

1 **Subtle effects of radiation on embryo development of the 3-spined stickleback**

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21 **Abstract**

22 The Chernobyl and Fukushima nuclear power plant (NPP) accidents that occurred in 1986 and
23 2011 respectively have led to many years of chronic radiation exposure of wildlife. However,
24 controversies remain on the dose threshold above which an impact on animal health occurs. Fish
25 have been highly exposed immediately after both accidents in freshwater systems around
26 Chernobyl and in freshwater and marine systems around Fukushima. The dose levels decreased
27 during the years after the accidents, however, little is known about the effects of environmental
28 low doses of radiation on fish health. The present laboratory study assesses the effects of an
29 environmentally relevant dose range of radiation (0.1, 1 and 10 mGy/day) on early life stages of
30 the 3-spined stickleback, *Gasterosteus aculeatus*.

31 The cardiac physiology and developmental features (head width, diameter, area) of high
32 exposed embryos (10 mGy/day) showed no significant change when compared to controls.
33 Embryos exposed to the medium and high dose were slower to hatch than the controls (between
34 166 and 195 hours post-fertilization). After 10 days of exposure (at 240 hours post-fertilization),
35 larvae exposed to the high dose displayed comparable growth to controls. High-throughput
36 sequence analysis of transcriptional changes at this time point revealed no significant changes in
37 gene regulation compared to controls regardless of exposure conditions. Our results suggest that
38 exposure of fish embryos to environmental radiation elicits subtle delays in hatching times, but
39 does not impair the overall growth and physiology, nor the gene expression patterns in the
40 recently hatched larvae.

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44 **Introduction**

45 Serious nuclear accidents at both the Chernobyl Nuclear Power Plant (NPP) in April 1986 and
46 the Fukushima NPP in March 2011 led to high levels of radiation exposure to wildlife. After the
47 Chernobyl accident, the dose to fish found in the cooling pond was estimated at 10 mGy/day¹
48 and then rapidly declined due to the decrease of short life radionuclides and sedimentation
49 processes².

50 Three decades after the Chernobyl NPP accident, the main radionuclides of concern are ⁹⁰Sr (a
51 β emitter) and ¹³⁷Cs (a β and γ emitter) due to their long radioactive half-life (28 and 30 years
52 respectively). Transuranium radioelements are also of concern due to their long radioactive half-
53 life and high energetic alpha particle emission. However, their contribution to the total dose to
54 fish at Chernobyl is very low³. In a highly contaminated lake called Glubokoye, located near the
55 Chernobyl NPP, the total dose rate to perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) 30
56 years after the accident was up to 16 and 14 μ Gy/h respectively (less than 0.4 mGy/day)³.

57 ³²P is a radioisotope of 14.26 days half-life that emits β particles of high energy (1.7 MeV)
58 whose track length is a few millimetres in water. A β -particle directly ionizes molecules by the
59 removal of an electron, whereas α particles (and X-ray) ionize molecules by generating a series
60 of fast electrons (effectively β particles) after first hitting molecules, therefore, the use of a β -
61 emitter is relevant to assess the effect of environmental radiation. In order to assess the effects of
62 ionising radiation exposure on fish embryos under laboratory conditions, ³²P was selected due to
63 its short half-life minimising the radioactive waste and its high energy β particles.

64 ³²P is used in research laboratories, medical procedures and industry that may lead to discharge
65 into freshwater systems. The Krasnoyarsk Mining and Chemical Industrial Complex in Russia
66 discharged significant amounts of ³²P in the Yenisei river that were found to have accumulated in

67 fish, with ^{32}P activities of 2.2 Bq/L in water and 2900 Bq/kg in fish found at 200 km from the
68 industrial site⁴. In the Columbia River contaminated by the cooling water from the Hanford
69 plutonium reactors, the estimated level of ^{32}P in water between 1950 and 1971 varied from 0.1 to
70 7.7 Bq/L⁵ and the activity of ^{32}P in fish was varied from 0.7×10^3 to 22×10^3 Bq/kg between
71 1962 and 1964⁶. Waterborne uptake of ^{32}P was found to be negligible as compared to uptake via
72 the dietary route in fish^{7,8}.

73 The 3-spined stickleback (*Gasterosteus aculeatus*) is a vertebrate model used in laboratory
74 settings to assess the mechanistic effects of pollutants and in field surveys using multi-biomarker
75 approaches⁹⁻¹⁰. The early stages of fish embryonic development are sensitive to ionising
76 radiation¹¹.

77 Numerous studies describe the effects of acute exposure to radiation on fish embryos.
78 However, much less is known about the effects of low doses of radiation in the environmental
79 range of 0.1 to 10 mGy/day. Importantly, results from the literature on the effects of low doses
80 on organism health differ¹². For instance, whilst some studies did not find evidence of radiation
81 effects on populations of aquatic invertebrates¹³, fish³ or mammals¹⁴ at Chernobyl, others found
82 adverse effects of radiation on the abundance of invertebrates¹⁵, birds¹⁶ and mammals¹⁷ at
83 Chernobyl and Fukushima. Thus, the dose at which significant damage to wildlife populations
84 occurs remains an open question¹².

85 Several studies have previously examined the effects of high doses of radiation (approximately
86 1000-fold above the environmental range) on the morphology of fish embryos. Exposure to acute
87 dose of radiation induced various morphological abnormalities in embryos of zebrafish (*Danio*
88 *rerio*)^{18,19,20,21}, mangrove killifish (*Kryptolebias marmoratus*)²² and medaka (*Oryzias latipes*)²³.

89 Numerous authors studied the effects of exposure to high doses of radiation on the hatching
90 success of embryos but the results differed according to the type and nature of exposure. No
91 difference in the percentage of embryos that hatched was observed in medaka embryos exposed
92 to 35.42 mGy/h of tritiated water²³ and in zebrafish embryos exposed to 0.01 and 0.05 Gy of γ
93 radiation²¹. However, other studies found significant effects on the hatching success. The
94 percentage of zebrafish embryos that hatched decreased significantly after exposure to a total
95 dose range of 0.1 - 10 Gy^{21,24}, and to a dose rate range of 0.3 - 2 Gy/day of γ radiation¹⁸. The
96 percentage of mangrove killifish embryos that hatched decreased significantly after exposure to a
97 total dose range of 2.5 - 10 Gy²². The hatching time is significantly delayed as compared to
98 controls in zebrafish embryos exposed to a dose range of 0.1 - 10 Gy²⁴ and a dose rate range of
99 0.3 - 2 Gy/day of γ radiation¹⁸.

100 However, whilst many studies have examined the effects of high doses of radiation on
101 hatching, fewer studies have examined hatching processes after exposure to lower doses, and no
102 clear patterns of such dose effects has been observed. An acceleration of hatching was observed
103 in zebrafish embryos exposed to a γ radiation dose of 10 and 1000 mGy/day, but no change was
104 recorded after exposure to 1 and 100 mGy/day¹⁸. Hatching process appears to depend on the
105 embryonic stage of exposure with some authors finding an acceleration of hatching in embryos
106 exposed from 3 hpf (blastula stage) but not from 24 hpf (segmentation stage) to 1-1000 mGy/day
107 of radiation¹¹.

108 Several studies investigated the effects of high doses of radiation on the cardiac physiology of
109 fish embryos. Studies reported no effect¹⁹ or a decreased heart rate²⁰ in zebrafish embryos. To
110 our knowledge, no studies in the literature have yet described the heart physiology of embryos
111 exposed to environmentally relevant doses of radiation.

112 Several recent studies explored the effects of short-term exposure to radiation (from a few
113 seconds to 96 hrs) on the transcriptional response of embryos. Gene expression changes were
114 described in zebrafish embryos exposed to a total dose of 10 mGy to 100 mGy during 11 to 110
115 secs respectively (dose rate: 79.2 Gy/day), and the number of the differentially expressed genes
116 was positively correlated to the dose¹⁹. Transcriptional response of genes involved in apoptosis²⁰
117 and DNA damage repair mechanisms^{20,25} were changed in zebrafish embryos exposed to 0.5 - 4
118 Gy of ⁵⁶Fe ion irradiation (Si et al. 2017)²⁰ or 9.6 and 96 mGy/day of γ radiation²⁵.

119 The present study is one of the few to assess of the effects of very low doses of radiation on
120 fish embryo development under laboratory conditions, combining both high throughput
121 molecular and biometric analyses. The aim was to investigate whether exposure to
122 environmental low doses of radiation induces developmental, physiological and transcriptional
123 changes in stickleback embryos.

124

125 **Methods**

126 *Fish maintenance*

127 Adult sticklebacks were kept in artificial reproduction conditions in order to generate the
128 embryos for *in vitro* fertilization. The artificial water composition and experimental conditions
129 were selected according to the OECD guideline for the testing of chemicals (CaCl₂: 294, MgSO₄:
130 123, NaHCO₃: 65, KCl: 6 mg/L, pH = 7.5, T°C = 19°C, photoperiod: 16h light/8h dark)²⁶. Four
131 distinct couples were used to generate the embryos. The fertilization procedure was performed
132 according to the protocol described in the OECD guideline for the testing of chemicals (test
133 number 236). In total, 54 embryos were used for monitoring growth and physiology (27 controls
134 and 27 exposed to the high dose), 108 for recording the hatching success (27 for each of the four

135 exposure conditions), 104 for next generation sequencing (NGS) analyses (Table S3) and 36 to
136 check ^{32}P uptake in the chorion, prolarvae and larvae (Table S2).

137 Three hours after fertilization, each embryo was placed in an individual glass tube and
138 waterborne exposed to 3 mL of a radioactive solution of 0.1, 1 and 10 mGy/day (or 4, 40 and 400
139 $\mu\text{Gy/h}$) using a solution of adenosine triphosphate labelled on the gamma phosphate group with
140 ^{32}P (PerkinElmer). The final concentration of ATP in control, low and medium experimental
141 tubes was adjusted to $1.1 \times 10^{-5} \mu\text{M}$ by addition of stable ATP. This ATP concentration is
142 negligible as compared to the concentration found in a typical cell of 5 mM^{27} . The doses
143 encompass the chronic low (L) (0.1 mGy/day) and medium (M) (1 mGy/day) doses encountered
144 in the environment at Chernobyl 30 years after the accident, and the initial high dose (H) to fish
145 after the accident (10 mGy/day). These doses span the Environmental Agency (EA) guidance
146 level of $40 \mu\text{Gy/h}$ (0.96 mGy/day) described in the radiological impacts on non- human species
147 report of the EA in 2011²⁸. Control embryos were kept in clean artificial water. 2.5 mL of the
148 water was renewed every 3 days. The embryos were exposed for 10 days and euthanized
149 according to schedule 1 of the Home Office Licence (ASPA, 1986)²⁹ using tricaine
150 methanesulfonate (Sigma).

151 *Dose calculation and monitoring*

152 Dose at the centre of the hemisphere was calculated from data in Berger (1971)³⁰ (Figure S1).
153 Activity of ^{32}P was measured using a HIDEX 300SL liquid scintillation counter and associated
154 MikroWin 2000 software (Version 4.43).

155 *Developmental and physiological measurements*

156 The morphological parameters were measured using a Zeiss axiozoom microscope and the Zen
157 Pro software. The physiological parameter was measured using an optical microscope. The

158 growth of embryos was recorded at 4 dpf (days post-fertilization) by measuring the diameter
159 (mm), area (mm²) and eye distance (mm). The hatching rate was calculated as the proportion of
160 fish that hatched to the total number of fish from the same condition for each observation time.
161 The cardiac physiology was assessed at 6 dpf by counting the heart beat rate (beats/min). The
162 growth of larvae was recorded at 10 dpf by measuring the length (mm) and head width (mm) of
163 the larvae through the glass tubes.

164

165 *Next generation sequencing (NGS)*

166 Differential expression analysis was conducted using NGS. For each of the 4 conditions
167 (control, low dose, medium dose and high dose), 3 biological replicates of pools of 4 to 10
168 embryos aged of 10 days were used for NGS analyses (Table S3). Total RNAs were extracted
169 using the High Pure RNA Tissue kit (Roche Diagnostics Ltd, West Sussex, U.K.) according to
170 the manufacturer's instructions. RNA quality and integrity were evaluated using a bioanalyzer
171 (Agilent, Santa Clara, USA). RNA integrity numbers ranged from 7.4 to 9.5 and showed low
172 RNA degradation rates. NGS libraries for each pool were generated using the Illumina TruSeq
173 mRNA library kit following the manufacturer's instructions. Libraries were sequenced using the
174 Illumina HiSeq 2500 analyser, generating 125 base, paired-end sequences from libraries yielding
175 an average of 33.4 ± 5.2 M paired reads per sample (Table S4). Quality control of raw fastq
176 reads was conducted using fastQC (bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were
177 trimmed using the Trim Galore script to remove adapter sequences and low quality sequence
178 tails (bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were mapped against
179 the BROAD S1 *Gasterosteus aculeatus* 3-spined stickleback genome from Ensembl³⁰ using the
180 STAR universal RNA seq aligner³¹ with parameters '--outSAMmultNmax 300'. Reads that

181 mapped uniquely to the genome in a proper pair with mapping quality score greater than 20 were
182 used in downstream analyses.

183 *Differential expression analyses*

184 Differential expression analyses were conducted using the DESeq2 package³³ in R. Gene
185 models were taken from Ensembl version 82³¹, and read counts over unique genes were
186 quantified using the “summarizeOverlaps()” function in the GenomicAlignments package³⁴
187 using mode “Union”. Raw read counts were normalised using the regularised log transformation
188 in DESeq2 for visualisation (Figure S2). P values were adjusted for multiple testing by using the
189 Benjamini and Hochberg correction³⁵. To account for potential confounding effects of lineage
190 due to a systematic difference in the pooling for replicate 3 compared to replicates 1 and 2 (Table
191 S3), lineage was included as a covariate in the analysis. Significant differentially expressed
192 genes were identified based on a fold-change of 2-fold or greater (up- or down-regulated) and an
193 adjusted p-value less than 0.05. To avoid over-representing differential expression in low-
194 abundance genes, significant genes were further filtered to remove those whose normalised
195 expression was less than 1 for both the exposed and control groups. Gene ontology analysis was
196 conducted using the clusterProfiler package³⁶.

197 *Statistical analyses*

198 Statistical analyses were performed using R version 3.1.2. After satisfying the assumptions of
199 the normal distribution of the residuals, linear models were used to assess the potential
200 differences. If the normality of the residuals wasn't respected a Kruskal-Wallis rank test was
201 applied. A Fisher exact test was applied to assess any difference between hatching success at
202 different times post-fertilization. When significant, post-hoc tests were performed and a
203 Bonferroni correction of the α error was applied.

204

205 **Results**

206 *Dose monitoring*

207 The mean activity of ^{32}P solutions measured during the 10 days exposure experiment were 0.7
208 ± 0.2 , 27 ± 3 , 253 ± 24 and 2588 ± 199 Bq/mL ($n = 12$) (Table S1) and in accordance with the
209 targeted activity of 2500, 250, 25 and 0 Bq/mL respectively, in each glass tube. After 3 days of
210 exposure, the activities measured in the chorion were 1 ± 0 Bq for controls and low exposure
211 conditions, and 3 ± 2 and 42 ± 11 Bq for the medium and high exposure conditions (mean \pm SD,
212 $n = 3$). The activities measured in dechorionated embryos were 1 ± 0 Bq for control, low and
213 medium exposure conditions and 3 ± 2 Bq for the high exposure condition (mean \pm SD, $n = 3$).
214 After 9 days of exposure, the activities measured in the larvae were 1 ± 0 for control, low and
215 medium exposure conditions and 2 ± 0 Bq for the high exposure condition (mean \pm SD, $n = 3$)
216 (Table S2).

217 *Developmental and physiological parameters*

218 Radiation exposure to 10 mGy/day did not significantly affect the growth of embryos. After 4
219 days, exposed embryos were equivalent in size. The diameter, surface and head width of exposed
220 embryos were 1.46 ± 0.01 mm, 1.68 ± 0.02 mm² and 0.85 ± 0.03 mm respectively. These
221 measures did not significantly differ from controls (diameter: 1.46 ± 0.01 mm, $p = 0.65$, area:
222 1.67 ± 0.02 mm², $p = 0.64$ and eye distance: 0.85 ± 0.03 mm, $p = 0.17$) (Table 1). After 6 days,
223 the cardiac physiology of exposed embryos was not disturbed by exposure to radiation. The
224 mean heart beats rate of exposed embryos were equal to 163.2 ± 12 beats/min and did not
225 significantly differ from controls (150.0 ± 3.1 beats/min, $p = 0.22$) (Table 1). After 10 days, the
226 development between exposed and control larvae remained similar. The length of exposed larvae

227 was 5.98 ± 0.06 mm and their head width 0.82 ± 0.01 mm. These values did not significantly
228 differ from controls (length: 5.99 ± 0.05 mm, $p = 0.88$ and head width: 0.81 ± 0.01 mm, $p =$
229 0.48) (Table 1).

230 During the hatching process, embryos exposed to 1 (M) and 10 (H) mGy/day were slower to
231 hatch at 170 hpf (hours post-fertilization), with 22% and 24% of the embryos respectively
232 displaying a delay in hatching ($p = 0.014$ and $p = 0.010$ respectively) (Figure 1). At 174 hpf, the
233 embryos exposed to the H condition reached 90% of hatching (HT₉₀) [169-176], 6 hours later
234 than the controls that hatched at 168 hpf [164-169] (based on 95% confidence interval overlap)
235 (Figure 2). There was no delay observed for the embryos exposed to L (HT₉₀: 171 hpf [168-173])
236 and M (HT₉₀: 172 hpf [166-175]) conditions as compared to the controls (Figure 2). Eventually,
237 at 195 hpf, no significant difference in hatching success was observed between conditions ($p =$
238 0.058) (Figure 1) and the hatching percentage reached 100% [88-100] for the control embryos
239 and 92%; 96% and 93% for the embryos exposed to 0.1 (L), 1 (M) and 10 (H) mGy/day
240 conditions respectively (Figure 1).

241 *Differential expression analysis*

242 Analysis of the NGS data quality identified these data as showing excellent base calling
243 qualities and post-filtering mapping rates of approximately 90% were seen throughout (Table
244 S4). Following gene abundance identification, principal component analysis identified little
245 difference between the dosage treatments in these data (Figure 3). A batch effect, resulting from
246 the parentage of individuals pooled in the different replicates was found (Table S3). This batch
247 effect was incorporated into the model for differential expression analysis.

248 Differential expression analysis was performed to identify genes whose expression was
249 significantly deregulated following radiation exposure by comparing each of the three dosed

250 treatments against a control treatment as described. No significant change in gene expression was
251 observed, with only a single gene showing significant differential expression in the low dosage
252 after filtering (Figure S2).

253 *Data availability*

254 The RNA sequencing data can be obtained from ArrayExpress
255 (<https://www.ebi.ac.uk/arrayexpress/>) with the accession number E-MTAB-7872.

256

257 **Discussion**

258 In the present study, embryo mortality was below 12% in each condition, in the range of what
259 is considered as normal in studies using zebrafish embryos. In the literature, zebrafish embryos
260 survival was reduced after exposures to higher doses such as 100 mGy¹⁹ and after 2 and 4 Gy of
261 exposure to ⁵⁶Fe²⁰.

262 During the hatching process, embryos exposed to 1 mGy/day (M) and 10 mGy/day (H)
263 conditions displayed reduced hatching successes, by 22 and 24% respectively. No change was
264 observed in embryos exposed to the lowest dose 0.1 mGy/day (L). At the end of the hatching
265 process, no significant difference on the hatching success of embryos was noticed between
266 controls and exposure conditions with percentages reaching 92% (L), 96% (M) and 93% (H).

267 Exposure to different dose and nature of radiation can accelerate hatching. For instance,
268 zebrafish embryos exposed to 9.6 mGy/h for 65h³⁶, 0.3 to 2 Gy/day¹⁸, 1-1000 mGy/day¹¹ and to
269 an X-ray dose of 25 mGy³⁷ hatched earlier than control embryos. The embryonic stage at which
270 radiation exposure occurs, appears to have consequences on the hatching sensitivity of embryos.
271 For instance, mangrove killifish embryos displayed a higher sensitivity when exposed at an early

272 stage to 2.5, 5, 7.5, and 10 Gy of γ radiation. In addition, the hatching success was significantly
273 decreased in embryos that were exposed early (10.5 hpf) for all doses²².

274 Other experiments showed that exposure to radiation induced a delay in hatching. Exposure to
275 an X-ray dose above 25 mGy delayed hatching zebrafish embryos³⁸. Waterborne exposure to 20
276 and 100 $\mu\text{g/L}$ of ²³³U induced a 12h delay in hatching time (HT₅₀: 59[54-66] and HT₅₀: 59[53-
277 68] respectively), as compared to controls (HT₅₀: 47[45-48])³⁹. In our study, exposure to
278 waterborne ³²P induced a 6h delay between embryos exposed to the highest dose, 10 mGy/day
279 (H), (HT₉₀: 174 hpf [169-176]) as compared to controls (HT₉₀: 168 hpf [164-169]). A study
280 performed on zebrafish embryos did not observe any modification of the hatching success of
281 embryos exposed to a radiation dose range of 1, 2, 5 and 10 Gy⁴⁰.

282 During hatching, biochemical and behavioural process are synchronised to destroy the
283 chorion⁴¹. Proteolytic enzymes and embryos movement contribute to the chorion disruption to
284 allow hatching. Hatching may be delayed because of potential changes induced by radiation to
285 those enzymes, as evidenced by studies on the effects of copper on rainbow trout eggs⁴¹. The
286 delay could also reflect a protective response to stress where the chorion would protect the
287 embryos from external hazard. A similar delay has been reported in zebrafish embryos exposed
288 to metals^{42,43}. The present data suggest that a delay in hatching is a good indicator of exposure to
289 environmental low dose of radiation in laboratory settings, which is in agreement with the study
290 by Bourrachot et al (2008)³⁹ that uses waterborne ²³³U. A developmental delay of maturing fish
291 eggs has also been observed in organism exposed in their natural environment. A delay of oocyte
292 growth has been evidenced in perch exposed to a total dose rate of 10-16 $\mu\text{Gy/h}$ (0.2-0.4
293 mGy/day) in exposed lakes at Chernobyl, 30 years after the accident, and was correlated to the
294 radiation dose³. The precise mechanism by which radiation induces this delay is unknown. At the

295 molecular level, a recent study found that transcriptional response of genes involved in the
296 circadian clock was modulated in zebrafish larvae (at 96 hpf) exposed to 0.4 and 4 mGy/h of
297 tritiated water²⁵.

298 In the present study, radiation exposure to 10 mGy/day did not significantly affect the growth
299 of embryos (no difference of diameter, area and eye distance at 4 dpf) and larvae (no difference
300 of head width and length at 10 dpf) respectively. Similarly, no deformity (short tail, spinal curve,
301 absence of pigment, failed hatching) and no length difference as compared to controls was
302 observed in embryos exposed to 9.6 mGy/h³⁷. A recent environmental study found that the length
303 and Fulton condition index of perch and roach were similar between lakes, in addition, no
304 malformation of gonads and oocytes was recorded revealing that fish were in good health in
305 general³. Other studies found a tail detachment in zebrafish exposed to 20 µg/L of ²³³U but not in
306 embryos exposed to 100 µg/L of ²³³U and a decrease of the body length for both exposures³⁹. A
307 reduction of body length was also found in zebrafish embryos exposed to high dose of radiation
308 (1 - 10 Gy)^{40,24}. Exposure to a γ radiation dose of 0.3 to 2 Gy/day induced morphological
309 abnormalities (tail atrophy and trunk axis malformations) in zebrafish embryos¹⁸. Using an acute
310 dose of radiation, deformities (including spinal curvature, pericardial cyst enlargement and
311 thoracic cavity variation) were noticed from 0.1 Gy, and hatching was reduced from 0.05 Gy¹⁹.
312 Malformations such as tail deformity, pericardial edema and spinal curve were found to increase
313 in zebrafish embryos exposed to 2 and 4 Gy of ⁵⁶Fe ion irradiation²⁰ and 0.01 to 1 Gy of γ
314 radiation²¹. Pericardial and yolk sac edema, curved notochord and thin caudal fin were observed
315 in the hermaphroditic fish embryos exposed to a total dose range of 2.5 - 10 Gy of γ radiation²².
316 Vertebral malformations were reported in medaka embryos exposed to 35.42 mGy/h of tritiated
317 water²³. Only a few studies have looked at the head development. Freeman et al. (2014)⁴⁰ found

318 that exposure to high dose of radiation (10 Gy) reduced eye diameter and head length. However,
319 this dose is higher than the environmental dose range used in the present study.

320 The physiology of 6 dpf embryos exposed to 10 mGy/day was not changed as compared to
321 controls based on the heart beats count. This is in agreement with a few previous studies that did
322 not observed any change in zebrafish embryos exposed to a total dose of 10 to 100 mGy for 10.9
323 s to 109 s respectively (dose rate of 79.2 Gy/day)¹⁹ or after exposure to 2 Gy of ⁵⁶Fe ion
324 irradiation (dose rate: 0.5 Gy/min)²⁰. The present work represents one of the rare studies that
325 have assessed this physiological criterion at environmental low doses.

326 Interestingly, no significant transcriptional changes were observed in 10 dpf larvae after
327 exposure to all dose levels. This may be a result of genes involved in protective mechanisms
328 already being activated before the larval stage to compensate for the negative effects of radiation
329 exposure, but returning to normal levels in later stages of development. In a recent transcriptomic
330 study assessing the effects of tritiated water on zebrafish embryos, it was suggested that the onset
331 of an early protective mechanism against oxidative stress may not be observed at the larval stage
332 of development (96 hpf)²⁵. Indeed, antioxidant defence mechanisms that are activated in embryos
333 may lead to a decrease in lipid peroxidation³⁷ and a reduction in DNA damage⁴⁴.

334 Another hypothesis is that the environmental doses used in the present study may be too low
335 for eliciting a differential gene expression change. The basal gene transcriptional levels may be
336 sufficient for the larvae to account for the effects of radiation. Results from the literature indicate
337 a change in gene expression for higher exposure. Kumar et al (2017)²⁴ found that exposure to 5
338 Gy of radiation induced transcriptional changes of *sox* genes involved in development. The
339 expression of genes seemed dependent on the embryonic stage of development and the dose
340 level. Transcriptional changes of genes involved in antioxidant defence were found in early stage

341 zebrafish embryos exposed to low doses, but not to high doses. These gene transcriptional levels
342 were unchanged at a later stage for both exposure conditions²⁵. The environmental doses used in
343 the present study and currently existing at Chernobyl may be too low to induce a significant
344 oxidative stress⁴⁵ and subsequent DNA damage. No genotoxic effect was evidenced as measured
345 by micronuclei in erythrocytes of perch, roach³ and catfish⁴⁶ exposed to environmental radiation
346 at Chernobyl.

347 These data suggest that low levels of radiation exposure have a negligible effect on gene
348 expression profiles and embryo growth and physiology but result in subtle delays to hatching
349 times that does not affect the final numbers of fish that hatched. The current levels of
350 environmental radiation at NPP sites are therefore unlikely to negatively impact embryonic
351 development of future offspring. These results support the findings from a previous large-scale
352 environmental study that found a delay in the maturation of perch eggs, and that fish were
353 otherwise in good general health³. Moreover, these results corroborate other environmental
354 studies led at Chernobyl on aquatic macro-invertebrate development and physiology^{45,46}. Finally,
355 this laboratory study is important as it provides environmentally relevant data to refine the
356 current thresholds for which an effect is observed.

357

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Figure and Table Legends.

Table 1. Morphological and physiological parameters recorded on 4 and 6 dpf embryos and 10 dpf larvae (mean \pm SEM, $n = 27$).

Figure 1. Hatching success of embryos exposed to control, Low (0.1 mGy/day), Medium (1 mGy/day) and High (10 mGy/day) conditions, recorded at 166, 168, 172 and 195 dpf (% , IC95, $n = 27$).

Figure 2. Time necessary to reach 90% of hatching for embryos exposed to control, Low (0.1 mGy/day), Medium (1 mGy/day) and High (10 mGy/day) conditions ($n = 27$, IC95).

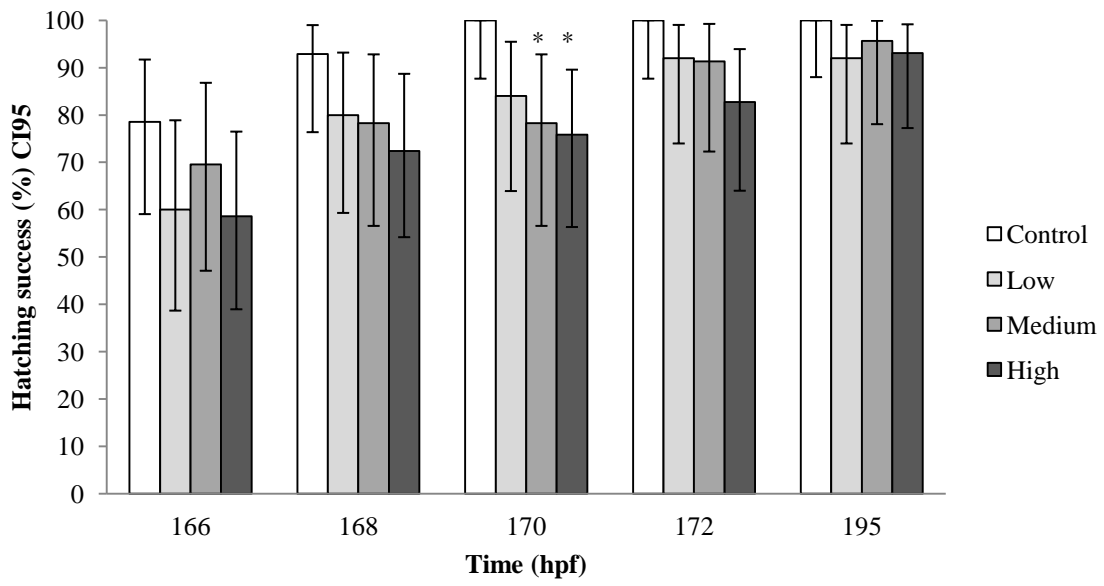
Figure 3. Principal component analysis showing the distribution of the 12 samples according to their gene expression profile over the top 500 genes based on their variance.

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586 **Table 1.**

Morphological and physiological parameters	Exposure condition	
	Control	10 mGy/day
Embryos 4 dpf		
Diameter (mm)	1.46 ± 0.01	1.46 ± 0.01
Area (mm ²)	1.67 ± 0.02	1.68 ± 0.02
Eye distance (mm)	0.91 ± 0.02	0.85 ± 0.03
Embryos 6 dpf		
Heart beats (beats/min)	150.0 ± 3.1	163.2 ± 12
Larvae 10 dpf		
Head width (mm)	0.81 ± 0.01	0.82 ± 0.01
Length (mm)	5.99 ± 0.05	5.98 ± 0.06

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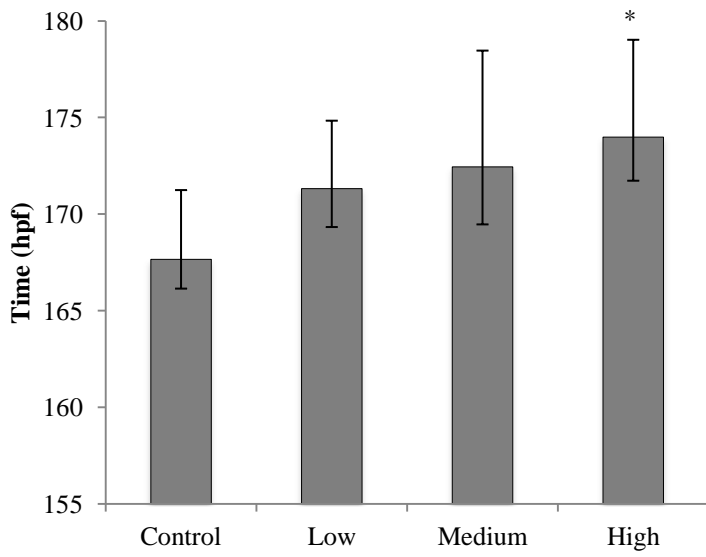
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603 **Figure 1.**



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Figure 2.



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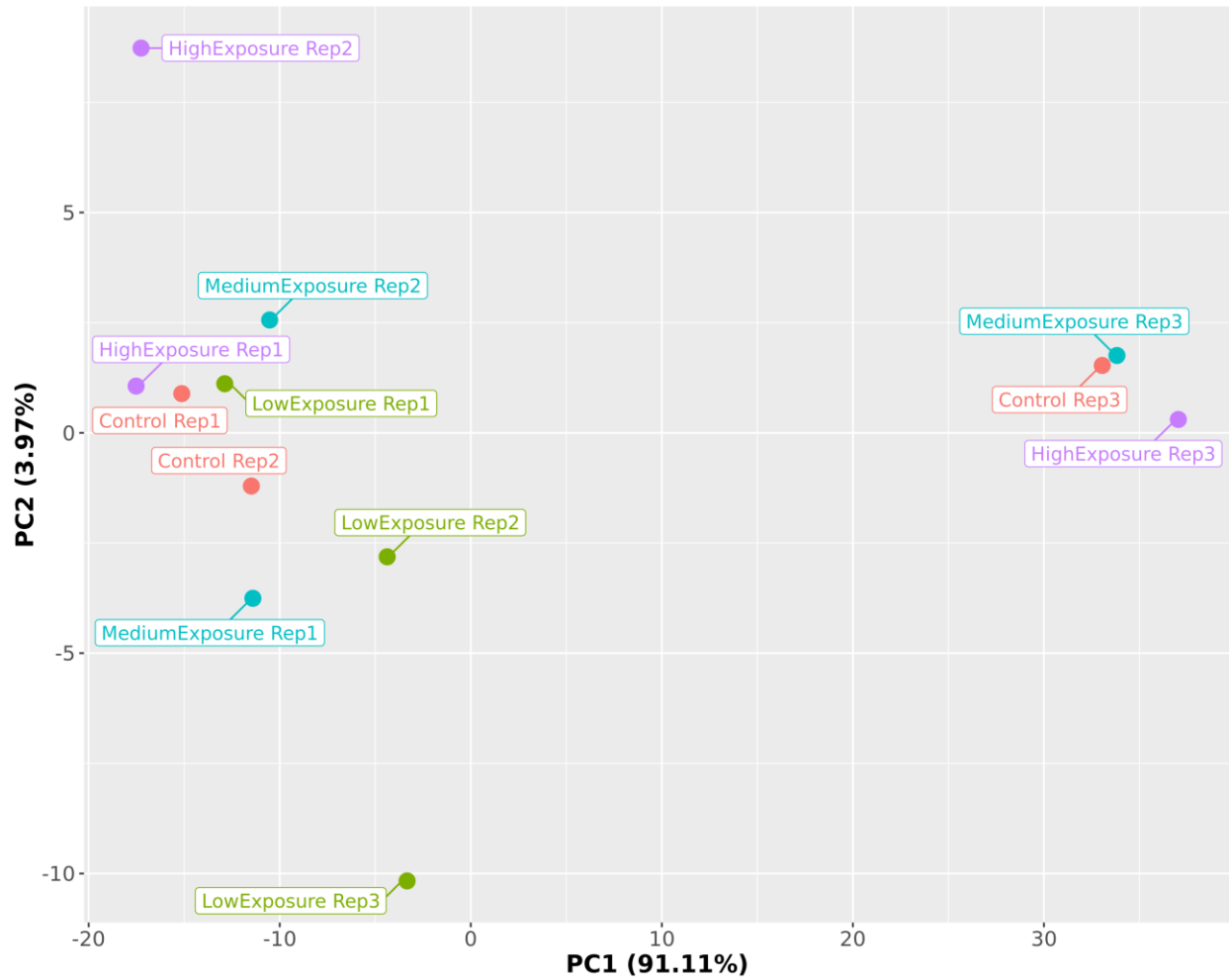
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635 **Figure 3.**

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645 **Supplemental Information.**

646 **Table S1.** Table showing the ^{32}P activity measured for each exposure solutions (corresponding to

647 the control, low: 0.1 mGy/day or 25 Bq/mL; medium: 1 mGy/day or 250 Bq/mL and high dose:

648 10 mGy/day or 2500 Bq/mL see Figure S1 for the dose calculation) renewed every 3 days (mean
649 \pm SD, $n = 3$). Measurements were performed using a HIDEX liquid scintillation counter.

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Day	Activity (Bq/mL)			
	Control	Low	Medium	High
0	0.6 \pm 0.1	22.6 \pm 0.5	229.0 \pm 6.8	2714.3 \pm 155.3
3	0.8 \pm 0.1	28.3 \pm 1.2	251.5 \pm 5.4	2533.9 \pm 31.3
6	0.6 \pm 0.1	31.4 \pm 1.2	290.0 \pm 3.2	2781.9 \pm 14.7
9	0.8 \pm 0.4	26.4 \pm 0.1	240.0 \pm 1.4	2320.5 \pm 19.8

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665 **Table S2.** Table showing the ^{32}P activity measured in the chorion and the dechorionated
666 embryos after 3 days of exposure to control, medium and high dose (mean \pm SD, $n = 3$) and in

667 the larvae after 9 days of exposure to control, medium and high dose (mean \pm SD, $n = 3$).

668 Measurements were performed using a HIDEX liquid scintillation counter.

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Tissue	Activity (Bq)			
	Control	Low	Medium	High
Chorion	1 \pm 0	1 \pm 0	3 \pm 2	42 \pm 11
Embryo	1 \pm 0	1 \pm 0	1 \pm 0	3 \pm 2
Larvae	1 \pm 0	1 \pm 0	1 \pm 0	2 \pm 0

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686 **Table S3.** Table showing the composition of the 12 different pools used for next generation

687 sequencing analyses.

Pool Id.	Treatment	Replicate (pool)	Number of embryos from each couple used to form the pool			
			Couple A	Couple B	Couple C	Couple D
1	Control	C01	3	3	3	1
2		C02	3	3	3	1
3		C03	1	0	0	5
4	Low	L11	3	3	3	1
5		L12	3	3	3	1
6		L13	2	2	2	4
7	Medium	M21	3	3	3	1
8		M22	3	3	3	1
9		M23	1	0	0	3
10	High	H31	3	3	3	1
11		H32	3	3	3	1
12		H33	0	0	0	4

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701 **Table S4.** Number of reads per condition throughout sample processing. Raw and trimmed

702 paired read counts are shown, along with the number of reads mapping to a unique locus

703 (“unique”) or multiple distinct loci (“multihits”). For multihits, the total number of hits and the
 704 number of distinct reads are shown in brackets. Reads were filtered to keep only those mapping
 705 uniquely in a proper pair with quality greater than 20 for downstream analyses.

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SampleName	NumReads	Trimmed	Unique	Multihits	Filtered	Mapped
Control_Rep1	2x 31,910,348	2x 31,801,110	57,429,028	10,218,142 (3,078,701)	57,422,444	90.30%
Control_Rep2	2x 38,110,879	2x 37,980,438	68,172,637	12,151,094 (4,032,549)	68,164,538	89.70%
Control_Rep3	2x 38,495,511	2x 38,344,466	67,359,423	19,103,766 (5,234,609)	67,350,294	87.80%
Low_Rep1	2x 29,136,639	2x 29,035,323	52,487,727	8,766,747 (2,746,623)	52,481,744	90.40%
Low_Rep2	2x 41,686,354	2x 41,527,406	74,369,507	13,157,459 (4,353,014)	74,359,872	89.50%
Low_Rep3	2x 31,456,558	2x 31,330,688	56,101,672	9,229,210 (3,025,834)	56,093,802	89.50%
Medium_Rep1	2x 37,321,589	2x 37,195,851	66,814,130	11518101 (3805997)	66,807,080	89.80%
Medium_Rep2	2x 36,890,705	2x 36,763,379	65,732,666	11977756 (4093513)	65,725,120	89.40%
Medium_Rep3	2x 31,668,750	2x 31,548,680	55,669,106	14570879 (4057295)	55,662,160	88.20%
High_Rep1	2x 22,580,848	2x 22,504,598	40,755,145	6789854 (2074281)	40,750,928	90.50%
High_Rep2	2x 32,552,649	2x 32,441,090	58,867,494	9306884 (2943724)	58,861,186	90.70%
High_Rep3	2x 29,308,498	2x 29,172,425	51,473,727	13017738 (3565734)	51,465,422	88.20%

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717 **Figure S1. Dose to fish egg.**

718 The mean dose to the egg calculated using the ERICA model is: 155 $\mu\text{Gy/h}$ per MBq/L in water.

719 The dose at the centre of the spherical egg calculated integrating the dose contribution from

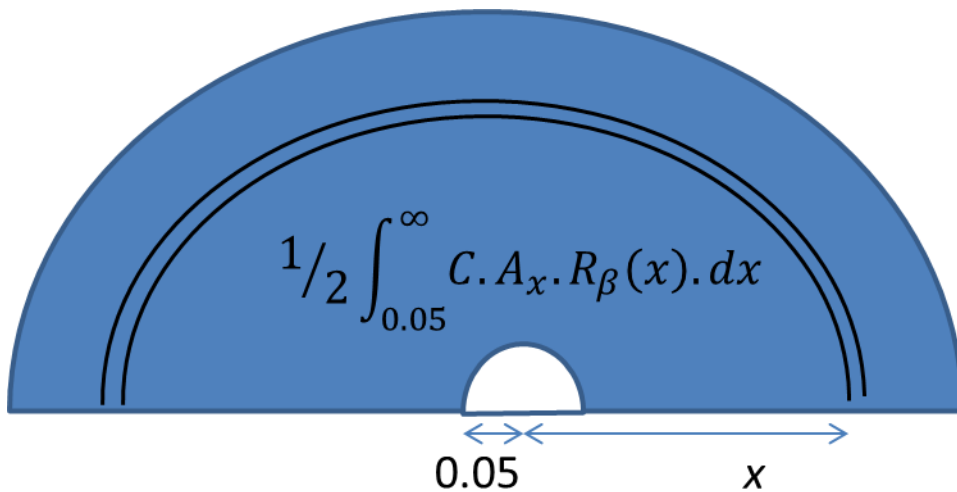
720 activity in water at different distances x (see Figure below) using data in Berger (1967) is 164

721 $\mu\text{Gy/h}$ per MBq/L.

722 C : Activity (Bq/L)

723 A : Area of hemisphere

724 R_β : Absorbed dose rate per Bq at a given distance x (Gy/s)



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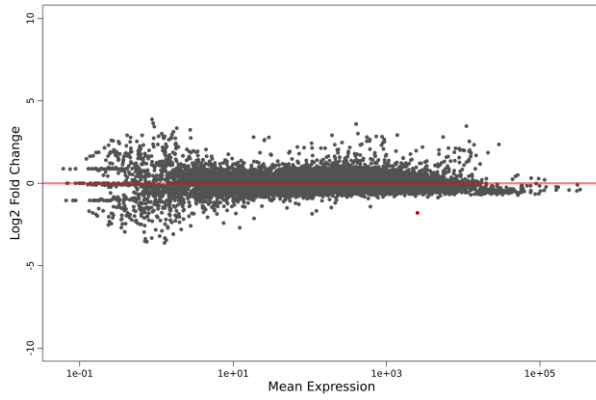
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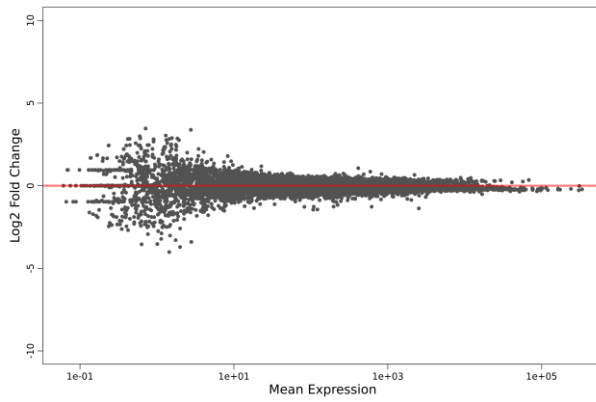
734 **Figure S2.** MvA plots for the DeSeq2-based differential expression analysis for a) low exposure,
735 b) medium exposure and c) high exposure samples.

736 a)



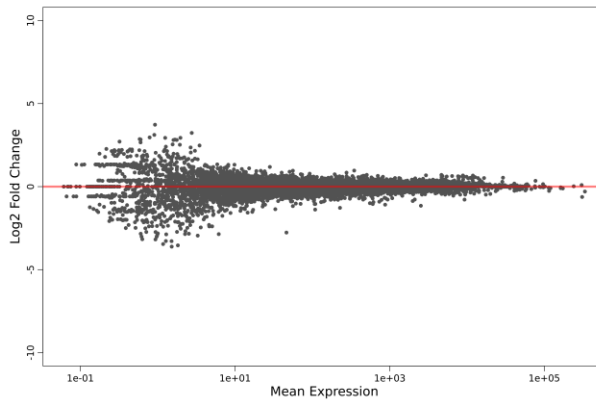
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738 b)



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745 **Figure S3.** Screen shot from the UCSC genome browser for the mapped read data for gene

746 ENSGACT00000015428.1, a homolog of POSTN.

