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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.

³²P is a radioisotope of 14.26 days half-life that emits β particles of high energy (1.7 MeV) whose track length is a few millimetres in water. A β -particle directly ionizes molecules by the removal of an electron, whereas α particles (and X-ray) ionize molecules by generating a series of fast electrons (effectively β particles) after first hitting molecules, therefore, the use of a β emitter is relevant to assess the effect of environmental radiation. In order to assess the effects of ionising radiation exposure on fish embryos under laboratory conditions, ³²P was selected due to 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 ³²P 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 Handford 69 plutonium reactors, the estimated level of ³²P in water between 1950 and 1971 varied from 0.1 to 70 7.7 Bq/L^5 and the activity of ³²P in fish was varied from 0.7 x 10³ to 22 x 10³ Bq/kg between 71 1962 and 1964⁶. Waterborne uptake of ³²P was found to be negligible as compared to uptake via 72 the dietary route in fish^{7,8}.

The 3-spined stickleback (*Gasterosteus aculeatus*) is a vertebrate model used in laboratory settings to assess the mechanistic effects of pollutants and in field surveys using multi-biomarker approaches⁹⁻¹⁰. The early stages of fish embryonic development are sensitive to ionising 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 on organism health differ¹². For instance, whilst some studies did not find evidence of radiation 80 effects on populations of aquatic invertebrates¹³, fish³ or mammals¹⁴ at Chernobyl, others found 81 adverse effects of radiation on the abundance of invertebrates¹⁵, birds¹⁶ and mammals¹⁷ at 82 83 Chernobyl and Fukushima. Thus, the dose at which significant damage to wildlife populations 84 occurs remains an open question 1^{12} .

Several studies have previously examined the effects of high doses of radiation (approximately 1000-fold above the environmental range) on the morphology of fish embryos. Exposure to acute dose of radiation induced various morphological abnormalities in embryos of zebrafish (*Danio rerio*)¹⁸¹⁹²⁰²¹, 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 to 35.42 mGy/h of tritiated water²³ and in zebrafish embryos exposed to 0.01 and 0.05 Gv of γ 92 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 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 95 96 percentage of mangrove killifish embryos that hatched decreased significantly after exposure to a total dose range of $2.5 - 10 \text{ Gy}^{22}$. The hatching time is significantly delayed as compared to 97 controls in zebrafish embryos exposed to a dose range of $0.1 - 10 \text{ Gy}^{24}$ and a dose rate range of 98 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 recorded after exposure to 1 and 100 mGy/day¹⁸. Hatching process appears to depend on the 104 105 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 of radiation¹¹. 107

Several studies investigated the effects of high doses of radiation on the cardiac physiology of fish embryos. Studies reported no effect¹⁹ or a decreased heart rate ²⁰ in zebrafish embryos. To our knowledge, no studies in the literature have yet described the heart physiology of embryos 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 was positively correlated to the dose¹⁹. Transcriptional response of genes involved in apoptosis²⁰ 116 and DNA damage repair mechanisms 20,25 were changed in zebrafish embryos exposed to 0.5 - 4 117 Gy of ⁵⁶Fe ion irradiation (Si et al. 2017)²⁰ or 9.6 and 96 mGy/day of γ radiation²⁵. 118 119 The present study is one of the few to assess of the effects of very low doses of radiation on

fish embryo development under laboratory conditions, combining both high throughput molecular and biometric analyses. The aim was to investigate whether exposure to environmental low doses of radiation induces developmental, physiological and transcriptional changes in stickleback embryos.

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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: 123, NaHCO₃: 65, KCl: 6 mg/L, pH = 7.5, $T^{\circ}C = 19^{\circ}C$, photoperiod: 16h light/8h dark)²⁶. Four 130 131 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

exposure conditions), 104 for next generation sequencing (NGS) analyses (Table S3) and 36 to
check ³²P 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 μ Gy/h) using a solution of adenosine triphosphate labelled on the gamma phosphate group with 140 ³²P (PerkinElmer). The final concentration of ATP in control, low and medium experimental tubes was adjusted to $1.1 \times 10^{-5} \mu M$ by addition of stable ATP. This ATP concentration is 141 negligible as compared to the concentration found in a typical cell of 5 mM^{27} . The doses 142 143 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 µGy/h (0.96 mGy/day) described in the radiological impacts on non- human species report of the EA in 2011^{28} . Control embryos were kept in clean artificial water. 2.5 mL of the 147 148 water was renewed every 3 days. The embryos were exposed for 10 days and euthanized according to schedule 1 of the Home Office Licence (ASPA, 1986)²⁹ using tricaine 149 150 methanesulfonate (Sigma).

151 *Dose calculation and monitoring*

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

155 Developmental and physiological measurements

156 The morphological parameters were measured using a Zeiss axiozoom microscope and the Zen157 Pro software. The physiological parameter was measured using an optical microscope. The

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

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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 that 20 were182 used in downstream analyses.

183 Differential expression analyses

Differential expression analyses were conducted using the DESeq2 package³³ in R. Gene 184 models were taken from Ensembl version 82^{31} , and read counts over unique genes were 185 quantified using the "summarizeOverlaps()" function in the GenomicAlignments package³⁴ 186 187 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 Benjamini and Hochberg correction³⁵. To account for potential confounding effects of lineage 189 190 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 conducted using the clusterProfiler package³⁶. 196

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.

205 **Results**

206 Dose monitoring

The mean activity of 32 P solutions measured during the 10 days exposure experiment were 0.7 207 208 \pm 0.2, 27 \pm 3, 253 \pm 24 and 2588 \pm 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 embryos were 1.46 \pm 0.01 mm, 1.68 \pm 0.02 mm² and 0.85 \pm 0.03 mm respectively. These 220 221 measures did not significantly differ from controls (diameter: 1.46 ± 0.01 mm, p = 0.65, area: $1.67 \pm 0.02 \text{ mm}^2$, p = 0.64 and eye distance: $0.85 \pm 0.03 \text{ mm}$, p = 0.17) (Table 1). After 6 days, 222 223 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 \pm 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

was 5.98 \pm 0.06 mm and their head width 0.82 \pm 0.01 mm. These values did not significantly differ from controls (length: 5.99 \pm 0.05 mm, p = 0.88 and head width: 0. 81 \pm 0.01 mm, p =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 (HT_{90}) [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 (HT_{90} : 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*

Analysis of the NGS data quality identified these data as showing excellent base calling qualities and post-filtering mapping rates of approximately 90% were seen throughout (Table S4). Following gene abundance identification, principal component analysis identified little difference between the dosage treatments in these data (Figure 3). A batch effect, resulting from the parentage of individuals pooled in the different replicates was found (Table S3). This batch 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 treatments against a control treatment as described. No significant change in gene expression was observed, with only a single gene showing significant differential expression in the low dosage after filtering (Figure S2).

253 Data availability

254The RNA sequencing data can be obtained from ArrayExpress255(https://www.ebi.ac.uk/arrayexpress/) with the accession number E-MTAB-7872.

256

257 Discussion

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

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

Exposure to different dose and nature of radiation can accelerate hatching. For instance, zebrafish embryos exposed to 9.6 mGy/h for 65h³⁶, 0.3 to 2 Gy/day¹⁸, 1-1000 mGy/day¹¹ and to an X-ray dose of 25 mGy³⁷ hatched earlier than control embryos. The embryonic stage at which radiation exposure occurs, appears to have consequences on the hatching sensitivity of embryos. For instance, mangrove killifish embryos displayed a higher sensitivity when exposed at an early stage to 2.5, 5, 7.5, and 10 Gy of γ radiation. In addition, the hatching success was significantly 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 an X-ray dose above 25 mGy delayed hatching zebrafish embryos³⁸. Waterborne exposure to 20 275 and 100 μ g/L of ²³³U induced a 12h delay in hatching time (HT₅₀: 59[54-66] and HT₅₀: 59[53-276 68] respectively), as compared to controls $(HT_{50}: 47[45-48])^{39}$. In our study, exposure to 277 waterborne ³²P induced a 6h delay between embryos exposed to the highest dose, 10 mGy/day 278 279 (H), (HT₉₀: 174 hpf [169-176]) as compared to controls (HT₉₀: 168 hpf [164-169]). A study 280 perfomed on zebrafish embryos did not observe any modification of the hatching success of embryos exposed to a radiation dose range of 1, 2, 5 and 10 Gy^{40} . 281

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 those enzymes, as evidenced by studies on the effects of copper on rainbow trout eggs⁴¹. The 285 286 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 to metals^{42,43}. The present data suggest that a delay in hatching is a good indicator of exposure to 288 289 environmental low dose of radiation in laboratory settings, which is in agreement with the study by Bourrachot et al (2008)³⁹ that uses waterborne ²³³U. A developmental delay of maturing fish 290 291 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 µGy/h (0.2-0.4 293 mGy/day) in exposed lakes at Chernobyl, 30 years after the accident, and was correlated to the radiation dose³. The precise mechanism by which radiation induces this delay is unknown. At the 294

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 observed in embryos exposed to 9.6 mGy/ h^{37} . A recent environmental study found that the length 302 303 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 general³. Other studies found a tail detachment in zebrafish exposed to 20 μ g/L of ²³³U but not in 305 embryos exposed to 100 μ g/L of ²³³U and a decrease of the body length for both exposures³⁹. A 306 307 reduction of body length was also found in zebrafish embryos exposed to high dose of radiation $(1 - 10 \text{ Gy})^{40,24}$. Exposure to a γ radiation dose of 0.3 to 2 Gy/day induced morphological 308 309 abnormalities (tail atrophia and trunk axis malformations) in zebrafish embryos¹⁸. Using an acute 310 dose of radiation, deformities (including spinal curvature, pericardial cyst enlargement and thoracic cavity variation) were noticed from 0.1 Gy, and hatching was reduced from 0.05 Gy¹⁹. 311 312 Malformations such as tail deformity, pericardial edema and spinal curve were found to increase in zebrafish embryos exposed to 2 and 4 Gy of 56 Fe ion irradiation 20 and 0.01 to 1 Gy of γ 313 radiation²¹. Pericardial and yolk sac edema, curved notochord and thin caudal fin were observed 314 315 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 water²³. Only a few studies have looked at the head development. Freeman et al. $(2014)^{40}$ found 317

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

The physiology of 6 dpf embryos exposed to 10 mGy/day was not changed as compared to controls based on the heart beats count. This is in agreement with a few previous studies that did not observed any change in zebrafish embryos exposed to a total dose of 10 to 100 mGy for 10.9 s to 109 s respectively (dose rate of 79.2 Gy/day)¹⁹ or after exposure to 2 Gy of ⁵⁶Fe ion irradiation (dose rate: 0.5 Gy/min)²⁰. The present work represents one of the rare studies that 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 of development (96 hpf)²⁵. Indeed, antioxidant defence mechanisms that are activated in embryos 332 may lead to a decrease in lipid peroxidation³⁷ and a reduction in DNA damage⁴⁴. 333

Another hypothesis is that the environmental doses used in the present study may be too low for eliciting a differential gene expression change. The basal gene transcriptional levels may be sufficient for the larvae to account for the effects of radiation. Results from the literature indicate a change in gene expression for higher exposure. Kumar et al (2017)²⁴ found that exposure to 5 Gy of radiation induced transcriptional changes of *sox* genes involved in development. The expression of genes seemed dependent on the embryonic stage of development and the dose 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 otherwise in good general health³. Moreover, these results corroborate other environmental 353 studies led at Chernobyl on aquatic macro-invertebrate development and physiology^{45,46}. Finally, 354 355 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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549		Freshwater	Crustacean	Asellus	Aquaticus	along	a	Gradient	of	Radionuclide
550		Contaminati	on at Chernol	byl. <i>Scienc</i>	ce of The Tot	al Envir	onm	ent 2018 , 6	528-0	629, 11–17.

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

566 Figure 1. Hatching success of embryos exposed to control, Low (0.1 mGy/day), Medium (1

567 mGy/day) and High (10 mGy/day) conditions, recorded at 166, 168, 172 and 195 dpf (%, IC95, *n*

568 = 27).

569 Figure 2. Time necessary to reach 90% of hatching for embryos exposed to control, Low (0.1

570 mGy/day), Medium (1 mGy/day) and High (10 mGy/day) conditions (n = 27, IC95).

571 **Figure 3.** Principal component analysis showing the distribution of the 12 samples according to

572 their gene expression profile over the top 500 genes based on their variance.

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physiological parametersControl10 mGy/dayEmbryos 4 dpfDiameter (mm) 1.46 ± 0.01 1.46 ± 0.01 Area (mm²) 1.67 ± 0.02 1.68 ± 0.02 Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpfHeart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpfHead width (mm) 0.81 ± 0.01 0.82 ± 0.01	physiological parametersControl10 mGy/dayEmbryos 4 dpf 1.46 ± 0.01 1.46 ± 0.01 Diameter (mm) 1.46 ± 0.01 1.46 ± 0.01 Area (mm²) 1.67 ± 0.02 1.68 ± 0.02 Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpf 163.2 ± 12 Larvae 10 dpf 142 ± 0.01 Head width (mm) 0.81 ± 0.01 0.82 ± 0.01 Length (mm) 5.99 ± 0.05 5.98 ± 0.06	Morphological and	Exposure condition						
Embryos 4 dpfDiameter (mm) 1.46 ± 0.01 1.46 ± 0.01 Area (mm²) 1.67 ± 0.02 1.68 ± 0.02 Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpfHeart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpfHead width (mm) 0.81 ± 0.01 0.82 ± 0.01	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	physiological parameters	Control	10 mGy/day					
Diameter (mm) 1.46 ± 0.01 1.46 ± 0.01 Area (mm²) 1.67 ± 0.02 1.68 ± 0.02 Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpfHeart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpfHead width (mm) 0.81 ± 0.01 0.82 ± 0.01	Diameter (mm) 1.46 ± 0.01 1.46 ± 0.01 Area (mm²) 1.67 ± 0.02 1.68 ± 0.02 Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpfHeart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpfHead width (mm) 0.81 ± 0.01 0.82 ± 0.01 Length (mm) 5.99 ± 0.05 5.98 ± 0.06	Embryos 4 dpf							
Area (mm²) 1.67 ± 0.02 1.68 ± 0.02 Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpf 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpf 0.81 ± 0.01 0.82 ± 0.01	Area (mm²) 1.67 ± 0.02 1.68 ± 0.02 Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpfHeart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpfHead width (mm) 0.81 ± 0.01 0.82 ± 0.01 Length (mm) 5.99 ± 0.05 5.98 ± 0.06	Diameter (mm)	1.46 ± 0.01	1.46 ± 0.01					
Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpf 150.0 ± 3.1 163.2 ± 12 Heart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpf 0.81 ± 0.01 0.82 ± 0.01	Eye distance (mm) 0.91 ± 0.02 0.85 ± 0.03 Embryos 6 dpf 1 Heart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpf 1 Head width (mm) 0.81 ± 0.01 0.82 ± 0.01 Length (mm) 5.99 ± 0.05 5.98 ± 0.06	Area (mm ²)	1.67 ± 0.02	1.68 ± 0.02					
Embryos 6 dpfHeart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpfHead width (mm) 0.81 ± 0.01 0.82 ± 0.01	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	Eye distance (mm)	0.91 ± 0.02	0.85 ± 0.03					
Heart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpf 163.2 \pm 0.01 10.82 \pm 0.01 Head width (mm) 0.81 ± 0.01 0.82 ± 0.01	Heart beats (beats/min) 150.0 ± 3.1 163.2 ± 12 Larvae 10 dpf 0.81 ± 0.01 0.82 ± 0.01 Head width (mm) 5.99 ± 0.05 5.98 ± 0.06	Embryos 6 dpf							
Larvae 10 dpf Head width (mm) 0.81 ± 0.01 0.82 ± 0.01	Larvae 10 dpf Head width (mm) 0.81 ± 0.01 0.82 ± 0.01 Length (mm) 5.99 ± 0.05 5.98 ± 0.06	Heart beats (beats/min)	150.0 ± 3.1	163.2 ± 12					
Head width (mm) $0.81 \pm 0.01 0.82 \pm 0.01$	Head width (mm) 0.81 ± 0.01 0.82 ± 0.01 Length (mm) 5.99 ± 0.05 5.98 ± 0.06	Larvae 10 dpf							
	Length (mm) 5.99 ± 0.05 5.98 ± 0.06	Head width (mm)	0.81 ± 0.01	0.82 ± 0.01					
Length (mm) $5.99 \pm 0.05 5.98 \pm 0.06$		Length (mm)	5.99 ± 0.05	5.98 ± 0.06					

Figure 1.



Figure 2.



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635	Figure 3.		
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Table S1. Table showing the ³²P activity measured for each exposure solutions (corresponding to
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

 \pm SD, n = 3). Measurements were performed using a HIDEX liquid scintillation counter.

	Day					Ac	tivity (B	q/mL)				-	-	• _	• _	_	-	-	-	_	_	_	_	-
	Day	y	Co	ntrol	Lo	W	Me	dium	Hi	gh														
	0		0.6	± 0.1	22.6	± 0.5	5 229.	0 ± 6.8	2714.3	± 155.3														
	3		0.8	± 0.1	28.3	± 1.2	2 251.	5 ± 5.4	2533.9	± 31.3														
	6		0.6	± 0.1	31.4	± 1.2	2 290.	0 ± 3.2	2781.9	± 14.7														
	9		0.8	± 0.4	26.4	± 0.1	240.	0 ± 1.4	2320.5	± 19.8														
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00		<i>34</i> .	1 0010	511U W 11	ig ine	Г	activity	measured	111 UIC		and	1	i une											

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.

	Tissuo	Activity (Bq)					
	1 issue	Control	Low	Medium	High		
	Chorion	1 ± 0	1 ± 0	3 ± 2	42 ± 11		
	Embryo	1 ± 0	1 ± 0	1 ± 0	3 ± 2		
	Larvae	1 ± 0	1 ± 0	1 ± 0	2 ± 0		
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	Tabla S3 Ta	bla chowing th	a composition	of the 12	different pools use		
	1 aute 55. 1 a	ore showing th	e composition	of the 12	unrerent pools use		
	sequencing an	alyses.					

Pool Id. Treatmer		Danliasta (naal)	Number of embryos from each couple used to form the pool							
Pool 1a.	1 reatment	Replicate (pool)	Couple A	Couple B	Couple C	Couple D				
1		C01	3	3	3	1				
2	Control	C02	3	3	3	1				
3		C03	1	0	0	5				
4		L11	3	3	3	1				
5	Low	L12	3	3	3	1				
6		L13	2	2	2	4				
7		M21	3	3	3	1				
8	Medium	M22	3	3	3	1				
9		M23	1	0	0	3				
10		H31	3	3	3	1				
11	High	H32	3	3	3	1				
12		H33	0	0	0	4				
Table S4	Number of	raade nar condi	tion through	ut comple proc	accing Dow and	trimmed				
paired re	ad counts are	e shown, along v	with the num	ber of reads ma	apping to a uni	que locus				

("unique") or multiple distinct loci ("multihits"). For multihits, the total number of hits and the
number of distinct reads are shown in brackets. Reads were filtered to keep only those mapping

uniquely in a proper pair with quality greater than 20 for downstream analyses.

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%

717 Figure S1. Dose to fish egg.

- The mean dose to the egg calculated using the ERICA model is: 155 μ Gy/h per MBq/L in water.
- 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
- μ Gy/h per MBq/L.
- *C*: Activity (Bq/L)
- 723 A: Area of hemisphere
- R_{β} : Absorbed dose rate per Bq at a given distance x (Gy/s)



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Figure S2. MvA plots for the DeSeq2-based differential expression analysis for a) low exposure,

b) medium exposure and c) high exposure samples.





746 ENSGACT00000015428.1, a homolog of POSTN.

