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Transcriptional Changes in the Ovaries of Perch from Chernobyl

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1 *Transcriptional changes in the ovaries of perch from*
2 *Chernobyl*

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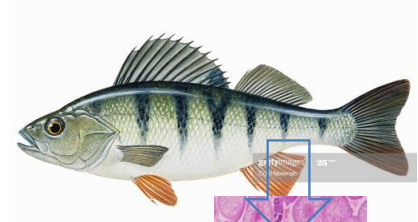
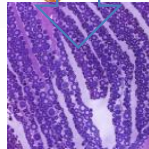
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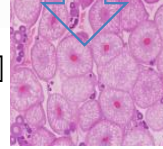
IRRADIATED Lakes



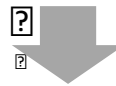
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Developed

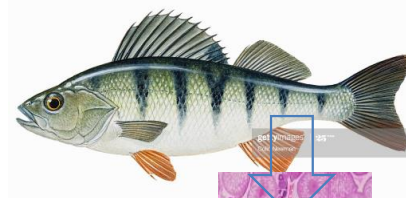


NGS & De novo transcriptome assembly

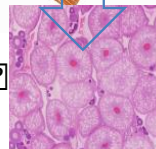


Differentially expressed genes

REFERENCE Lakes



Developed



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33 **Abstract**

34 Fish have been highly exposed to radiation in freshwater systems after the Chernobyl Nuclear
35 Power Plant (NPP) accident in 1986, and in freshwater and marine systems after the more recent
36 Fukushima NPP accident in 2011. In the years after the accident, the radioactivity levels rapidly
37 declined due to radioactive decay and environmental processes, but chronic lower dose
38 exposures persisted. In order to gain insights into the long-term effects of environmental low
39 dose radiation on fish ovaries development, a high-throughput transcriptomic approach including
40 a *de novo* assembly was applied to different gonad phenotypes of female perch: developed
41 gonads from reference lakes, developed/irradiated from medium contaminated lake and both
42 developed/irradiated and undeveloped from more highly contaminated lakes. This is the most
43 comprehensive analysis to date of the gene responses in wildlife reproductive system to
44 radiation. Some gene responses that were modulated in irradiated gonads were found to be
45 involved in biological processes including cell differentiation and proliferation (*ggnb2*, *mod5*,
46 *rergl*), cytoskeleton organization (*k1C18*, *mtpn*), gonad development (*nell2*, *tcp4*), lipid
47 metabolism (*ldah*, *at11b*, *nltp*), reproduction (*cyb5*, *cyp17A*, *ovos*), DNA damage repair (*wdhd1*,
48 *rad51*, *hus1*) and epigenetic mechanisms (*dmap1*). Identification of these genes provides a better
49 understanding of the underlying molecular mechanisms underpinning the development of the
50 gonad phenotypes of wild perch and how fish may respond to chronic exposure to radiation in
51 their natural environment, though causal attribution of gene responses remains unclear in the
52 undeveloped gonads.

53 **Introduction**

54 Whilst the biological effects of acute exposure to radiation in laboratory settings is well
55 documented (Frederica radiation database: www.frederica-online.org), little is known about the
56 long-term effects of low dose exposure to radiation on organisms in their natural environment.
57 Moreover, results from the literature on the health effects of low doses and the fate of wildlife in
58 the Chernobyl exclusion zone differ^{1,2}. For instance, some studies did not find evidence of
59 radiation effects on populations of aquatic³⁻⁶, and terrestrial⁷⁻¹⁰ animals whereas others found
60 adverse effects of radiation on the abundance of invertebrates¹¹, birds¹² and mammals¹³ at
61 Chernobyl. Thus, the dose at which significant damage to wildlife populations occurs remains an
62 open question².

63 Fish have been highly exposed at both Chernobyl and Fukushima, following Nuclear Power
64 Plant (NPP) accidents in 1986 and 2011 respectively. The highest dose rate to fish after the
65 Chernobyl accident was estimated to be 400 $\mu\text{Gy}/\text{h}$ ¹⁴. Thereafter, dose rates rapidly declined
66 mainly due to the decay of short-lived isotopes. Three decades after the Chernobyl Nuclear
67 Power Plant (NPP) accident, the main radionuclides of concern are ⁹⁰Sr (a β -emitter) and ¹³⁷Cs (a
68 β - and γ -emitter) due to their long radioactive half-lives (28 and 30 years respectively)⁴. The
69 contribution of transuranium radioelements to the total dose to fish at Chernobyl is very low⁴.
70 The total dose to perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) in 2015 varied from 0.8 to
71 14.1 and 1.7 to 15.7 $\mu\text{Gy}/\text{h}$ respectively⁴. Biomagnification of ¹³⁷Cs was observed between roach
72 (an omnivorous species) and perch (a carnivorous species) from several lakes in Belarus and
73 Ukraine, 30 years after the Chernobyl NPP accident⁴.

74 Morphological changes of the reproductive system of fish, such as asymmetry, sterility,
75 abnormal cells or absence of gonads, were observed several years after both the Chernobyl
76 accident and the Kyshtym disaster at the Mayak plutonium production site in Russia^{15,16}. In a

77 recent large-scale study of the effects of chronic radiation on fish from Chernobyl, the
78 reproductive system of the European perch, *P. fluviatilis*, appeared to be more sensitive to
79 radiation than an omnivorous species, the roach, *R. rutilus* for which no effect was recorded⁴. In
80 female perch remaining at Chernobyl, environmental radiation induced a delay of the maturation
81 of the gonads as evidenced by the positive correlation between the percentage of immature
82 oocytes composing the developed gonads of fish from exposed lakes and the gradient of
83 radiation dose across the seven lakes⁴. In addition, a significant proportion of the females had
84 undeveloped gonads in two of the three highly contaminated lakes⁴. However, since
85 undeveloped gonads were not seen in the third highly contaminated lake, it remains unclear
86 whether this phenotype is a direct result of radiation exposure or instead some other
87 environmental factors: the two lakes in which the effect was seen were artificial reservoirs
88 (Chernobyl Cooling Pond and Yanovsky Crawl) whereas the third lake was a natural floodplain
89 lake (Glubokoye).

90 To our knowledge, the effects of radiation on the transcriptome of fish have been assessed
91 through a few laboratory studies performed in adult Atlantic salmon (*Salmo salar*)¹⁷, embryos of
92 zebrafish (*Danio rerio*)^{18,19} and embryos of stickleback (*Gasterosteus aculeatus*)²⁰. However, the
93 effects of radiation on the transcriptome of aquatic organisms exposed in their natural
94 environment are still uncharacterised. Moreover, the effects of chronic environmental radiation
95 on the molecular mechanisms involved in gonad maturation of fish and potentially underlying
96 the delay in the gonad maturation process previously observed in perch at Chernobyl are
97 unknown.

98 In order to address this knowledge gap, we applied a high-throughput transcriptomic approach
99 to observed gonad phenotypes of female fish exposed to a gradient of radiation at Chernobyl.

100 The transcriptome of perch collected from lakes located inside and outside the Chernobyl
101 Exclusion Zone (CEZ) was sequenced, assembled *de novo* and annotated. Changes in the
102 transcriptional responses were assessed between developed female gonads from reference lakes,
103 developed/irradiated female gonads from lakes characterised by medium (M) (Svyatoye) and
104 high (H) (Chernobyl Cooling Pond and Yanovsky Crawl) levels of radioactive contamination,
105 and undeveloped/irradiated female gonads from lakes characterised by high (H) levels of
106 radioactive contamination.

107

108 **Methods**

109 **Fish collection.** 24 female European perch (*P. fluviatilis*) were selected from the September
110 2014 field work session (as described in Lerebours *et al.*, 2018⁴). Fish were collected from 6
111 lakes in Belarus and Ukraine using 3 gill nets of 20 m length and 21 mm mesh size to ensure the
112 capture of homogenous groups of mature fish. Perch were carefully removed from the nets and
113 placed into tanks containing aerated water. Fish fell unconscious by a blow to the head and were
114 then killed by performing a concussion of the brain to limit as much as possible the suffering as
115 recommended by UK Home Office procedures (ASPA, 1986)²¹. Scales were sampled for age
116 determination. The body weight, total length and presence of external signs of disease were
117 noted for each fish using methodology specified by the International Council for the Exploration
118 of the Sea (Feist *et al.*, 2004)²². The Fulton condition, hepatosomatic and gonadosomatic index
119 were determined. The fulton condition (K), hepatosomatic index (HSI), and gonadosomatic
120 index (GSI) were determined as follow: $K = \text{body weight} / (\text{total length (cm)}^3) \times 100$; $\text{HSI} = (\text{liver}$
121 $\text{weight/body weight}) \times 100$ and $\text{GSI} = (\text{gonad weight/body weight}) \times 100$, respectively. (Table
122 1).

123 **Sampling sites.** Six lakes situated in Belarus and Ukraine were selected according to their
124 hydrological properties and the long-term exposure to a gradient of radiation doses (Table S1).
125 Lakes were classified into three exposure groups, reference (R) for Gorova, Dvoriche and
126 Stoyacheye lakes, medium (M) for Svyatoye lake, and high (H) for Yanovsky and Cooling Pond
127 lakes based on the total dose rates to perch in 2015. The lakes are situated at distances from 1.5
128 to 225 km of the Chernobyl NPP (Supplementary Table S1).

129 **Water chemistry.** Multiple chemical parameters were measured to assess the presence of
130 potential confounding abiotic factors in each lake during all sampling sessions. The pH,
131 temperature, dissolved oxygen (DO), conductivity ($\mu\text{S}/\text{cm}$), major alkali and alkali-earth element
132 water concentrations (Na, Mg, S, K and Ca), trace elements concentrations (As, Sr, Cd, Cs, Pb
133 and U) and nutrients (NO_3^- , NO_2^- and PO_4^{3-}) were measured in the 6 lakes (Table S2).

134 **Activity measurements of ^{137}Cs and ^{90}Sr and dose calculation.** Measurement of ^{137}Cs and
135 ^{90}Sr in the body were performed for 5 individual fish from each lake and doses rates were
136 calculated as described in Lerebours *et al.* (2018)⁴ (Table S1).

137 **Histological analyses.** Histological analyses were performed in order to determine the gonadal
138 maturation stage. A standardised cross section of gonad was removed for each fish and fixed for
139 24 h in 10% neutral buffered formalin before transfer to 70% ethanol for subsequent histological
140 assessment. Gonads were processed in a vacuum infiltration processor using standard
141 histological protocols²² and embedded in paraffin wax. Using a rotary microtome, sections of 5-7
142 μm were taken and subsequently stained with haematoxylin and eosin (H&E). The gonadal stage
143 was defined by determining the percentage of immature (perinuclear) and mature (cortical
144 alveolar) oocytes observed in a defined surface area at magnification 10x using a microscope
145 (Zeiss axiozoom) (Figure 1).

146 **Next generation sequencing (NGS).** For each fish, the section adjacent to the section
147 dedicated to histological analyses was dissected and immediately snap frozen in liquid nitrogen
148 for transcriptomic analyses. 6 pools of female perch gonads were formed as followed: 1 pool of 3
149 developed/irradiated and 1 pool of 3 undeveloped gonads from fish collected in Yanovsky (H)
150 lake; 1 pool of 3 developed/irradiated and 1 pool of 3 underdeveloped gonads from fish collected
151 in Cooling Pond (H); 1 pool of 3 developed/irradiated gonads from fish collected in Svyatoye
152 (M) and 1 pool of 9 developed/reference gonads from Gorova, Dvoriche and Stoyacheye (R)
153 lakes (3 gonads from each) (Table S3). Total RNAs were extracted using the High Pure RNA
154 Tissue kit (Roche Diagnostics Ltd, West Sussex, U.K.) according to the supplier's instructions.
155 RNA quality and integrity were evaluated using a bioanalyzer. RNA integrity numbers ranged
156 from 7.8 to 9.3. A total of 6 pools were used for NGS analyses. Sequencing libraries were
157 prepared for each pool using the TruSeq Stranded mRNA protocol (Illumina, San Diego,
158 California, USA) and sequenced using the Illumina HiSeq 2500 analyser using a 125 base
159 paired-end run (Genepool facility, Edinburgh, UK).

160 **Assembly and Annotation of the European Perch.** The quality of the raw reads was assessed
161 using FastQC v0.11.7. Sample contamination was assessed by mapping raw reads against the
162 European Perch (*P. fluviatilis*) genome from Ozerov *et al.*, (2018)²³, as well as genomes from
163 multiple species taken from the Ensembl database version 91²⁴ using Fastq Screen²⁵. Adapter
164 sequences and poor-quality bases (poly-N ambiguous base calls, or contiguous base calls with
165 quality score less than 20) were trimmed from the raw reads using Trim Galore v0.4.4.
166 Transcripts from all samples were combined and assembled into a putative transcriptome using
167 Trinity v2.5.1²⁶. The parameters used are described in Supplementary Text. Putative coding
168 regions within assembled transcripts were identified using TransDecoder v5.0.2 using default

169 parameters to identify open reading frames of 100 amino acids or more. Transcripts were
170 annotated by using Trinotate v3.02, which combines multiple sources of transcript annotation
171 including annotation against the Universal Protein Knowledge Base (UniProtKB) database (The
172 UniProt Consortium) using BLAST, the Protein family (Pfam) database²⁷ using HMMER
173 (HMMER Web Server), Clusters of Orthologous Groups of proteins (eggNOG) database²⁸, the
174 Kyoto Encyclopedia of Genes and Genomes (KEGG) database²⁹, and Gene Ontology (GO)
175 database³⁰. For each transcript, the annotated gene name was taken from either the BLAST
176 results of the longest TransDecoder peptide sequence (blastp), or from the translated nucleotide
177 sequence directly if no open reading frame was identified (blastx). The corresponding species
178 and gene name from UniProtKB/Swiss-Prot were used for annotation and gene filtering.

179 **Differential Expression Analysis.** Transcript abundance was estimated for each of the
180 individual samples against the assembled transcriptome using Kallisto v0.43³¹ with parameters “-
181 -rf-stranded”. All assembled transcripts were considered in the primary analysis and quality
182 control stage. After assessing sample quality based on the complete transcriptome, the transcripts
183 were filtered to keep only the transcripts 1) greater than 500 bases, 2) with a complete open
184 reading frame, and 3) with a normalised fragment per kilobase mapped (FPKM) abundance
185 above 1 in at least one of the six samples (to avoid low abundance transcripts). In addition,
186 transcripts most closely matching Archaeal, Bacterial or Viral species were removed (Figure S1).
187 Differential expression analysis between the different conditions was conducted using DESeq2³²
188 package in R (R Core team 2017)³³. Comparisons were made between the phenotypes
189 (developed/reference, developed/irradiated, undeveloped/irradiated) by combining pools
190 (described in Table S3) from different sampling sites as replicates. Resulting *p*-values were
191 adjusted for multiple testing using the Benjamini and Hochberg false discovery rate correction³⁴.

192 Genes were identified as differentially expressed between conditions if they showed a fold
193 change greater than 2-fold (upregulated or downregulated), with an adjusted p value less than
194 0.05. In addition, to avoid over-inflating changes in low abundance transcripts, differentially
195 expressed genes were chosen such that the normalized FPKM expression measure was greater
196 than 1 for at least one of the two conditions being compared. Careful attention should be paid
197 when reading the gene expression results between two phenotypes in Table S8 especially when
198 the comparison with a FPKM value of 0 is indicated. Indeed, this can result in very high
199 $\log_2\text{FoldChange}$. Therefore, the consideration of the sense of the expression (up- or
200 downregulated) rather than the expression value is recommended. Significantly enriched gene
201 ontology terms and pathways were identified against the *D. rerio* database by PANTHER
202 v14.1³⁵.

203 **Quantitative Real-Time RT-PCR.** For each individual fish gonad, first strand cDNAs were
204 synthesized from 1 μg of total RNA using the AffinityScript Multiple Temperature cDNA
205 Synthesis Kit (Agilent Technologies, Stockport, U. K.) and according to the supplier's
206 instructions. Primer pairs and FAMTM-TAMRATM dye probes used to amplify the target
207 sequences (Table S4) were designed using the Prime Express software (Applied Biosystem). 10
208 ng of the reverse transcribed product was used as a template for subsequent polymerase chain
209 reaction (PCR) in a 20 μL final volume using 1x of TaqMan® Fast Advanced Master Mix (Life
210 technologies, Paisley, U.K.), primers and probe at a final concentration of 900 nM and 250 nM
211 respectively and following the supplier's protocol. PCR reactions were performed in the Applied
212 BiosystemsTM ViiATM 7 Real-Time PCR System using the following programme: one cycle at
213 95°C for 20 s and 40 amplification cycles at 95°C for 3 s and 60°C for 30 s. Primer efficiencies
214 were determined by 10 times dilution series of the cDNA template and were about 100%. The

215 stability of the expressions of 3 reference genes (*efl*, *actb*, *tbb*) was tested on all the samples and
216 analysed using NormFinder. The expression of the β actin gene displayed the highest stability.

217 **Statistical analyses.** Statistical analyses were performed using R version 3.1.2 (R Core team
218 2017)³³. After satisfying the assumptions of the normal distribution of the residuals using the
219 Shapiro-Wilk test, ANOVA models were used to assess the potential differences. The
220 significance of parameters at the different sites was assessed using the contrast method with the
221 esticon function in R (DoBy package)^{36,37}. If the normality of the residuals was not respected a
222 Kruskal-Wallis rank test was applied. When significant, post-hoc tests were performed and a
223 Bonferroni correction of the α error was applied.

224 **Data availability.** Raw sequencing data were deposited in the NCBI Sequence Read Archive
225 (SRA) and the complete transcriptome was deposited in the Transcriptome Shotgun Assembly
226 (TSA) database. These data have been collected under BioProject accession code PRJNA556365
227 (<https://www.ncbi.nlm.nih.gov/bioproject>).

228 **Results**

229 **General health condition.** The body weight, total length and hepatosomatic index of fish
230 displaying the different phenotypes were similar across lakes ($p > 0.05$) (Table 1). The Fulton
231 condition (K) index of fish from Cooling Pond (H) and Yanovsky (H) lakes were decreased as
232 compared to the Fulton condition index of fish with developed gonads from reference lakes ($p <$
233 0.05) (Table 1). There was not any difference between the K index of fish from Svyatoye lake
234 (M) and fish from the reference lakes (Table 1).

235 **Reproductive status.** Histological analyses showed that the undeveloped gonads were
236 composed of immature oocytes only, and that developed gonads, from both reference and
237 irradiated lakes, of both immature and maturing oocytes. The distributions of immature oocytes

238 in the developed gonads were 55 % in fish from the reference lakes, 61 % in fish from the
239 medium (M) irradiated lake and 68 % in fish from high (H) irradiated lakes (Table 1, Figure 1).
240 The weights of undeveloped gonads in fish from Cooling pond and Yanovsky lake were
241 significantly lower than the weights of developed/irradiated gonads in fish from the same lakes,
242 Svyatoye lake, and the weights of developed gonads in fish from the reference lakes ($p < 0.05$)
243 (Table 1). The gonadosomatic index (GSI) of fish with undeveloped gonads was significantly
244 lower than the GSI of fish with developed/irradiated and developed/reference gonads ($p < 0.05$)
245 (Table 1). The GSI of fish with developed/irradiated gonads from Cooling Pond, Yanovsky lake
246 and the GSI of fish with developed gonads from reference lakes are similar ($p > 0.05$) (Table 1).
247 The GSI of fish with developed/irradiated gonads from Svyatoye is significantly lower than GSI
248 of fish from reference lakes ($p = 0.027$) (Table 1).

249 **De novo transcriptome assembly.** Illumina sequencing generated a total of 151,785,472
250 paired-end reads. The number of paired-end reads for each of the 6 sample pools can be seen in
251 Supplementary Table S5. Reads were aligned against the European Perch (*P. fluviatilis*) genome
252 from Ozerov *et al.*, (2018)²³, as well as multiple model genome species taken from the Ensembl
253 database v91²⁴. The vast majority (more than 95 %) of all reads mapped to the perch genome,
254 with the remaining reads either showing no hits, or mapping to related fish species (Figure S2).
255 The base calling (method for determining the base sequence of a given nucleotide sequence)
256 quality scores were consistently high throughout the data set. Trimming to remove adapters,
257 ambiguous base calls (of unknown value) and poor-quality base calls (below some pre-defined
258 threshold) removed only a small proportion (0.1%) of reads. In total, 151,590,007 quality
259 trimmed paired-end reads were used for *de novo* transcriptome assembly. The *P. fluviatilis*
260 transcriptome was assembled *de novo* as described by combining all paired-end reads from

261 across the dataset. The final assembly consists of 534,099 transcripts (clustered into 309,937
262 “genes”) ranging from 201 to 24,533 bases in size (further filtered later for short fragment < 500
263 bases). The mean transcript size was 871.5 bases, the N50 score (a weighted median of the
264 assembly such that 50% of the assembly is contained within contigs equal to or larger than this
265 score) was 1,879 bases and the GC content was 46.31 % (Table S6). Mapping of the reads for the
266 individual samples back to this assembled transcriptome resulted in mapping rates of 73.7-85.1
267 % (Table S6).

268 In total, 155,437 (29.1 %) of the assembled transcripts were annotated against the
269 UniProtKB/Swiss-prot database as described in supplementary material. 33,043 filtered
270 transcripts were produced, of which 31,029 (93.9 %) were annotated to a total of 10,662 unique
271 genes, suggesting that there is some redundancy in the assembly (Figure S1).

272 **Functional classification of transcriptome.** Gene Ontology (GO) analysis was used to
273 identify enriched categories of functional classes amongst the expressed genes. Potential GO
274 classes were identified for 9,415 (88.3%) of the 10,662 annotated protein coding genes identified
275 in the perch transcriptome. Gene ontology analysis showed enrichment for classes such as
276 chromosome organization, cell cycle, cytoskeleton, response to stress, lipid binding, cell
277 differentiation and proliferation, anatomical structure development and morphogenesis
278 (Supplementary text, Figure S4).

279 **Gene expression analysis.** Principal component analysis (PCA) of the top changing genes in
280 the data set showed that the largest source of variation (accounting for 37.7 % of the variance)
281 was between the undeveloped and developed phenotype (Figure S5). The second greatest source
282 of variation (18.0 %) highlighted differences between developed/reference and
283 developed/irradiated samples (Figure S5). The normalised gene expression for the 33,043 filtered

284 transcripts across the samples represented on the heatmap (Figure S6) showed a cluster of the
285 undeveloped gonads in fish from high irradiated lakes and a cluster of developed gonads in fish
286 from high exposed, medium exposed and reference lakes. Sub-clusters are observed between the
287 developed gonads exposed to a gradient of radiation across the lakes (Figure S6).

288 Transcripts expressed in each sample were identified based on a normalised FPKM abundance
289 greater than 1, and a comparison between all samples showed that 10,897 out of 33,043 filtered
290 transcripts were expressed in all samples and a smaller proportion of transcripts were expressed
291 specifically in one sample (Figure S7). The developed/reference samples showed 1,166 uniquely
292 expressed transcripts. In contrast, the developed/irradiated and undeveloped/irradiated samples
293 showed 4,770 and 3,690 uniquely expressed transcripts respectively. The irradiated samples
294 showed an increase in the number of transcripts expressed.

295 **Quantitative real-time PCR validation.** Gene expression changes of five random genes
296 (*ska1*, *gthb1*, *cp2g1*, *star* and *hus1*) using quantitative real-time PCR technique were of similar
297 direction to the changes obtained by the NGS technique (Table S7).

298 **Gene expression changes between irradiated phenotypes (undeveloped and developed)**
299 **and developed/reference gonads.** Analysis of genes showing differential expression between
300 undeveloped/irradiated gonads and developed/reference gonads identified a total of 1825
301 transcripts (Figure 2) among which 836 (732 genes) were upregulated and 989 (769 genes) were
302 downregulated in undeveloped gonads (Table S8).

303 Analysis of genes showing differential expression between developed/irradiated fish gonads
304 sampled from the three contaminated lakes and developed/reference fish gonads sampled from
305 the reference lakes identified a total of 610 transcripts (Figure 2) among which 552 (496 genes)
306 were upregulated and 58 (57 genes) downregulated (Figure S8c, Table S8). The gene expression

307 profiles varied between Svyatoye Lake (M), Yanovsky Lake (H) and Cooling Pond (H) (Figure
308 S8c).

309 **Common differentially expressed genes between two or three phenotype comparisons,**
310 **potentially involved in gonadal growth and response to radiation exposure.** Examples
311 include genes belonging to enriched classes such lipid metabolism (*at11b*, *ldah*, *nltf*),
312 development (*prgr*, *vegfc*), reproduction (*cytb5*, *cyp17a*, *ovos*, *odo1*), development timing (*nell2*,
313 *tcp4*), cytoskeleton (*k1c18*, *mtpn*), cell proliferation and differentiation (*ggnb2*, *mod5*, *rerpl*,
314 *EIF31*, *gps2*), DNA damage repair (*wdhd1*, *rad51*, *hus1*) and epigenetic mechanisms (*dmap1*)
315 (Table 2, S8, S9).

316

317 **Discussion**

318 Long-term exposure to stress can inhibit gonad development in fish and explain the presence
319 of undeveloped phenotypes but causal factors are rarely described in the literature. This
320 undeveloped phenotype is not necessarily causally connected to radiation as it was not found in
321 female fish from the third most contaminated lake, Glubokoye, in 2015⁴. Other factors might be
322 involved such as food availability and social interactions. Populations may also have adapted to
323 long-term exposure to radiation through generations that have passed since the accident,
324 potentially having led to the disappearance of the disadvantageous phenotype in some lakes. In
325 the present study, perch from the medium and highly contaminated lakes are able to form
326 developed gonads similarly to perch from reference lakes as shown by similar gonadosomatic
327 index. However, this adaptation may not be without consequences; at the cellular level, a delay
328 of the maturation of the oocytes was significant and correlated to the gradient of radiation dose to
329 fish⁴.

330 In order to better understand the long-term effects of exposure to radiation on the significant
331 delay of oocyte development previously observed in Lerebours *et al.* (2018)⁴, the current study
332 looked at the differential gene expression changes between developed/irradiated and
333 developed/reference gonads and some genes in common with the undeveloped/irradiated gonads.

334 Interestingly, some genes were found to be involved in gonad maturation, lipid metabolism,
335 reproduction, cell structure, development timing, cell proliferation and differentiation, DNA
336 repair and epigenetics (further bibliographical information on the selected genes can be found in
337 Supplementary Table S10).

338 **Differential expression of genes potentially involved in gonadal growth.** Some
339 differentially expressed genes involved in energetic metabolism, may play a role in gonadal
340 growth by ensuring the supply of energetic demand during this developmental process. For
341 instance, the protein encoding for *odo1* gene, overexpressed in irradiated samples, catalyzes the
342 conversion of 2-oxoglutarate, an intermediate of the Krebs cycle. Other genes involved in lipid
343 metabolism, an essential process ensuring fish oocyte growth and reproduction were found
344 differentially expressed. The *nntp*, *ldah* and *at11b* genes associated to the transport and
345 transformation of lipids^{38,39} are overexpressed in developed/irradiated gonads compared to
346 reference. The synthesis of NLTP and LDAH proteins may be stimulated by the mobilisation of
347 fatty acids and oil droplets required in perch maturing oocytes³⁸. Transcripts involved in lipid
348 metabolism were found modulated in bank voles inhabiting Chernobyl and were suggested to
349 allow the small mammals to cope with the stress induced by radiation, however it was unclear
350 whether this mechanism was changed as the result of the different diet available at the different
351 geographical location or exposure to radiation⁴⁰. Moreover, other confounding factors such as the
352 reproductive stage of the individuals were not considered.

353 Other genes involved in reproduction such as *cyb5*, *cyp17a* and *ovos* were differentially
354 expressed. CYB5 regulates the activity of CYP17A^{41,42} which play a role in gonadal
355 steroidogenesis. *Cyp17a* gene was found differentially expressed in female gonads of yellow
356 catfish (*Pelteobagrus fulvidraco*)⁴³ and Japanese medaka (*Oryzias latipes*)⁴⁴ exposed to
357 endocrine disruptors. The *ovos* gene has not been previously described in fish but in the
358 developing reproductive system of hens⁴⁵. *Cyp17a* and *ovos* genes were found downregulated in
359 the underdeveloped phenotype and may contribute to the inhibition of gonad development. Other
360 differentially regulated genes appeared to be involved in oocyte growth. *Vegfc* and *Prgr* genes
361 were found consistently downregulated in underdeveloped gonads. The *vegfc* gene was found
362 up-regulated in human mature oocytes as compared to immature oocytes⁴⁶. The *prgr* gene
363 encode the progesterone receptor protein previously found involved in oocyte maturation in the
364 fish, Nile tilapia (*Oreochromis niloticus*)⁴⁷. A depletion of progesterone (and subsequent receptor
365 activity) resulted in failure of gonad development in female fish kutum (*Rutilus frisii kutum*)⁴⁸.
366 Therefore, those results suggest a potential role played by *vegfc* and *prgr* genes in the inhibition
367 of gonad development of perch. Finally, some genes involved in cytoskeletal changes such as
368 *kIC18* were found downregulated in the undeveloped phenotypes which is consistent with the
369 up-regulation described in mature oocytes (vitellogenic) as compared to previtellogenic oocytes
370 in the Senegalese⁴⁹. Those genes may therefore be linked to a delay of oocyte growth and
371 differentiation.

372 Transcriptional responses of *nell2* and *tcp4* genes involved in timing of reproduction and organ
373 development were decreased in the undeveloped phenotype. Interestingly, NELL2 regulates the
374 release of gonadotropin-releasing hormone⁵⁰, a major regulator of oocyte maturation in teleost

375 fish⁵¹. Therefore, these results suggest that a biological timing may refrain the gonad maturation
376 in the undeveloped phenotype.

377 Some genes involved in cell proliferation and differentiation such as *mod5*, *gps2*, *rergl*, *EIF3*
378 and *ggnb2* were found differentially expressed across the different phenotype comparisons.
379 *Mod5*, *gps2* and *rergl* genes act as negative regulators in carcinogenesis⁵²⁻⁵⁴, therefore, their
380 upregulation observed in the present study may result in a slower cell division and explain the
381 delay of gonad maturation in both irradiated phenotypes compared to the reference gonads.

382 The percentage of immature oocytes of the developed gonads samples follows a gradient with
383 55%, 61% and 68% recorded in fish from reference, medium and highly contaminated lakes
384 respectively. The genes described above may be responsible for this delay of gonad maturation
385 that was evidenced in female perch exposed to 10-16 $\mu\text{Gy/h}$ at Chernobyl (Lerebours *et al.*,
386 2018)⁴ and the subtle delay in hatching recently highlighted in stickleback embryos waterborne
387 exposed to 400 $\mu\text{Gy/h}$ ²⁰.

388 **Differential expression of genes involved in pathways previously found associated to**
389 **radiation exposure.** DNA damage and oxidative stress have been the focus of many studies led
390 on the effects of radiation on fauna and reduced animal models, therefore some gene responses
391 involved in those pathways were sought-after. No gene encoding for the classical oxidative stress
392 biomarkers of radiation exposure/effect used in previous studies such as the superoxide
393 dismutase or catalase were found differentially expressed. In the present study, some genes
394 involved in checkpoint regulation and DNA damage repair, *wdhd1*, *hus1* and *rad51* were found
395 upregulated in both irradiated gonads compared to reference. Their overexpression has been
396 shown to confer resistance to DNA-damaging agents⁵⁵⁻⁵⁷. Interestingly, HUS1 and RAD51 have
397 been involved in adaptive response to ionising radiation in female reproductive system of

398 invertebrate⁵⁵ and during oocytes maturation in mammals^{58,59} respectively. Those findings
399 suggest that the upregulation of *wdhd1*, *hus1* and *rad51* genes in irradiated gonads may
400 constitute an adaptive response to radiation exposure by protecting the oocytes of fish from a
401 potential increase of DNA damage. This is consistent with previous work that did not observe
402 any genotoxic effects (based on the number of micronuclei) in several fish species living at
403 Chernobyl^{60,4}.

404 The area of epigenetics in radioecology has elicited a recent interest⁶¹⁻⁶³. In the present study,
405 the *dm1* gene encoding for DNA methyltransferase 1-associated protein 1 was found
406 upregulated in both irradiated gonad phenotypes. DMAT1 maintains methylation pattern during
407 DNA replication and is involved in fish development⁶⁴. DNA methylation imprints are required
408 during oocytes maturation to set the developmental programme for embryogenesis^{65,66}. The
409 effect of environmental contaminants and the long-term consequences on the oocyte epigenome
410 is largely unknown. Certain DNA methylation patterns are expected at different stages of oocytes
411 development in wild fish exposed to chemical contaminants⁶⁷ and have been found in ovaries in
412 zebrafish exposed to γ radiation but were not passed through generations⁶². Another laboratory
413 study showed that exposure to γ radiation induces methylation changes in the F2 and F3
414 generations of the aquatic crustacean *Daphnia magna*⁶¹. However, no effect on growth, brood
415 size or survivals on these generations were recorded⁶¹ and how methylation changes affect gene
416 expressions remain an unanswered question especially in germ cells. The present study
417 highlights the consistent upregulation of a gene encoding for an enzyme involved in methylation
418 changes in irradiated gonads but this does not necessary support the hypothesis of a concrete
419 epigenetic change happening since post-transcriptional changes can occur. However, this area of

420 research would deserve future interest since epigenetic changes occur under exposure to
421 radiation and could be involved in the transmission of various effects through generations.

422 Fish have become resilient with perch able to produce seemingly normal gonads however the
423 delay in oocyte development observed in these seemingly developed samples, suggest that
424 adaptation to environmental radiation may require a higher energy cost to maintain reproduction.
425 Perch uses a considerable amount of energy, mainly lipids, from endogenous reserves and food
426 for ovarian maturation and spawning⁶⁸ and can expand up to 22,8% of its total body energy
427 (3,481 kJ) during the spawning period⁶⁹.

428 The present study addresses the effects of long-term environmental exposure to radiation
429 on the transcriptome of perch collected in high contaminated lakes in the 10 km Chernobyl
430 exclusion zone as compared to fish collected in reference lakes close to natural radiation
431 background. The study is the most comprehensive to date of gene responses to radiation in fish
432 in natural ecosystems. It revealed differences of transcripts abundance involved in gonad
433 development and pathways previously found modulated under exposure to radiation. There is
434 good evidence that radiation is the causal factor for the subtle delay observed in oocyte
435 development in developed/irradiated phenotypes⁴. However, the undeveloped phenotype could
436 not be causally linked to radiation, though this is a possible cause. The study highlighted a great
437 number of differentially expressed genes that may point towards additional directions of further
438 research on the encoded proteins (that represent a higher level of biological significance)
439 especially those involved the delay of the maturation of oocytes in female fish.

440 **Supporting Information.** The Supporting Information is available online. The file is composed
441 of pages S1 to S19 and more specifically: Supplementary Text, Supplementary Tables: Tables S1
442 to S10 and Supplementary Figures: Figures S1 to S8.

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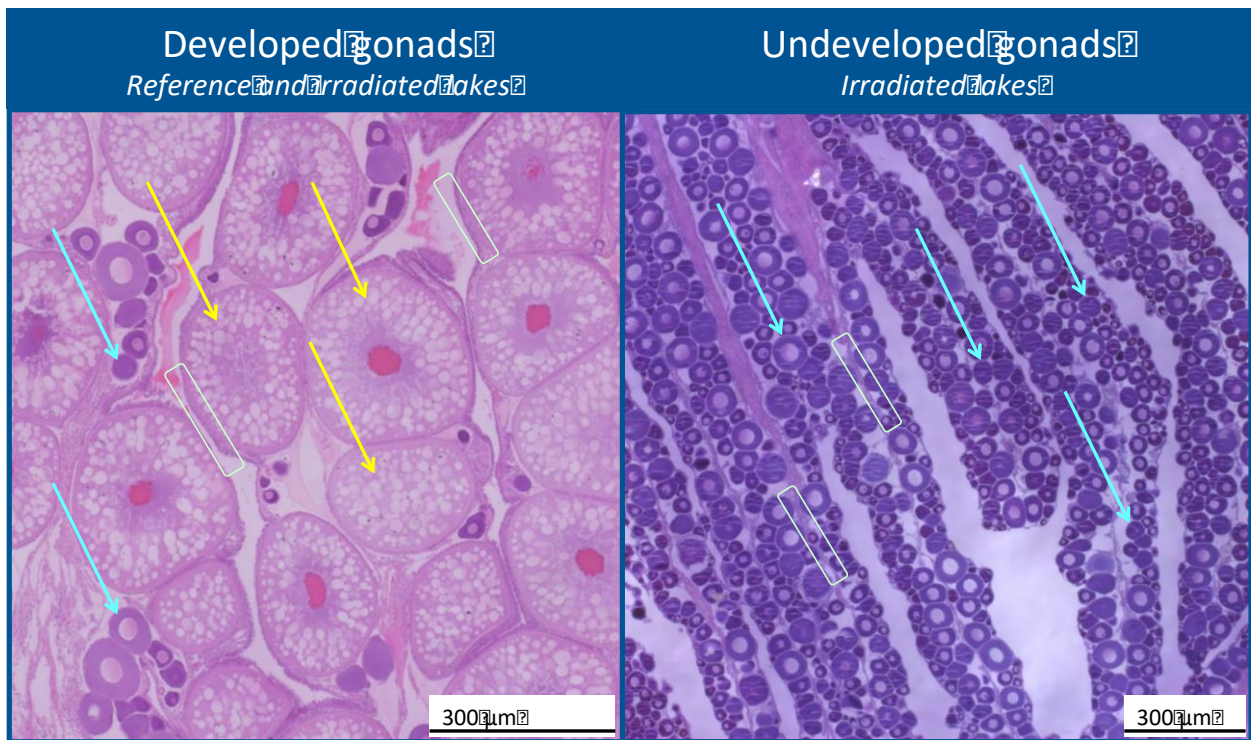
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680 **Figure and Table Legend.**

681 **Figures**

682 **Figure 1.** Histological sections of ovaries of female perch. The picture on the left shows the
683 developed gonad phenotype composed of maturing oocytes (cortical alveolar oocytes) and
684 immature oocytes (perinuclear oocytes) found in fish from reference and irradiated lakes. The
685 picture on the right shows the undeveloped gonad phenotype composed of immature oocytes
686 (perinuclear oocytes) found in fish from irradiated lakes. The yellow arrows show maturing
687 oocytes. The blue arrows show immature oocytes. Each oocyte will form an egg. The light green
688 rectangles indicate portions of the follicular epithelium (round-shaped structure sheltering the
689 oocytes).

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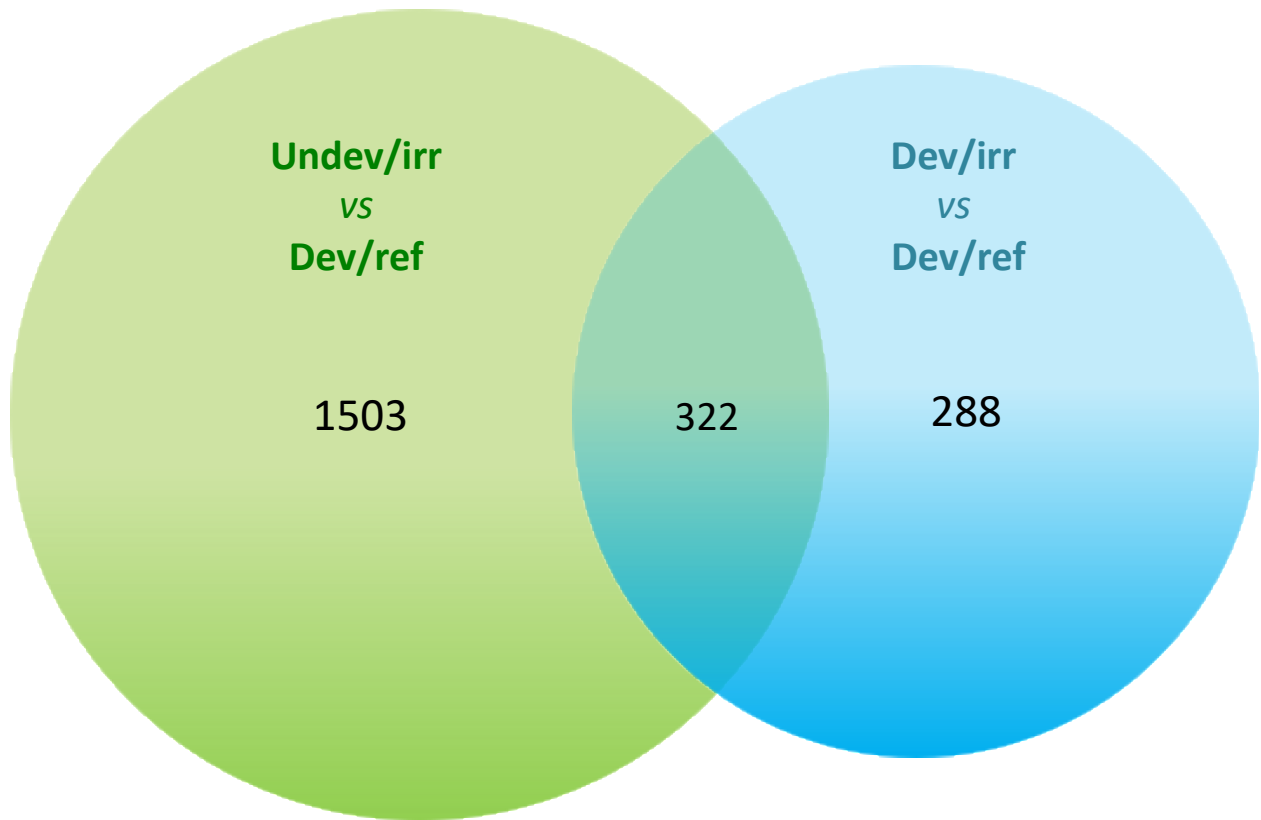
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695 **Figure 2:** Venn diagram showing the number of unique and common differentially expressed
696 transcripts between the following comparisons: undeveloped/irradiated *versus*
697 developed/reference (undev/irr vs dev/ref) and developed/irradiated *versus* developed/reference
698 (dev/irr vs dev/ref).



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704 **Tables**

705 **Table 1:** Fulton condition, hepatosomatic index, gonadosomatic index, age, liver, gonad and
 706 body weight (g), and total length (cm) (mean \pm SD) measured in female perch with
 707 Developed/Irradiated and Undeveloped gonads from Yanovsky (H) (Developed/Irradiated/: $n =$
 708 3; Undeveloped: $n = 3$) and Cooling Pond (H) (Developed/Irradiated: $n = 3$; Undeveloped: $n =$
 709 3), Developed/Irradiated gonads from Svyatoye (M) and Developed gonads from Reference (L)
 710 lakes ($n = 9$).

	Yanovsky (H)		Cooling Pond (H)		Svyatoye (M)	Reference
	<i>Developed/ irradiated</i>	<i>Undeveloped</i>	<i>Developed/ irradiated</i>	<i>Undeveloped</i>	<i>Developed/ irradiated</i>	<i>Developed</i>
Fulton Condition	1 \pm 0.1	1.1 \pm 0.1	1 \pm 0.7	1 \pm 0.2	1.2 \pm 0.1	1.3 \pm 0.1
Body weight (g)	64 \pm 7	85 \pm 38	86 \pm 26	67 \pm 9	93 \pm 8	79 \pm 18
Total length (cm)	18 \pm 1	20 \pm 2	21 \pm 2	19 \pm 1	20 \pm 1	18 \pm 1
Hepatosomatic Index	1.3 \pm 0.3	1.2 \pm 0.6	1.6 \pm 0.3	0.9 \pm 0.1	0.5 \pm 0.1	1.2 \pm 0.5
Liver weight (g)	0.8 \pm 0.2	1.1 \pm 1.0	1.3 \pm 0.5	0.6 \pm 0.1	0.5 \pm 0.1	1 \pm 0.5
Gonadosomatic Index	4.8 \pm 0.3	0.7 \pm 0.1	4.3 \pm 0.5	0.5 \pm 0.1	2.4 \pm 0.4	3.9 \pm 1.3
Gonad weight (g)	3.1 \pm 0.5	0.5 \pm 0.2	3.6 \pm 0.7	0.4 \pm 0.1	2.3 \pm 0.1	3 \pm 0.8
Immature Oocytes (%)	68	100	68	100	61	55
Age (years)	5.3 \pm 0.7		4.5 \pm 0.6		/	5.6 \pm 0.8

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720 **Table 2:** Table displaying some of the common differentially expressed genes between two or
 721 three phenotype comparisons and potentially involved in gonad maturation, lipid metabolism,
 722 development, reproduction, development timing, cytoskeleton, cell proliferation and
 723 differentiation, DNA damage repair and epigenetic mechanisms.

	IRRADIATED Lakes Undeveloped	IRRADIATED Lakes Developed
REFERENCE Lakes Developed	Up-regulated: 836; Down-regulated: 989. Proposed common selected diff. genes involved in gonad maturation: <i>rpc4</i> (↗10), lipid metabolism: <i>ldah</i> (↗3), development: <i>prgr</i> (↘9), <i>vegfc</i> (↘23), reproduction: <i>cyb5</i> (↗11), <i>cyp17a</i> (↘7), <i>ovos</i> (↘7), <i>odo1</i> (↗9), development timing: <i>nell2</i> (↘5), <i>tcp4</i> (↘10), cytoskeleton: <i>k1C18</i> (↘6), cell prolif. and diff.: <i>gps2</i> (↗9), <i>ggnb2</i> (↗1), <i>mod5</i> (↗10), <i>eif31</i> (↘10), <i>rergl</i> (↗6), DNA damage repair: <i>wdhd1</i> (↗11), <i>rad51</i> (↗4), <i>hus1</i> (↗10) and epigenetic: <i>dmap1</i> (↗10).	Up-regulated: 552; Down-regulated: 58. Proposed common selected diff. genes involved in gonad maturation: <i>rpc4</i> (↗12), lipid metabolism: <i>ldah</i> (↗3), <i>at11b</i> (↘10), <i>nltf</i> (↗21), reproduction: <i>cyb5</i> (↘2), <i>odo1</i> (↗9), cytoskeleton: <i>k1C18</i> (↗5), cell prolif. and diff.: <i>mod5</i> (↗10), <i>gps2</i> (↗10), <i>rergl</i> (↗6), DNA damage repair: <i>wdhd1</i> (↗10), <i>rad51</i> (↗3), <i>hus1</i> (↗10) and epigenetic: <i>dmap1</i> (↗9).

