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

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20

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/day<sup>1</sup>  
48 and then rapidly declined due to the decrease of short life radionuclides and sedimentation  
49 processes<sup>2</sup>.

50 Three decades after the Chernobyl NPP accident, the main radionuclides of concern are <sup>90</sup>Sr (a  
51  $\beta$  emitter) and <sup>137</sup>Cs (a  $\beta$  and  $\gamma$  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 low<sup>3</sup>. 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  $\mu$ Gy/h respectively (less than 0.4 mGy/day)<sup>3</sup>.

57 <sup>32</sup>P is a radioisotope of 14.26 days half-life that emits  $\beta$  particles of high energy (1.7 MeV)  
58 whose track length is a few millimetres in water. A  $\beta$ -particle directly ionizes molecules by the  
59 removal of an electron, whereas  $\alpha$  particles (and X-ray) ionize molecules by generating a series  
60 of fast electrons (effectively  $\beta$  particles) after first hitting molecules, therefore, the use of a  $\beta$ -  
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, <sup>32</sup>P was selected due to  
63 its short half-life minimising the radioactive waste and its high energy  $\beta$  particles.

64 <sup>32</sup>P 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 <sup>32</sup>P in the Yenisei river that were found to have accumulated in

67 fish, with  $^{32}\text{P}$  activities of 2.2 Bq/L in water and 2900 Bq/kg in fish found at 200 km from the  
68 industrial site<sup>4</sup>. In the Columbia River contaminated by the cooling water from the Hanford  
69 plutonium reactors, the estimated level of  $^{32}\text{P}$  in water between 1950 and 1971 varied from 0.1 to  
70 7.7 Bq/L<sup>5</sup> and the activity of  $^{32}\text{P}$  in fish was varied from  $0.7 \times 10^3$  to  $22 \times 10^3$  Bq/kg between  
71 1962 and 1964<sup>6</sup>. Waterborne uptake of  $^{32}\text{P}$  was found to be negligible as compared to uptake via  
72 the dietary route in fish<sup>7,8</sup>.

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 approaches<sup>9-10</sup>. The early stages of fish embryonic development are sensitive to ionising  
76 radiation<sup>11</sup>.

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 differ<sup>12</sup>. For instance, whilst some studies did not find evidence of radiation  
81 effects on populations of aquatic invertebrates<sup>13</sup>, fish<sup>3</sup> or mammals<sup>14</sup> at Chernobyl, others found  
82 adverse effects of radiation on the abundance of invertebrates<sup>15</sup>, birds<sup>16</sup> and mammals<sup>17</sup> at  
83 Chernobyl and Fukushima. Thus, the dose at which significant damage to wildlife populations  
84 occurs remains an open question<sup>12</sup>.

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*)<sup>18,19,20,21</sup>, mangrove killifish (*Kryptolebias marmoratus*)<sup>22</sup> and medaka (*Oryzias latipes*)<sup>23</sup>.

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 water<sup>23</sup> and in zebrafish embryos exposed to 0.01 and 0.05 Gy of  $\gamma$   
93 radiation<sup>21</sup>. 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<sup>21,24</sup>, and to a dose rate range of 0.3 - 2 Gy/day of  $\gamma$  radiation<sup>18</sup>. The  
96 percentage of mangrove killifish embryos that hatched decreased significantly after exposure to a  
97 total dose range of 2.5 - 10 Gy<sup>22</sup>. The hatching time is significantly delayed as compared to  
98 controls in zebrafish embryos exposed to a dose range of 0.1 - 10 Gy<sup>24</sup> and a dose rate range of  
99 0.3 - 2 Gy/day of  $\gamma$  radiation<sup>18</sup>.

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  $\gamma$  radiation dose of 10 and 1000 mGy/day, but no change was  
104 recorded after exposure to 1 and 100 mGy/day<sup>18</sup>. 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 radiation<sup>11</sup>.

108 Several studies investigated the effects of high doses of radiation on the cardiac physiology of  
109 fish embryos. Studies reported no effect<sup>19</sup> or a decreased heart rate<sup>20</sup> 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 dose<sup>19</sup>. Transcriptional response of genes involved in apoptosis<sup>20</sup>  
117 and DNA damage repair mechanisms<sup>20,25</sup> were changed in zebrafish embryos exposed to 0.5 - 4  
118 Gy of <sup>56</sup>Fe ion irradiation (Si et al. 2017)<sup>20</sup> or 9.6 and 96 mGy/day of  $\gamma$  radiation<sup>25</sup>.

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 (CaCl<sub>2</sub>: 294, MgSO<sub>4</sub>:  
130 123, NaHCO<sub>3</sub>: 65, KCl: 6 mg/L, pH = 7.5, T°C = 19°C, photoperiod: 16h light/8h dark)<sup>26</sup>. 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  $^{32}\text{P}$  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  $^{32}\text{P}$  (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  $5 \text{ mM}^{27}$ . 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 2011<sup>28</sup>. 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)<sup>29</sup> 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)<sup>30</sup> (Figure S1).  
153 Activity of  $^{32}\text{P}$  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 (mm<sup>2</sup>) 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  $33.4 \pm 5.2$  M paired reads per sample (Table S4). Quality control of raw fastq  
176 reads was conducted using fastQC ([bioinformatics.babraham.ac.uk/projects/fastqc/](http://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/](http://bioinformatics.babraham.ac.uk/projects/trim_galore/)). Trimmed reads were mapped against  
179 the BROAD S1 *Gasterosteus aculeatus* 3-spined stickleback genome from Ensembl<sup>30</sup> using the  
180 STAR universal RNA seq aligner<sup>31</sup> 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 package<sup>33</sup> in R. Gene  
185 models were taken from Ensembl version 82<sup>31</sup>, and read counts over unique genes were  
186 quantified using the “summarizeOverlaps()” function in the GenomicAlignments package<sup>34</sup>  
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 correction<sup>35</sup>. 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 package<sup>36</sup>.

### 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  $\alpha$  error was applied.

204

## 205 **Results**

### 206 *Dose monitoring*

207 The mean activity of  $^{32}\text{P}$  solutions measured during the 10 days exposure experiment were 0.7  
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  $1 \pm 0$  Bq for controls and low exposure  
211 conditions, and  $3 \pm 2$  and  $42 \pm 11$  Bq for the medium and high exposure conditions (mean  $\pm$  SD,  
212  $n = 3$ ). The activities measured in dechorionated embryos were  $1 \pm 0$  Bq for control, low and  
213 medium exposure conditions and  $3 \pm 2$  Bq for the high exposure condition (mean  $\pm$  SD,  $n = 3$ ).  
214 After 9 days of exposure, the activities measured in the larvae were  $1 \pm 0$  for control, low and  
215 medium exposure conditions and  $2 \pm 0$  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  $1.46 \pm 0.01$  mm,  $1.68 \pm 0.02$  mm<sup>2</sup> and  $0.85 \pm 0.03$  mm respectively. These  
221 measures did not significantly differ from controls (diameter:  $1.46 \pm 0.01$  mm,  $p = 0.65$ , area:  
222  $1.67 \pm 0.02$  mm<sup>2</sup>,  $p = 0.64$  and eye distance:  $0.85 \pm 0.03$  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  $163.2 \pm 12$  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

227 was  $5.98 \pm 0.06$  mm and their head width  $0.82 \pm 0.01$  mm. These values did not significantly  
228 differ from controls (length:  $5.99 \pm 0.05$  mm,  $p = 0.88$  and head width:  $0.81 \pm 0.01$  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 ( $HT_{90}$ ) [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 ( $HT_{90}$ : 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 mGy<sup>19</sup> and after 2 and 4 Gy of  
261 exposure to <sup>56</sup>Fe<sup>20</sup>.

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 65h<sup>36</sup>, 0.3 to 2 Gy/day<sup>18</sup>, 1-1000 mGy/day<sup>11</sup> and to  
269 an X-ray dose of 25 mGy<sup>37</sup> 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  $\gamma$  radiation. In addition, the hatching success was significantly  
273 decreased in embryos that were exposed early (10.5 hpf) for all doses<sup>22</sup>.

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 embryos<sup>38</sup>. Waterborne exposure to 20  
276 and 100  $\mu\text{g/L}$  of <sup>233</sup>U induced a 12h delay in hatching time (HT<sub>50</sub>: 59[54-66] and HT<sub>50</sub>: 59[53-  
277 68] respectively), as compared to controls (HT<sub>50</sub>: 47[45-48])<sup>39</sup>. In our study, exposure to  
278 waterborne <sup>32</sup>P induced a 6h delay between embryos exposed to the highest dose, 10 mGy/day  
279 (H), (HT<sub>90</sub>: 174 hpf [169-176]) as compared to controls (HT<sub>90</sub>: 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 Gy<sup>40</sup>.

282 During hatching, biochemical and behavioural process are synchronised to destroy the  
283 chorion<sup>41</sup>. 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 eggs<sup>41</sup>. 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<sup>42,43</sup>. 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)<sup>39</sup> that uses waterborne <sup>233</sup>U. 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 dose<sup>3</sup>. 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 water<sup>25</sup>.

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/h<sup>37</sup>. 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 general<sup>3</sup>. Other studies found a tail detachment in zebrafish exposed to 20 µg/L of <sup>233</sup>U but not in  
306 embryos exposed to 100 µg/L of <sup>233</sup>U and a decrease of the body length for both exposures<sup>39</sup>. A  
307 reduction of body length was also found in zebrafish embryos exposed to high dose of radiation  
308 (1 - 10 Gy)<sup>40,24</sup>. 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 embryos<sup>18</sup>. 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 Gy<sup>19</sup>.  
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 <sup>56</sup>Fe ion irradiation<sup>20</sup> and 0.01 to 1 Gy of γ  
314 radiation<sup>21</sup>. 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 radiation<sup>22</sup>.  
316 Vertebral malformations were reported in medaka embryos exposed to 35.42 mGy/h of tritiated  
317 water<sup>23</sup>. Only a few studies have looked at the head development. Freeman et al. (2014)<sup>40</sup> 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)<sup>19</sup> or after exposure to 2 Gy of <sup>56</sup>Fe ion  
324 irradiation (dose rate: 0.5 Gy/min)<sup>20</sup>. 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)<sup>25</sup>. Indeed, antioxidant defence mechanisms that are activated in embryos  
333 may lead to a decrease in lipid peroxidation<sup>37</sup> and a reduction in DNA damage<sup>44</sup>.

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)<sup>24</sup> 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 conditions<sup>25</sup>. The environmental doses used in  
343 the present study and currently existing at Chernobyl may be too low to induce a significant  
344 oxidative stress<sup>45</sup> and subsequent DNA damage. No genotoxic effect was evidenced as measured  
345 by micronuclei in erythrocytes of perch, roach<sup>3</sup> and catfish<sup>46</sup> 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 health<sup>3</sup>. Moreover, these results corroborate other environmental  
354 studies led at Chernobyl on aquatic macro-invertebrate development and physiology<sup>45,46</sup>. 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).

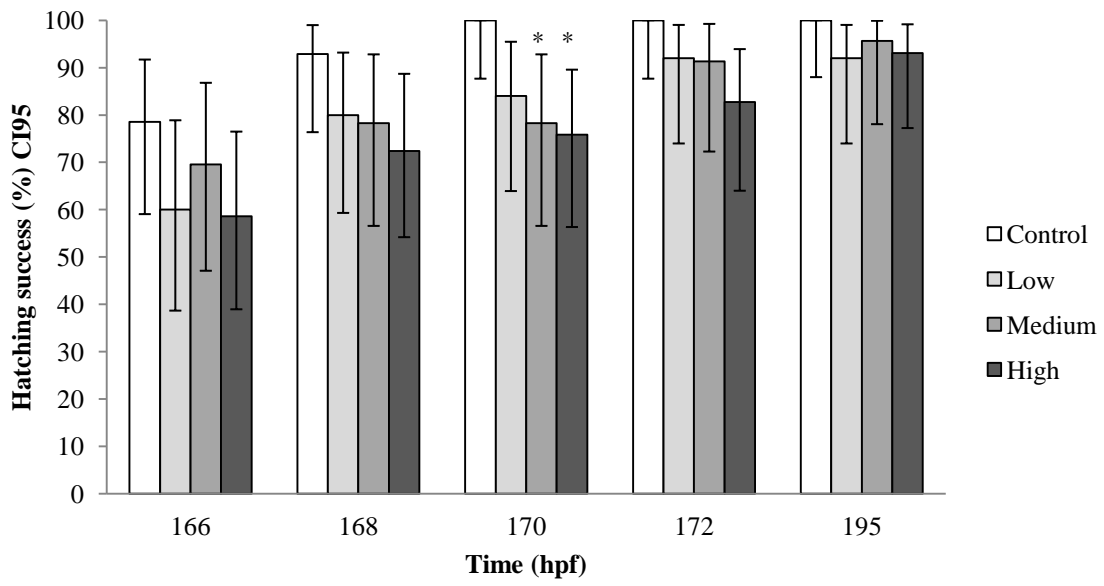
**Figure 3.** Principal component analysis showing the distribution of the 12 samples according to their gene expression profile over the top 500 genes based on their variance.

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586 **Table 1.**

<b>Morphological and physiological parameters</b>	<b>Exposure condition</b>	
	<b>Control</b>	<b>10 mGy/day</b>
<b>Embryos 4 dpf</b>		
Diameter (mm)	1.46 ± 0.01	1.46 ± 0.01
Area (mm <sup>2</sup> )	1.67 ± 0.02	1.68 ± 0.02
Eye distance (mm)	0.91 ± 0.02	0.85 ± 0.03
<b>Embryos 6 dpf</b>		
Heart beats (beats/min)	150.0 ± 3.1	163.2 ± 12
<b>Larvae 10 dpf</b>		
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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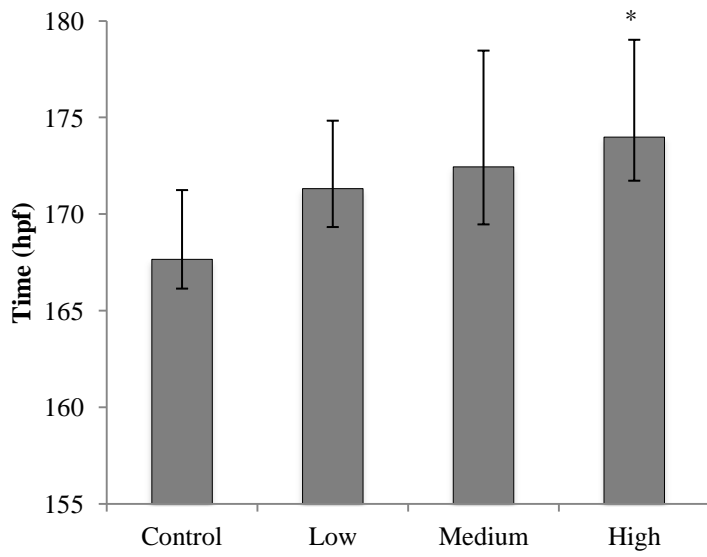
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603 **Figure 1.**



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**Figure 2.**



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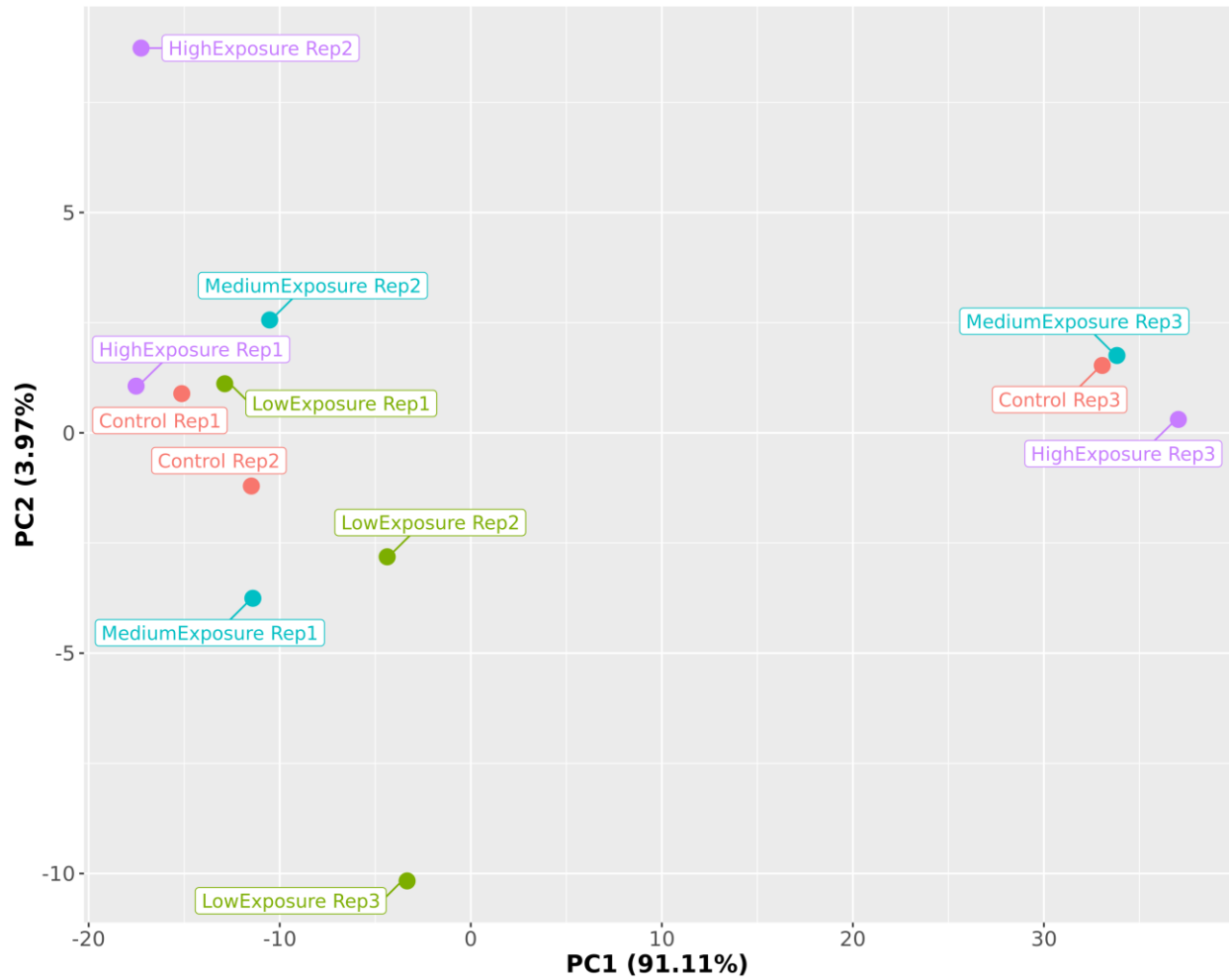
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635 **Figure 3.**

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645 **Supplemental Information.**

646 **Table S1.** Table showing the  $^{32}\text{P}$  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  $^{32}\text{P}$  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.

669

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

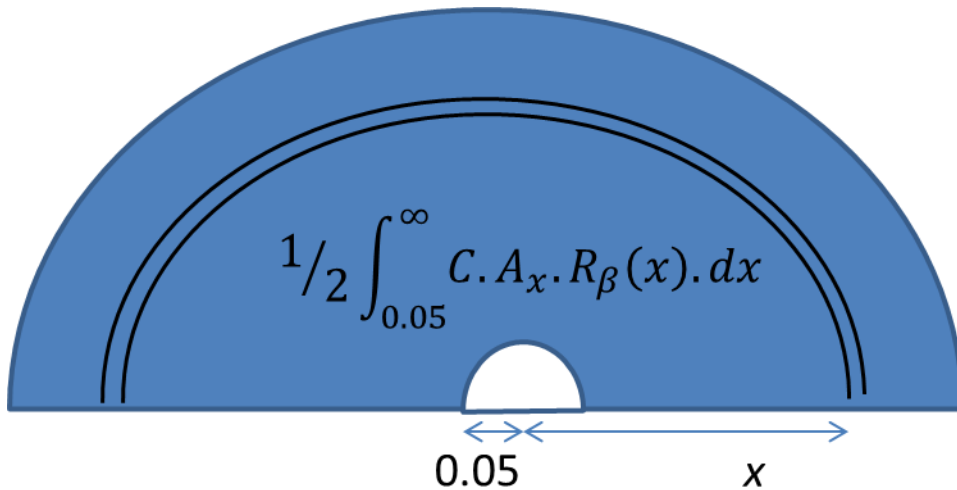
720 activity in water at different distances  $x$  (see Figure below) using data in Berger (1967) is 164

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

722  $C$ : Activity (Bq/L)

723  $A$ : Area of hemisphere

724  $R_\beta$ : Absorbed dose rate per Bq at a given distance  $x$  (Gy/s)



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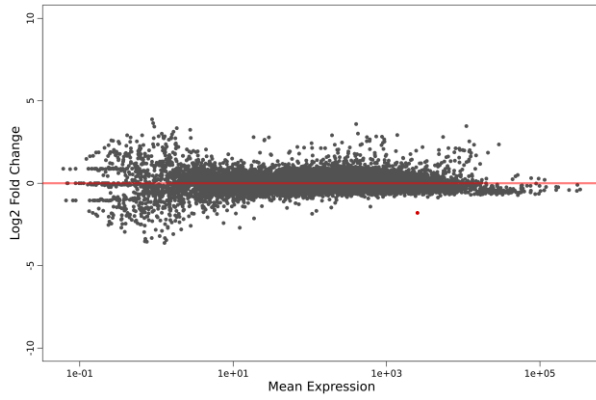
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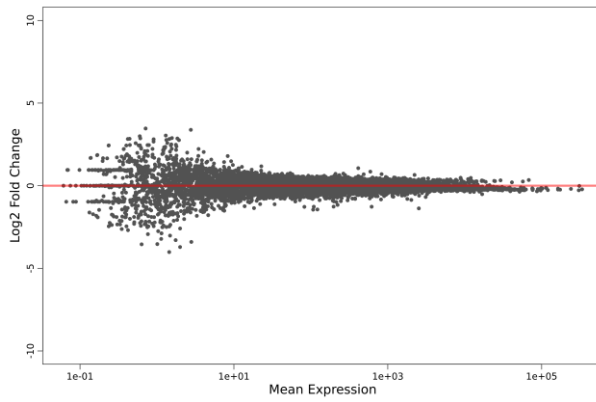
734 **Figure S2.** MvA plots for the DeSeq2-based differential expression analysis for a) low exposure,  
735 b) medium exposure and c) high exposure samples.

736 a)



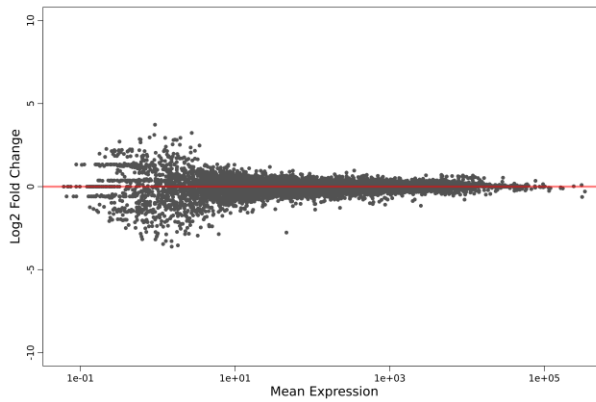
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738 b)



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740 c)



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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.

