}Cs gamma source (either
148 liquid in a polystyrene tube (20 MBq in HCl 0.1 M) or solid (1.85 GBq)). Eight plates were
149 used for each source. Only 3 or 5 wells on each plate were used in order that each well
150 received a similar dose rate of gamma irradiation, depending on their position towards the
151 source. The same experimental design was used for controls (25 well-plate, 5 eggs in 2 mL).
152 Therefore, 160 eggs were used for each condition. Eggs/larvae were randomly sampled in
153 different wells for all analyses. Dose rates of the three sources were characterized using RPL
154 glass dosimeter measurements (ChiyoadaTechnologies, Japan) at means of 3.3×10^1 , 1.34×10^2
155 and 1.24×10^3 $\mu\text{Gy/h}$ (Dubourg, pers. comm.). Experiments lasted 10 days. Water was

156 renewed after 3 and 7 days of experiments. From days 6 to 10, a special feed for larvae was
157 given to each well (GemmaMicro 75, Planktovie, France).

158

159 **2.4 Monitoring of embryonic developmental parameters**

160 Mortality was monitored daily. At 2 dpf, larvae started to hatch and were monitored each day
161 until 10 dpf (Frayse et al. 2006). Hatching time 50% (HT₅₀), which is the time needed to
162 reach 50% of hatched eggs, was calculated using REGTOX ®
163 (http://www.normalesup.org/~vindimian/fr_index.html).

164 At 1, 2, 3, 4, 7 and 10 dpf, pictures were acquired in order to measure total length, and egg
165 and yolk bag diameters and analyzed on individual eggs or larvae as described before
166 (Gagnaire et al. 2015b).

167

168 **2.5 Comet assay**

169 The comet assay in alkaline conditions was used to detect DNA strand breaks (single- and
170 double-strand breaks, and alkali-labile sites) as described before (Gagnaire et al. 2015b) on
171 eggs and larvae controls and irradiated according to the procedure of Singh et al. (1988)
172 modified by Devaux et al. (1998). Three pools of ten 1 dpf-eggs and ten individual 4, 7 and 10
173 dpf larvae were used for each condition (control and 3 dose rates of gamma irradiation). The
174 tail moment (measurement of fluorescence intensity in comet tail) was determined for all
175 samples.

176

177 **2.6 ROS production**

178 Production of Reactive Oxygen Species (ROS), used here as a biomarker of immunotoxicity,
179 was *in vivo* measured on 4, 7 and 10 dpf larvae, as described before (Gagnaire et al. 2013).
180 Briefly, 20 larvae per condition were individually placed in HBSS buffer in a black 96-well
181 plate. All larvae received H₂DCFDA. In order to determine basal and stimulated activities,
182 half of the larvae received DMSO and the other half received PMA. Fluorescence was
183 monitored during 300 min (TECAN Infinite M1000). The ROS production index was
184 calculated (PMA/DMSO).

185

186 **2.7 Gene expression**

187 RNA was extracted from three pools of fifteen 4, 7 and 10 dpf larvae per condition and the
188 methodology described in Gagnaire et al. (2015b) was used to obtain the gene expression
189 data. Briefly, Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix was used in addition of

190 cDNA sample, primers (reverse and forward) and a referent dye on a Mx3000P real-time PCR
191 machine (Stratagene, Agilent). 35 cycles of amplification were used (30 sec at 95°C, 1 min at
192 60°C and 1 min at 72°C). The expression of several genes involved in immune response
193 (lysozyme C), apoptosis (bcl2-associated X protein), oxidative stress (myeloid specific
194 peroxidase), detoxication (cytochrome P450 CYP1A, glutathion-S-transferase,
195 metallothionein) and nervous system (acetylcholinesterase, choline acetyltransferase) were
196 followed. The gene of elongation factor 1 was followed as a reference gene. The list of
197 primers used has been previously published in Gagnaire et al. (2015b). The relative
198 expression ratio of mRNA of every gene normalized by the reference gene was calculated
199 using the threshold cycle Ct on REST-384© software (Pfaffl et al. 2002). The expression of
200 the housekeeping gene efl (elongation factor 1) was validated between all conditions: Ct
201 means \pm SE for controls and 4, 7 and 10 dpf larvae irradiated at the three dose rates were not
202 different (18.9 ± 0.6 for controls and 19.2 ± 0.8 for $3.3 \times 10^1 \mu\text{Gy/h}$ ($p=0.07$, t test); 18.8 ± 0.6 for
203 controls, 18.8 ± 0.6 for $1.34 \times 10^2 \mu\text{Gy/h}$ and 19.1 ± 0.4 for $1.24 \times 10^3 \mu\text{Gy/h}$ ($p=0.21$, ANOVA).

204

205 **2.8 Histological observations**

206 Three larvae aged of 4, 7 and 10 dpf of each condition (controls and irradiated groups) were
207 sampled for microscopical observations and individually analyzed as described before
208 (Gagnaire et al. 2020a, Gagnaire et al. 2015b). However, for technical reasons, not all larvae
209 of all ages and all groups could be analyzed.

210

211 **2.9 Statistical analyses**

212 Results (ROS and developmental parameters data) were expressed as means \pm standard error
213 (se=standard deviation/square root(n)). Analyses were realized using STATISTICA Software
214 v12 (StatSoft, Inc., Tulsa, OK, USA), with a significance level of $p \leq 0.05$. Normality was
215 checked using Shapiro–Wilk tests. In the case of non-normal data, data were Boxcox-
216 transformed. For normal data, differences between conditions (controls and irradiated larvae)
217 were tested with t-tests or ANOVAs followed by an *a posteriori* post-hoc LSD (least
218 significant difference) test in the case of rejection of H_0 . For non-normal data, Kruskal-Wallis
219 or Mann-Whitney U tests were used.

220 For statistical analysis of comet data, we used previously described method using R software
221 (R_Core_Team 2017) on raw data of the individual cell tail moment (Gagnaire et al. 2020a).
222 Briefly, we constructed a linear mixed-effects model using the *nlme* package
223 (<https://CRAN.R-project.org/package=nlme>) with condition (control or irradiated larvae) as

224 fixed factor and replicates (slides) as random factor. Then, random permutation tests were
225 applied using the *pgirmess* packages (<https://CRAN.R-project.org/package=pgirmess>).
226

227 **3. Results**

228

229 **3.1 Mortality, hatching and embryonic development**

230 Mortality and hatching of embryos were monitored every day. No significant difference was
231 shown neither for mortality, nor for hatching in controls and irradiated groups (data not
232 shown).

233 Larvae irradiated to 3.3×10^1 $\mu\text{Gy/h}$ presented an increase of 11% of yolk-bag diameter only at
234 10 dpf compared to controls (t test, $p=0.0066$) (Table 1). Larvae irradiated to 1.34×10^2 and
235 1.24×10^3 $\mu\text{Gy/h}$ presented variations for earlier stages: 1 dpf egg diameter was decreased of
236 11% (Kruskal Wallis test, $p=0$) and 4 dpf length was increased of 5% for larvae irradiated to
237 1.24×10^3 $\mu\text{Gy/h}$ compared to other conditions (ANOVA, $p=0.0056$) (Table 1). At 7 dpf,
238 larvae irradiated to 1.34×10^2 and 1.24×10^3 $\mu\text{Gy/h}$ had a yolk-bag diameter decreased of 18%
239 and 11% compared to controls, respectively (ANOVA, $p=0.034$), but no more effect was
240 shown on larval length (Table 1). No significant difference was shown at 10 dpf.

241

242 **3.2 DNA damages**

243 DNA damages were measured by comet assay at 1, 4, 7 and 10 dpf. Larvae irradiated to the
244 three dose rates showed no significant differences compared to controls for all sampling
245 points (Table 2).

246

247 **3.3 ROS production**

248 ROS production was also evaluated at 1, 4, 7 and 10 dpf. At 7 dpf, larvae irradiated to 3.3×10^1
249 $\mu\text{Gy/h}$ presented a significantly higher ROS basal activity compared to controls (U Mann
250 Whitney test, $p=0.027$); at 10 dpf, these larvae presented a ROS production index
251 significantly higher than controls (t test, $p=0.0148$), related to an increase of stimulated
252 activity (U Mann Whitney test, $p=0.0014$) (Table 3). Larvae irradiated to 1.34×10^2 and
253 1.24×10^3 $\mu\text{Gy/h}$ presented a ROS production index significantly higher than controls only at 4
254 dpf (ANOVA, $p=0.0035$), also related to an increase of stimulated activity (Kruskal Wallis
255 test, $p=0.002$) (Table 3 **Table 3**).

256

257 **3.4 Muscle histopathology**

258 For technical reasons, all samples could not be analyzed. Larvae irradiated to 1.34×10^2 $\mu\text{Gy/h}$
259 showed visible effects on mitochondria and myofibrils at 4 and 7 dpf (Figure 1B and C).
260 Larvae irradiated to 1.24×10^3 $\mu\text{Gy/h}$ showed more important effects compared to 1.34×10^2

261 $\mu\text{Gy/h}$ at 7 and 10 dpf, with missing mitochondria and lesions on the myofibrils attachment
262 point (Figure 1D and E).

263

264 3.5 Gene expression

265 The expression of the eight genes (and elongation factor) was followed in 1, 4, 7 and 10 dpf
266 larvae. At $3.3 \times 10^1 \mu\text{Gy/h}$, only *bax* gene was significantly repressed in 7 dpf larvae compared
267 to controls; in 10 dpf larvae, the same non-significant trend was observed (Table 4). At
268 $1.34 \times 10^2 \mu\text{Gy/h}$, *ache* and *mt2* genes were repressed at 7 dpf and *cyp1a* gene was repressed at
269 10 dpf in irradiated larvae compared to controls; for *cyp1a* gene, the same non-significant
270 trend was observed at 7 dpf (Table 4). At $1.24 \times 10^3 \mu\text{Gy/h}$, *mpx* gene was repressed at 7 dpf
271 and overexpressed at 10 dpf, while *gstp1* gene was repressed at 10 dpf in irradiated larvae
272 compared to controls (Table 4).

273

274 All of the results concerning tritium and gamma irradiation are presented in Tables 5 and 6,
275 respectively. HTO data were previously published (Gagnaire et al. 2020a) but the dose rate
276 was recalculated here without the weighting factors (see explanations in (Gagnaire et al.
277 2020a)) and results were summed up here to allow a direct comparison with gamma
278 irradiation results. For HTO, dose rates of 3.43×10^1 , 1.36×10^2 and $1.26 \times 10^3 \mu\text{Gy/h}$ for
279 nominal concentrations of HTO of 1.22×10^4 , 1.22×10^5 and $1.22 \times 10^6 \text{ Bq/mL}$, respectively
280 (Table 5), so very close to dose rates studied in gamma irradiation (3.3×10^1 , 1.34×10^2 and
281 $1.24 \times 10^3 \mu\text{Gy/h}$, Table 6).

282

283 4. Discussion

284

285 4.1 Effects of gamma irradiation on embryonic development, DNA damage, ROS 286 production, gene expression and muscle integrity

287 In the present work, no effect of gamma irradiation was seen on mortality or hatching.
288 Zebrafish exposed to 38 mGy/h presented an increase in mortality (Hurem et al. 2017b),
289 showing that this endpoint is sensitive as higher dose rates than the one tested in our study.
290 However, an increase of 4 dpf larval length was shown at 1.34×10^2 and 1.24×10^3 $\mu\text{Gy/h}$
291 and a decrease of yolk bag diameter was shown in 7 dpf larvae at 1.34×10^2 $\mu\text{Gy/h}$. This result
292 was previously observed in zebrafish larvae irradiated in the same conditions at 2.4×10^4
293 $\mu\text{Gy/h}$ (Gagnaire et al. 2015b). Differences on larval length in our study was transient and
294 may be due do a difference in kinetic of vitellin reserve consumption in the different
295 conditions. Malformations were also observed in 4 dpf zebrafish exposed at 0.4 mGy/h
296 (Hurem et al. 2017b). It would be interesting to continue the observation of animals to see if
297 this increase of 11% of yolk bag diameter has an impact on age at metamorphosis or on adult
298 stage. Previous experiments showed an acceleration of hatching in zebrafish larvae irradiated
299 to dose rates comprised between 3.3×10^1 and 4.1×10^4 $\mu\text{Gy/h}$ (Gagnaire et al. 2015b, Pereira et
300 al. 2011, Simon et al. 2011), while a decrease was observed at 1.5×10^4 $\mu\text{Gy/h}$ when eggs were
301 irradiated earlier (2 hpf) (Hurem et al. 2017b) and an absence of effects was shown after
302 exposure to 0.5-50 mGy/h on zebrafish larvae irradiated starting at 1 hpf (Murat El
303 Houdiguet al. 2019). These whole results seem to indicate that gamma irradiation induces an
304 increase in consumption of energetic reserves and therefore an increase of development and of
305 global metabolism of larvae. It would be interesting to deep further in these results at the
306 physiological level using DEBtox approaches to characterize which mechanism leading to this
307 altered phenotype is impaired (Kooijman et al. 2009).

308 Gamma irradiation has often been shown to induce DNA damages in zebrafish larvae at
309 different dose rates, such as 3.3×10^1 $\mu\text{Gy/h}$ (Gagnaire et al. 2015b) and from 4.16×10^2 to
310 4.16×10^5 $\mu\text{Gy/h}$ (Simon et al. 2011). DNA damages also appeared in progeny when parents
311 were exposed to gamma irradiation (Guirandy et al. 2019) or when exposure occur during
312 gametogenesis (Hurem et al. 2018). However, at 2.4×10^4 $\mu\text{Gy/h}$, DNA damages increased at 1
313 dpf and decreased at 4 dpf, suggesting that repair mechanisms occurred (Gagnaire et al.
314 2015b). The absence of effects of gamma irradiation on DNA damage in the present work is
315 unexpected, as DNA damages are one of the first effect observed in animals exposed to
316 radiations. DNA strand breaks are known to repair very quickly, therefore we can hypothesize

317 that for older larvae (7 and 10 dpf), the repair mechanisms suspected in the previous study on
318 4 dpf larvae could have been induced to a large extent, as physiological functions are more
319 developed in older animals. Moreover, the background DNA damage levels was high in
320 controls for 1 and 4 dpf, explaining the difficulty to show some effects for 1.34×10^2 and
321 1.24×10^3 $\mu\text{Gy/h}$. An interesting complement would be to study the repair mechanisms
322 initiated for example by gamma H2AX protein, which has been shown to be a sensitive
323 endpoint in fish early life stages exposed to gamma irradiation (Honjo and Ichinohe 2019,
324 Sayed et al. 2017).

325 In 10 dpf larvae exposed to 3.3×10^1 $\mu\text{Gy/h}$, an increase in ROS production due to a
326 decrease of ROS basal level was observed, while no effect was seen on stimulated level. The
327 exact same kind of result was already shown for other pollutants, such as endocrine disruptors
328 (Bado-Nilles et al. 2014b) and uranium (Gagnaire et al. 2015a, Gagnaire et al. 2014, Le
329 Guernic et al. 2016). However, an increase of ROS basal level was shown in zebrafish
330 progeny when parents were exposed to gamma irradiation (Guirandy et al. 2019, Hurem et al.
331 2017a). On the contrary, 4 dpf larvae exposed to 1.34×10^2 and 1.24×10^3 $\mu\text{Gy/h}$ showed also
332 an increase of ROS production index, but this was due to an increase of the stimulated level,
333 as shown before for agricultural products (Bado-Nilles et al. 2014a). However, for both dose
334 rates, no effect was shown at 7 and 10 dpf. Larvae irradiated to high dose rates of gamma
335 irradiation seem therefore able to regulate ROS production, hypothetically by the
336 development of compensatory mechanisms. In previous experiments, irradiation to 2.4×10^4
337 $\mu\text{Gy/h}$ did not induce ROS production, which confirms this hypothesis (Gagnaire et al.
338 2015b). However, at low dose rate (3.3×10^1 $\mu\text{Gy/h}$), effects persist at 10 dpf and ROS
339 production index was decreased at 4 dpf (Gagnaire et al. 2015b). ROS production is a global
340 indicator of stress, used to assess the exposure to stressful conditions (including pollutants),
341 but the response can vary during time and can be transient (Hakim 1993). Our results show
342 the non-linearity of responses of ROS production after a gamma irradiation.

343 Several histological alterations were shown for larvae exposed to the highest dose
344 rates. Degradation of myofibrils with alteration of actin and myosin filaments were already
345 shown in previous studies after irradiation at 3.3×10^1 et 2.4×10^4 $\mu\text{Gy/h}$ during 4 dpf, but
346 without any effects on the mitochondria (Gagnaire et al. 2015b). These results showed tissue
347 alterations that can affect muscle contractility and cellular respiration and have consequences
348 on larvae motility. At other dose rates, zebrafish larvae locomotion was impacted (Murat El
349 Houdigui et al. 2019). It would be interesting to complete the results with behavioural tests of
350 motility and response to a stimulus to confirm this hypothesis.

351 Genes involved in detoxication were also repressed in the late stages of zebrafish
352 larvae (*Cyp1a*, *gstp1* and *mt2*) at different dose rates, which was already seen before (Hurem
353 et al. 2017b). Moreover, similarly to results previously obtained, *mpx* and *ache* seem to be
354 the most modulated by gamma irradiation (Gagnaire et al. 2015b). It is interesting to note that
355 at 1.24×10^3 $\mu\text{Gy/h}$, *mpx* gene modulation happened at 7 and 10 dpf, where no effect was
356 shown on ROS production, and did not happen at 4 dpf, where the ROS production index was
357 increased. This result could be explained by a delay of response between gene expression and
358 ROS production. Taken together, these results confirm the induction of an oxidative stress by
359 gamma irradiation.

360 It is important to underline that in their majority, obtained responses followed a non-
361 monotonous dose-response. To explain this result, the hypothesis of plurality of molecular
362 targets could be made: responses at low dose rates could be different from responses at high
363 dose rates because molecular targets could be differentially activated depending on the dose,
364 therefore producing different kinds of responses, as it was shown for endocrine disruptors
365 (Lagarde et al. 2015). It seems important to take into account these non-linear responses in
366 risk assessment, as NOEC or LOEC values are mostly defined for monotonous dose-response
367 relationships.

368

369 **4.2 Comparison of results for HTO/tritiated thymidine/gamma radiation** 370 **exposure**

371 In our previous work on HTO, dose rates were calculated by applying dose coefficients to
372 external and internal dose rates, according to the hypothesis of an homogeneous tritium
373 distribution in tissues (Gagnaire et al. 2020a). A ponderation coefficient of 3 was applied in
374 order to take into account beta radiation efficiency as recommended (Brown et al. 2008).
375 However, in the present paper, we removed the ponderation coefficient of 3 to calculation of
376 doses rates, in order to allow direct comparison between HTO and gamma irradiation results.

377 Our results showed that gamma irradiation at high dose rate accelerated hatching,
378 while HTO had no effect on this parameter; on the contrary, tritiated thymidine induced a
379 delay of hatching for equivalent dose rates (dose rates of 1.8×10^2 - 4.4×10^2 - 2.4×10^3 $\mu\text{Gy/h}$) and
380 also some malformations. Developmental parameters, even if they were modified in a
381 transitory way, confirmed the hypothesis of an acceleration of development for gamma
382 irradiation and a slowing of development for tritium. However, for tritium, effects differed
383 according to the form used. Developmental parameters do not seem to be relevant indicators
384 of HTO effects on zebrafish larvae, contrarily to gamma irradiation and tritiated thymidine.

385 DNA damages were observed, mostly at 4 dpf, for both types of ionizing radiation and
386 chemical forms studied. Increase of ROS production was also shown for the different
387 stressors, but not for all stages or all dose rates studied. These results confirm the known data
388 on the effects of ionizing radiation in terms of oxidizing stress and genotoxicity (Adam-
389 Guillermin et al. 2012, Gagnaire et al. 2017, Gagnaire et al. 2020b, Hu et al. 2016).

390 For both gamma irradiation and tritium, genes involved in detoxication and
391 neurotransmission were punctually altered, but only gamma irradiation induced effects on
392 EROD and AChE activities (Gagnaire et al. 2015b). Gamma irradiation at 5×10^3 and 5×10^4
393 $\mu\text{Gy/h}$ also induced modification of expression of genes involved in nervous central system
394 and decrease of locomotion when exposure happened in the gastrulation stage of zebrafish
395 (Murat El Houdigui et al. 2019). The nervous system did not seem to be a target of HTO in
396 zebrafish larvae, contrary to gamma irradiation. However, AChE activity was modified in
397 adult fathead minnows exposed to HTO after depuration (Gagnaire et al. 2020b). Moreover,
398 genes involved in muscle contraction were modified by HTO (Arcanjo et al. 2018), and
399 swimming behavior of larvae was altered but only for one dose rate (Arcanjo et al. 2020).
400 Gamma irradiation seemed to induce more deleterious effects on nervous system compared to
401 HTO, but the pathway of alteration was similar for both stressors.

402 In the present study, only HTO modified the expression of *lyz* gene, involved in
403 immune response. Immune parameters were also modified in adult minnows exposed to HTO
404 (Gagnaire et al. 2020b). These results showed on the basis of these parameters some
405 differences of responses to different kind of radioactive stressors.

406 Regarding the tissue level, gamma irradiation (Gagnaire et al. 2015b), as well as HTO
407 (Gagnaire et al. 2020a), were previously shown to induce myofibril alterations on 4 dpf
408 larvae. In the present study, important alterations were observed on mitochondria. This result
409 could be related to the increase of ROS production also seen in the present study. The exact
410 same results were observed after exposure to HTO at 7 and 10 dpf (Gagnaire et al. 2020a).
411 Therefore, the mechanisms of action of these different kinds of radioactive stressors on
412 zebrafish tail muscle were similar in early life stages.

413 Therefore, ROS production, DNA damages and detoxication process, as well as
414 histology of muscle, responding similarly to both to tritium and gamma irradiation, seemed to
415 be relevant and could be used in further studies in the context of environmental risk
416 assessment of ionizing radiation. Our worked also showed some specific response to tritium
417 (immune system) and gamma irradiation (nervous system).

418 It is important to take into account the fact that tools available to calculate dose rates are
419 based on the hypothesis of a homogeneous distribution of dose deposition, which may be the
420 case for HTO and gamma irradiation but not for tritiated thymidine that is incorporated in
421 DNA; however, as we have no other tool, we assume that tritium results obtained can be
422 compared to gamma irradiation. An interesting perspective would be to better characterize
423 tritium internal distribution at the cellular level (DNA molecule) and, with the help of
424 modelisation (microdosimetry and toxicodynamics), to better understand the link between
425 dose rates and effects (Sakata et al. 2019, Tang et al. 2019).

426

427 **4.3 Comparison between the effects of gamma and beta ionizing radiation**

428

429 In the present work, we obtained data that were missing for the same dose rates as the
430 ones already tested for HTO. The study of molecular responses showed that oxidative stress
431 and DNA damages are the major toxic mode of action of gamma irradiation as well as tritium.
432 For both stressors, the tail muscle integrity was also altered in the same way. However, these
433 effects are generally not dose-dependent, which could indicate a compensation of several
434 mechanisms of defense and toxicity, or the overwhelming of cell repair capacity. The effects
435 seemed more pronounced for gamma irradiation than for tritium for equivalent dose rates (e.g.
436 muscular alterations). Some molecular responses seemed more specific of gamma irradiation
437 (e.g. AChE pattern modification at several biological levels) compared to tritium
438 (modification of immune response). The whole results suggest some common pattern of
439 responses, but also some differences of mode of action between HTO, tritiated thymidine
440 (internal contamination) and gamma irradiation (external irradiation) on zebrafish larvae.

441 For beta emitters and in particular for tritium, a RBE of 3 was recommended (Adam-
442 Guillermin et al. 2012, ASN 2010, Brown et al. 2008). However, there is no consensus on this
443 value in the whole scientific community, due to the uncertainty of models (Harrison et al.
444 2002, Melintescu et al. 2007). The recent work of ICRP on biological effects of tritium on
445 non-human species showed that RBE values obtained are of 1.5-2 towards X rays and 2-2.5
446 towards gamma rays (ICRP 2019). For environmental radioprotection, ICRP proposed to
447 weight absorbed dose rates resulting of exposure of biota to radiation of low linear energy
448 transfer (including beta radiation from tritium) by a RBE of 1. However, they recommend,
449 when the level of exposure to tritium is susceptible to induce potentially deleterious effect on
450 a population of plants or animals, that the use of a higher RBE can be considered (ICRP
451 2019).

452 We designed the work presented in this paper in order to calculate a RBE for tritium.
453 Unfortunately, the RBE could not be calculated because i) the responses for ROS and DNA
454 damages did not increase in a monotonous manner with the dose rate, both for tritium as for
455 gamma irradiation, and ii) some responses were specific. Moreover, dose rates inducing no
456 effects on followed parameters (NOEDR) are missing. However, on the basis of obtained
457 results in the present study and in previous studies, the value of 1 proposed by ICRP seems to
458 be relevant, as effects seemed to be more important after gamma irradiation than after tritium
459 exposure for equivalent dose rates.

460

461 **5. Conclusions**

462 The effects of a gamma irradiation were studied on zebrafish larvae during the critical
463 period of the larval development until 10 dpf for three dose rates, the lowest one (3.3×10^1
464 $\mu\text{Gy/h}$) being close to the benchmark value for ecosystem protection towards radiations (10
465 $\mu\text{Gy/h}$). Effects on mortality, hatching, development, DNA damages, immune responses, gene
466 expression and tissue integrity were searched.

467 The work presented here, added with previous work on gamma irradiation and tritium,
468 allowed to show that ionizing radiation induced effects on zebrafish early life stages. Some
469 pathways were seen to be shared with the different kinds of ionizing radiations tested, as
470 effects on muscular tissue. However, the effects can differ considering the form studied for a
471 same dose rate. Indeed, effects of tritium were not the same for HTO (transitory
472 morphometric modifications) and tritiated thymidine (slowing of hatching, malformations of
473 larvae). The form of tritium used plays a preponderant role in its toxicity.

474 These results allow to show the interest of using molecular responses in complement to
475 macroscopical observations better understand the mechanisms activated during zebrafish
476 larvae exposure to different kinds of ionizing radiations. Some results still need to be acquired
477 in order to calculate a RBE for tritium, with the help of microdosimetry to better characterize
478 the internal dose.

479

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Figure captions

Figure 1: muscular tissue of zebrafish larvae observed by TEM in control (A) and irradiated at 1.34×10^2 $\mu\text{Gy/h}$ at 4 (B) and 7 (C) dpf. E: endomysium, M: mitochondria, My: myofibrils, S: sarcomere, Z: Z stria.

Table 1: measurements in mm of egg diameter, total length and yolk bag (YB) diameter in zebrafish eggs and larvae control and irradiated to 3.3×10^1 , 1.34×10^2 and 1.24×10^3 $\mu\text{Gy/h}$. Values are expressed as means of at least 10 replicates. Bold values indicate a significant difference between conditions. $p \leq 0.05$; a, b: significantly different, $a < b$. n.d.: not determined.

Table 2: mean tail moments (comet assay) obtained for 1 dpf zebrafish embryos (n=3 pools of 10) and 4, 7 and 10 dpf larvae (n=10) control and irradiated to 3.3×10^1 , 1.34×10^2 and 1.24×10^3 $\mu\text{Gy/h}$. Mix model (R), $p \leq 0.001$. n.d.: not determined in the present study.

Table 3: ROS basal and stimulated levels and stimulation index in zebrafish larvae irradiated at 3.3×10^1 , 1.34×10^2 and 1.24×10^3 $\mu\text{Gy/h}$ during 10 days. Values are expressed as means of 20 replicates (10 for stimulated levels and 10 for basal levels); standard error is presented. $p < 0.05$; Bold values indicate a significant difference between conditions. a, b, c: significantly different, $a < b < c$.

Table 4: relative gene expression (normalized to reference gene *ef1*) compared to control in zebrafish larvae irradiated at 3.3×10^1 , 1.34×10^2 and 1.24×10^3 $\mu\text{Gy/h}$ during 10 days (n=9). Analysis with REST-384©, $p \leq 0.05$; bold values indicate a significant difference compared to

519 control; underlined values indicate a non-significant difference compared between exposed
520 and control of a factor 2 or superior.

521

522 **Table 5:** synthesis of results obtained for exposition of zebrafish larvae to tritiated thymidine
523 and HTO (adapted from Adam-Guillermin et al. (2013) and Gagnaire et al. (2020a)). For
524 explanations concerning weighting factors, please refer to Gagnaire et al. (2020a). C: control;
525 \emptyset : no significant effect; -: not relevant; n.d.: not determined. Diam: Diameter; YB: Yolk Bag.

526

527 **Table 6:** synthesis of results obtained for exposition of zebrafish larvae to five dose rates of
528 gamma irradiation. C: control; \emptyset : no significant effect; -: not relevant; n.d.: not determined.

529 Diam: Diameter; YB: Yolk Bag; HT₅₀: hatching time 50%.

530

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