Environmental Science & Technology

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Transcriptional Changes in the Ovaries of Perch from Chernobyl

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Cite This: *Environ. Sci. Technol.* 2020, 54, 10078−10087

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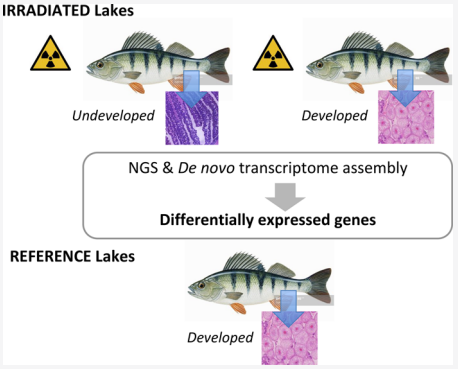
ABSTRACT: Fish have been highly exposed to radiation in freshwater systems after the Chernobyl Nuclear Power Plant (NPP) accident in 1986 and in freshwater and marine systems after the more recent Fukushima NPP accident in 2011. In the years after the accident, the radioactivity levels rapidly declined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



INTRODUCTION

While the biological effects of acute exposure to radiation in laboratory settings is well documented (Frederica radiation database: www.frederica-online.org), little is known about the long-term effects of low dose exposure to radiation on organisms in their natural environment. Moreover, results from the literature on the health effects of low doses and the fate of wildlife in the Chernobyl exclusion zone (CEZ) differ.[1,2] For instance, some studies did not find evidence of radiation effects on populations of aquatic[3−6] and terrestrial[7−10] animals, whereas others found adverse effects of radiation on the abundance of invertebrates,[11] birds,[12] and mammals[13] at Chernobyl. Thus, the dose at which significant damage to wildlife populations occurs remains an open question.[2]

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

Morphological changes of the reproductive system of fish, such as asymmetry, sterility, abnormal cells, or absence of gonads, were observed several years after both the Chernobyl accident and the Kyshtym disaster at the Mayak plutonium production site in Russia.[15,16] In a recent large-scale study of the effects of chronic radiation on fish from Chernobyl, the reproductive system of the European perch, *P. fluviatilis*, appeared to be more sensitive to radiation than an omnivorous species, the roach, *R. rutilus* for which no effect was recorded.[4] In female perch remaining at Chernobyl, environmental radiation induced a delay of the maturation of the gonads as

Received: April 24, 2020
Revised: July 17, 2020
Accepted: July 20, 2020
Published: July 20, 2020

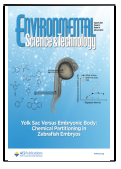


Table 1. Fulton Condition, Hepatosomatic Index, Gonadosomatic Index, Age, Liver, Gonad and Body Weight (g), and Total Length (cm) (Mean ± SD) Measured in Female Perch with Developed/Irradiated and Undeveloped Gonads from Yanovsky (H) (Developed/Irradiated: n = 3; Undeveloped: n = 3) and Cooling Pond (H) (Developed/Irradiated: n = 3; Undeveloped: n = 3), Developed/Irradiated Gonads from Svyatoye (M) and Developed Gonads from Reference (L) Lakes (n = 9)

	Yanovsky (H)		Cooling Pond (H)		Svyatoye (M)	reference
	developed/irradiated	undeveloped	developed/irradiated	undeveloped	developed/irradiated	developed
Fulton condition	1 ± 0.1	1.1 ± 0.1	1 ± 0.7	1 ± 0.2	1.2 ± 0.1	1.3 ± 0.1
body weight (g)	64 ± 7	85 ± 38	86 ± 26	67 ± 9	93 ± 8	79 ± 18
total length (cm)	18 ± 1	20 ± 2	21 ± 2	19 ± 1	20 ± 1	18 ± 1
hepasomatic index	1.3 ± 0.3	1.2 ± 0.6	1.6 ± 0.3	0.9 ± 0.1	0.5 ± 0.1	1.2 ± 0.5
liver weight (g)	0.8 ± 0.2	1.1 ± 1.0	1.3 ± 0.5	0.6 ± 0.1	0.5 ± 0.1	1 ± 0.5
gonadosomatic index	4.8 ± 0.3	0.7 ± 0.1	4.3 ± 0.5	0.5 ± 0.1	2.4 ± 0.4	3.9 ± 1.3
gonad weight (g)	3.1 ± 0.5	0.5 ± 0.2	3.6 ± 0.7	0.4 ± 0.1	2.3 ± 0.1	3 ± 0.8
immature oocytes (%)	68	100	68	100	61	55
age (years)	5.3 ± 0.7		4.5 ± 0.6			5.6 ± 0.8

evidenced by the positive correlation between the percentage of immature oocytes composing the developed gonads of fish from exposed lakes and the gradient of radiation dose across the seven lakes.[4] In addition, a significant proportion of the females had undeveloped gonads in two of the three highly contaminated lakes.[4] However, since undeveloped gonads were not seen in the third highly contaminated lake, it remains unclear whether this phenotype is a direct result of radiation exposure or instead some other environmental factors: the two lakes in which the effect was seen were artificial reservoirs (Chernobyl Cooling Pond and Yanovsky Crawl), whereas the third lake was a natural floodplain lake (Glubokoye).

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

To address this knowledge gap, we applied a high-throughput transcriptomic approach to observed gonad phenotypes of female fish exposed to a gradient of radiation at Chernobyl. The transcriptome of perch collected from lakes located inside and outside the Chernobyl exclusion zone (CEZ) was sequenced, assembled de novo, and annotated. Changes in the transcriptional responses were assessed between developed female gonads from reference lakes, developed/irradiated female gonads from lakes characterized by medium (M) (Svyatoye) and high (H) (Chernobyl Cooling Pond and Yanovsky Crawl) levels of radioactive contamination, and undeveloped/irradiated female gonads from lakes characterized by high (H) levels of radioactive contamination.

METHODS

Fish Collection. Twenty-four female European perch (*P. fluviatilis*) were selected from the September 2014 field work session (as described in Lerebours et al.[4]). Fish were collected from six lakes in Belarus and Ukraine using three gill nets of 20 m length and 21 mm mesh size to ensure the capture of homogenous groups of mature fish. Perch were carefully removed from the nets and placed into tanks containing aerated water. Fish fell unconscious by a blow to the head and were then killed by performing a concussion of the brain to limit as much as possible the suffering as recommended by UK Home Office procedures (ASPA, 1986).[21] Scales were sampled for age determination. The body weight, total length, and presence of external signs of disease were noted for each fish using methodology specified by the International Council for the Exploration of the Sea (Feist et al.).[22] The Fulton condition (K), hepatosomatic index (HSI), and gonadosomatic index (GSI) were determined as follows: K = body weight/(total length (cm)^3) × 100; HSI = (liver weight/body weight) × 100; and GSI = (gonad weight/body weight) × 100, respectively (Table 1).

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

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

Activity Measurements of ^{137}Cs and ^{90}Sr and Dose Calculation. Measurement of ^{137}Cs and ^{90}Sr in the body were performed for five individual fish from each lake, and doses rates were calculated as described in Lerebours et al.[4] (Table S1).

Histological Analyses. Histological analyses were performed to determine the gonadal maturation stage. A standardized cross section of gonad was removed for each fish and fixed for 24 h in 10% neutral buffered formalin before transfer to 70% ethanol for subsequent histological assessment. Gonads were processed in a vacuum infiltration processor using standard histological protocols[22] and embedded in paraffin wax. Using a rotary microtome, sections of 5−7 μm were taken and subsequently stained with hematoxylin and eosin (H&E). The gonadal stage was defined by determining the percentage of immature (perinuclear) and mature (cortical

https://dx.doi.org/10.1021/acs.est.0c02575
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alveolar) oocytes observed in a defined surface area at magnification 10× using a microscope (Zeiss axiozoom) (Figure 1).

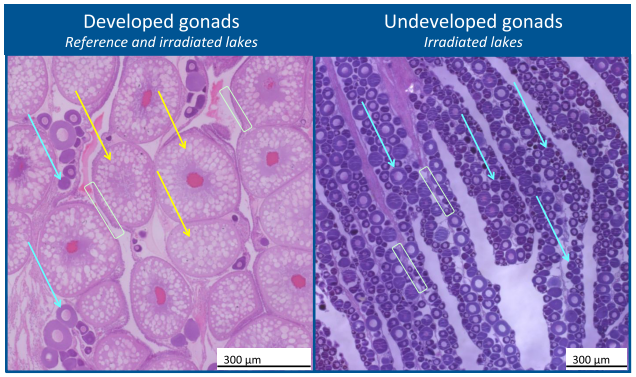

Figure 1. Histological sections of ovaries of female perch. The picture on the left shows the developed gonad phenotype composed of maturing oocytes (cortical alveolar oocytes) and immature oocytes (perinuclear oocytes) found in fish from reference and irradiated lakes. The picture on the right shows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

Next-Generation Sequencing (NGS). For each fish, the section adjacent to the section dedicated to histological analyses was dissected and immediately snap-frozen in liquid nitrogen for transcriptomic analyses. Six pools of female perch gonads were formed as followed: one pool of three developed/irradiated and one pool of three undeveloped gonads from fish collected in Yanovsky (H) lake; one pool of three developed/irradiated and one pool of three underdeveloped gonads from fish collected in Cooling Pond (H); one pool of three developed/irradiated gonads from fish collected in Svyatoye (M) and one pool of nine developed/reference gonads from Gorova, Dvoriche, and Stoyacheye (R) lakes (three gonads from each) (Table S3). Total RNAs were extracted using the high pure RNA tissue kit (Roche Diagnostics Ltd, West Sussex, U.K.), according to the supplier's instructions. RNA quality and integrity were evaluated using a bioanalyzer. RNA integrity numbers ranged from 7.8 to 9.3. A total of six pools were used for NGS analyses. Sequencing libraries were prepared for each pool using the TruSeq Stranded mRNA protocol (Illumina, San Diego, CA) and sequenced using the Illumina HiSeq. 2500 analyzer using a 125 base paired-end run (Genepool facility, Edinburgh, U.K.).

Assembly and Annotation of the European Perch. The quality of the raw reads was assessed using FastQC v0.11.7. Sample contamination was assessed by mapping raw reads against the European Perch (*P. fluviatilis*) genome from Ozerov, et al.,[23] as well as genomes from multiple species taken from the Ensembl database version 91[24] using Fastq Screen.[25] Adapter sequences and poor-quality bases (poly-N ambiguous base calls, or contiguous base calls with a quality score less than 20) were trimmed from the raw reads using Trim Galore v0.4.4. Transcripts from all samples were combined and assembled into a putative transcriptome using Trinity v2.5.1.[26] The parameters used are described in the Supporting Text. Putative coding regions within assembled transcripts were identified using TransDecoder v5.0.2 using default parameters to identify open reading frames of 100 amino acids or more. Transcripts were annotated using Trinotate v3.02, which combines multiple sources of transcript annotation including annotation against the Universal Protein Knowledge Base (UniProtKB) database (The UniProt Consortium) using BLAST, the Protein family (Pfam) database[27] using HMMER (HMMER Web Server), Clusters of Orthologous Groups of proteins (eggNOG) database,[28] the Kyoto Encyclopedia of Genes and Genomes (KEGG) database,[29] and Gene Ontology (GO) database.[30] For each transcript, the annotated gene name was taken from either the BLAST results of the longest TransDecoder peptide sequence (blastp) or from the translated nucleotide sequence directly if no open reading frame was identified (blastx). The corresponding species and gene name from UniProtKB/Swiss-Prot were used for annotation and gene filtering.

Differential Expression Analysis. Transcript abundance was estimated for each of the individual samples against the assembled transcriptome using Kallisto v0.43[31] with parameters "--rf-stranded". All assembled transcripts were considered in the primary analysis and quality control stage. After assessing sample quality based on the complete transcriptome, the transcripts were filtered to keep only the transcripts (1) greater than 500 bases, (2) with a complete open reading frame, and (3) with a normalized fragment per kilobase mapped (FPKM) abundance above 1 in at least one of the six samples (to avoid low abundance transcripts). In addition, transcripts most closely matching archaeal, bacterial, or viral species were removed (Figure S1). Differential expression analysis between the different conditions was conducted using DESeq.2[32] package in R (R Core team 2017).[33] Comparisons were made between the phenotypes (developed/reference, developed/irradiated, undeveloped/irradiated) by combining pools (described in Table S3) from different sampling sites as replicates. Resulting *p*-values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate correction.[34] Genes were identified as differentially expressed between conditions if they showed a fold change greater than twofold (upregulated or downregulated), with an adjusted *p*-value less than 0.05. In addition, to avoid overinflating changes in low abundance transcripts, differentially expressed genes were chosen such that the normalized FPKM expression measure was greater than 1 for at least one of the two conditions being compared. Careful attention should be paid when reading the gene expression results between two phenotypes in Table S8 especially when the comparison with a FPKM value of 0 is indicated. Indeed, this can result in very high log2FoldChange. Therefore, the consideration of the sense of the expression (up- or downregulated) rather than the expression value is recommended. Significantly enriched gene ontology terms and pathways were identified against the *D. rerio* database by PANTHER v14.1.[35]

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR). For each individual fish gonad, first-strand cDNAs were synthesized from 1 μg of total RNA using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Stockport, U.K.) and according to the supplier's instructions. Primer pairs and FAM−TAMRA dye probes used to amplify the target sequences (Table S4) were designed using the Prime Express software (Applied Biosystem). Ten nanograms of the reverse transcribed product was used as a template for subsequent polymerase chain

https://dx.doi.org/10.1021/acs.est.0c02575
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reaction (PCR) in a 20 μL of final volume using 1× of TaqMan Fast Advanced Master Mix (Life Technologies, Paisley, U.K.), primers and probe at a final concentration of 900 and 250 nM, respectively, and following the supplier's protocol. PCR reactions were performed in the Applied Biosystems ViiA 7 Real-Time PCR System using the following program: one cycle at 95 °C for 20 s and 40 amplification cycles at 95 °C for 3 s and 60 °C for 30 s. Primer efficiencies were determined by 10 times dilution series of the cDNA template and were about 100%. The stability of the expressions of three reference genes (*ef1*, *actb*, *tbb*) was tested on all of the samples and analyzed using NormFinder. The expression of the β actin gene displayed the highest stability.

Statistical Analyses. Statistical analyses were performed using R version 3.1.2 (R Core Team 2017).[33] After satisfying the assumptions of the normal distribution of the residuals using the Shapiro−Wilk test, analysis of variance (ANOVA) models were used to assess the potential differences. The significance of parameters at the different sites was assessed using the contrast method with the esticon function in R (DoBy package).[36,37] If the normality of the residuals was not respected, a Kruskal−Wallis rank test was applied. When significant, post hoc tests were performed and a Bonferroni correction of the α error was applied.

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

RESULTS

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

Reproductive Status. Histological analyses showed that the undeveloped gonads were composed of immature oocytes only, and that developed gonads, from both reference and irradiated lakes, of both immature and maturing oocytes. The distributions of immature oocytes in the developed gonads were 55% in fish from the reference lakes, 61% in fish from the medium (M) irradiated lake, and 68% in fish from high (H) irradiated lakes (Table 1 and Figure 1). The weights of undeveloped gonads in fish from Cooling Pond and Yanovsky lake were significantly lower than the weights of developed/irradiated gonads in fish from the same lakes, Syatoye lake, and the weights of developed gonads in fish from the reference lakes ($p < 0.05$) (Table 1). The gonadosomatic index (GSI) of fish with undeveloped gonads was significantly lower than the GSI of fish with developed/irradiated and developed/reference gonads ($p < 0.05$) (Table 1). The GSI of fish with developed/irradiated gonads from Cooling Pond, Yanovsky lake and the GSI of fish with developed gonads from reference lakes are similar ($p > 0.05$) (Table 1). The GSI of fish with developed/irradiated gonads from Svyatoye is significantly lower than GSI of fish from reference lakes ($p = 0.027$) (Table 1).

De Novo Transcriptome Assembly. Illumina sequencing generated a total of 151 785 472 paired-end reads. The number of paired-end reads for each of the six sample pools can be seen in Supporting Table S5. Reads were aligned against the European Perch (*P. fluviatilis*) genome from Ozerov, et al.,[23] as well as multiple model genome species taken from the Ensembl database v91.[24] The vast majority (more than 95%) of all reads mapped to the perch genome, with the remaining reads either showing no hits or mapping to related fish species (Figure S2). The base calling (method for determining the base sequence of a given nucleotide sequence) quality scores were consistently high throughout the data set. Trimming to remove adapters, ambiguous base calls (of unknown value) and poor-quality base calls (below some predefined threshold) removed only a small proportion (0.1%) of reads. In total, 151 590 007 quality trimmed paired-end reads were used for de novo transcriptome assembly. The *P. fluviatilis* transcriptome was assembled de novo as described by combining all paired-end reads from across the data set. The final assembly consists of 534 099 transcripts (clustered into 309 937 "genes") ranging from 201 to 24 533 bases in size (further filtered later for short fragment < 500 bases). The mean transcript size was 871.5 bases, the N50 score (a weighted median of the assembly such that 50% of the assembly is contained within contigs equal to or larger than this score) was 1879 bases, and the GC content was 46.31% (Table S6). Mapping of the reads for the individual samples back to this assembled transcriptome resulted in mapping rates of 73.7−85.1% (Table S6).

In total, 155 437 (29.1%) of the assembled transcripts were annotated against the UniProtKB/Swiss-prot database as described in Supporting Information. Thirty-three thousand forty-three filtered transcripts were produced, of which 31 029 (93.9%) were annotated to a total of 10 662 unique genes, suggesting that there is some redundancy in the assembly (Figure S1).

Functional Classification of Transcriptome. Gene ontology (GO) analysis was used to identify enriched categories of functional classes among the expressed genes. Potential GO classes were identified for 9415 (88.3%) of the 10 662 annotated protein-coding genes identified in the perch transcriptome. Gene ontology analysis showed enrichment for classes such as chromosome organization, cell cycle, cytoskeleton, response to stress, lipid binding, cell differentiation and proliferation, anatomical structure development, and morphogenesis (Supporting Text, Figure S4).

Gene Expression Analysis. Principal component analysis (PCA) of the top changing genes in the data set showed that the largest source of variation (accounting for 37.7% of the variance) was between the undeveloped and developed phenotype (Figure S5). The second greatest source of variation (18.0%) highlighted differences between developed/reference and developed/irradiated samples (Figure S5). The normalized gene expression for the 33 043 filtered transcripts across the samples represented on the heatmap (Figure S6) showed a cluster of the undeveloped gonads in fish from high irradiated lakes and a cluster of developed gonads in fish from high exposed, medium exposed, and reference lakes. Subclusters are observed between the developed gonads exposed to a gradient of radiation across the lakes (Figure S6).

Transcripts expressed in each sample were identified based on a normalized FPKM abundance greater than 1, and a comparison between all samples showed that 10 897 out of 33 043 filtered transcripts were expressed in all samples and a

https://dx.doi.org/10.1021/acs.est.0c02575
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smaller proportion of transcripts were expressed specifically in one sample (Figure S7). The developed/reference samples showed 1166 uniquely expressed transcripts. In contrast, the developed/irradiated and undeveloped/irradiated samples showed 4770 and 3690 uniquely expressed transcripts, respectively. The irradiated samples showed an increase in the number of transcripts expressed.

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

Gene Expression Changes between Irradiated Phenotypes (Undeveloped and Developed) and Developed/Reference Gonads. Analysis of genes showing differential expression between undeveloped/irradiated gonads and developed/reference gonads identified a total of 1825 transcripts (Figure 2), among which 836 (732 genes) were upregulated and 989 (769 genes) were downregulated in undeveloped gonads (Table S8).

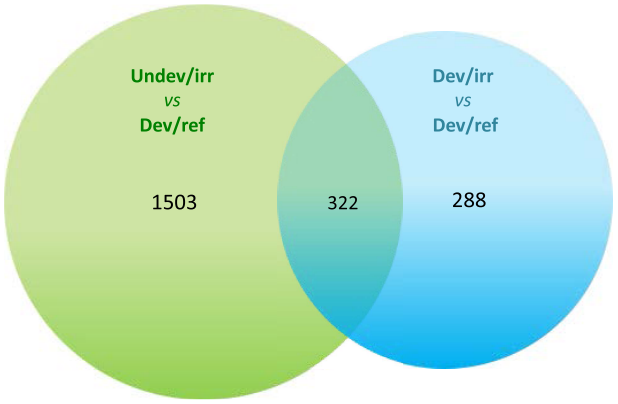

Figure 2. Venn diagram showing the number of unique and common differentially expressed transcripts between the following comparisons: undeveloped/irradiated vs developed/reference (undev/irr vs dev/ref) and developed/irradiated vs developed/reference (dev/irr vs dev/ref).

Analysis of genes showing differential expression between developed/irradiated fish gonads sampled from the three contaminated lakes and developed/reference fish gonads sampled from the reference lakes identified a total of 610 transcripts (Figure 2), among which 552 (496 genes) were upregulated and 58 (57 genes) downregulated (Figure S8c and Table S8). The gene expression profiles varied between Svyatoye Lake (M), Yanovsky Lake (H), and Cooling Pond (H) (Figure S8c).

Common Differentially Expressed Genes between Two or Three Phenotype Comparisons, Potentially Involved in Gonadal Growth and Response to Radiation Exposure. Examples include genes belonging to enriched classes such lipid metabolism (*at11b*, *ldah*, *nltp*), development (*prgr*, *vegfc*), reproduction (*cytb5*, *cyp17a*, *ovos*, *odo1*), development timing (*nell2*, *tcp4*), cytoskeleton (*k1C18*, *mtpn*), cell proliferation and differentiation (*ggnb2*, *mod5*, *rergl*, *eif31*, *gps2*), DNA damage repair (*wdhd1*, *rad51*, *hus1*), and epigenetic mechanisms (*dmap1*) (Tables 2, S8, and S9).

DISCUSSION

Long-term exposure to stress can inhibit gonad development in fish and explain the presence of undeveloped phenotypes, but causal factors are rarely described in the literature. This undeveloped phenotype is not necessarily causally connected to radiation as it was not found in female fish from the third most contaminated lake, Glubokoye, in 2015.[4] Other factors might be involved such as food availability and social interactions. Populations may also have adapted to long-term exposure to radiation through generations that have passed since the accident, potentially having led to the disappearance of the disadvantageous phenotype in some lakes. In the present study, perch from the medium and highly contaminated lakes are able to form developed gonads similar to perch from reference lakes as shown by the similar gonadosomatic index. However, this adaptation may not be without consequences; at the cellular level, a delay of the maturation of the oocytes was significant and correlated with the gradient of radiation dose to fish.[4]

To better understand the long-term effects of exposure to radiation on the significant delay of oocyte development previously observed in Lerebours, et al.,[4] the current study

Table 2. Table Displaying Some of the Common Differentially Expressed Genes between Two Phenotype Comparisons and Potentially Involved in Gonad Maturation, Lipid Metabolism, Development, Reproduction, Development Timing, Cytoskeleton, Cell Proliferation and 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**: *rpc4* (↗10), **lipid metabolism**: *ldah* (↗3), **development**: *prgr* (↘9), *vegfc* (↘23), **reproduction**: *cyb5* (↗11), *cyp17a* (↘7), *ovos* (↘7), *odo1* (↗9), **development timing**: *nell2* (↘5), *tcp4* (↘10), **cytoskeleton**: *k1C18* (↘6), **cell prolif. and diff.**: *gps2* (↗9), *ggnb2* (↗1), *mod5* (↗10), *eif31* (↘10), *rergl* (↗6), DNA **damage repair**: *wdhd1* (↗11), rad51 (↗4), hus1 (↗10) and **epigenetic**: *dmap1* (↗10).	Up-regulated: 552; Down-regulated: 58. Proposed common selected diff. genes involved in **gonad maturation**: *rpc4* (↗12), **lipid metabolism**: *ldah* (↗3), *at11b* (↘10), *nltp* (↗21), **reproduction**: *cyb5* (↘2), *odo1* (↗9), **cytoskeleton**: *k1C18* (↗5), **cell prolif. and diff.**: *mod5* (↗10), *gps2* (↗10), *rergl* (↗6), **DNA damage repair**: *wdhd1* (↗10), rad51 (↗3), hus1 (↗10) and **epigenetic**: *dmap1* (↗9).

https://dx.doi.org/10.1021/acs.est.0c02575
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looked at the differential gene expression changes between developed/irradiated and developed/reference gonads and some genes in common with the undeveloped/irradiated gonads.

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

Differential Expression of Genes Potentially Involved in Gonadal Growth. Some differentially expressed genes involved in energetic metabolism may play a role in gonadal growth by ensuring the supply of energetic demand during this developmental process. For instance, the protein encoding for *odo1* gene, overexpressed in irradiated samples, catalyzes the conversion of 2-oxoglutarate, an intermediate of the Krebs cycle. Other genes involved in lipid metabolism, an essential process ensuring fish oocyte growth and reproduction, were found differentially expressed. The *nltp*, *ldah*, and *at11b* genes associated with the transport and transformation of lipids[38,39] are overexpressed in developed/irradiated gonads compared to reference. The synthesis of NLTP and LDAH proteins may be stimulated by the mobilization of fatty acids and oil droplets required in perch maturing oocytes.[38] Transcripts involved in lipid metabolism were found modulated in bank voles inhabiting Chernobyl and were suggested to allow the small mammals to cope with the stress induced by radiation; however, it was unclear whether this mechanism was changed as the result of the different diet available at the different geographical location or exposure to radiation.[40] Moreover, other confounding factors such as the reproductive stage of the individuals were not considered.

Other genes involved in reproduction such as *cyb5*, *cyp17a*, and *ovos* were differentially expressed. CYB5 regulates the activity of CYP17A[41,42] that play a role in gonadal steroidogenesis. *Cyp17a* gene was found differentially expressed in female gonads of yellow catfish (*Pelteobagrus fulvidraco*)[43] and Japanese medaka (*Oryzias latipes*)[44] exposed to endocrine disruptors. The *ovos* gene has not been previously described in fish but in the developing reproductive system of hens.[45] *Cyp17a* and *ovos* genes were found downregulated in the underdeveloped phenotype and may contribute to the inhibition of gonad development. Other differentially regulated genes appeared to be involved in oocyte growth. *Vegfc* and *Prgr* genes were found consistently downregulated in underdeveloped gonads. The *vegfc* gene was found upregulated in human mature oocytes as compared to immature oocytes.[46] The *prgr* gene encode the progesterone receptor protein previously found involved in oocyte maturation in the fish, Nile tilapia (*Oreochromis niloticus*).[47] A depletion of progesterone (and subsequent receptor activity) resulted in failure of gonad development in female fish kutum (*Rutilus frisii kutum*).[48] Therefore, these results suggest a potential role played by *vegfc* and *prgr* genes in the inhibition of gonad development of perch. Finally, some genes involved in cytoskeletal changes such as *k1C18* were found downregulated in the undeveloped phenotypes, which is consistent with the upregulation described in mature oocytes (vitellogenic) as compared to previtellogenic oocytes in the Senegalese.[49] These genes may therefore be linked to a delay of oocyte growth and differentiation.

Transcriptional responses of *nell2* and *tcp4* genes involved in the timing of reproduction and organ development were decreased in the undeveloped phenotype. Interestingly, NELL2 regulates the release of gonadotropin-releasing hormone,[50] a major regulator of oocyte maturation in teleost fish.[51] Therefore, these results suggest that a biological timing may refrain the gonad maturation in the undeveloped phenotype.

Some genes involved in cell proliferation and differentiation such as *mod5*, *gps2*, *rergl*, *eif3*, and *ggnb2* were found differentially expressed across the different phenotype comparisons. *Mod5*, *gps2*, and *rergl* genes act as negative regulators in carcinogenesis;[52−54] therefore, their upregulation observed in the present study may result in a slower cell division and explain the delay of gonad maturation in both irradiated phenotypes compared to the reference gonads.

The percentage of immature oocytes of the developed gonad samples follows a gradient with 55, 61, and 68% recorded in fish from reference, medium, and highly contaminated lakes, respectively. The genes described above may be responsible for this delay of gonad maturation that was evidenced in female perch exposed to 10−16 μGy/h at Chernobyl (Lerebours, et al.)[4] and the subtle delay in hatching recently highlighted in stickleback embryos waterborne exposed to 400 μGy/h.[20]

Differential Expression of Genes Involved in Pathways Previously Found Associated with Radiation Exposure. DNA damage and oxidative stress have been the focus of many studies that led on the effects of radiation on fauna and reduced animal models; therefore, some gene responses involved in those pathways were sought after. No gene encoding for the classical oxidative stress biomarkers of radiation exposure/effect used in previous studies such as the superoxide dismutase or catalase were found differentially expressed. In the present study, some genes involved in checkpoint regulation and DNA damage repair; *wdhd1*, *hus1*, and *rad51* were found upregulated in both irradiated gonads compared to reference. Their overexpression has been shown to confer resistance to DNA-damaging agents.[55−57] Interestingly, HUS1 and RAD51 have been involved in the adaptive response to ionizing radiation in the female reproductive system of invertebrate[55] and during oocytes maturation in mammals,[58,59] respectively. Those findings suggest that the upregulation of *wdhd1*, *hus1*, and *rad51* genes in irradiated gonads may constitute an adaptive response to radiation exposure by protecting the oocytes of fish from a potential increase of DNA damage. This is consistent with previous work that did not observe any genotoxic effects (based on the number of micronuclei) in several fish species living at Chernobyl.[4,60]

The area of epigenetics in radioecology has elicited a recent interest.[61−63] In the present study, the *dmat1* gene encoding for DNA methyltransferase 1-associated protein 1 was found upregulated in both irradiated gonad phenotypes. DMAT1 maintains methylation pattern during DNA replication and is involved in fish development.[64] DNA methylation imprints are required during oocytes maturation to set the developmental program for embryogenesis.[65,66] The effect of environmental contaminants and the long-term consequences on the oocyte epigenome is largely unknown. Certain DNA methylation patterns are expected at different stages of oocytes development in wild fish exposed to chemical contaminants[67] and have been found in ovaries in zebrafish exposed to γ radiation but were not passed through generations.[62] Another laboratory

https://dx.doi.org/10.1021/acs.est.0c02575
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study showed that exposure to γ radiation induces methylation changes in the F2 and F3 generations of the aquatic crustacean *Daphnia magna*.[61] However, no effect on growth, brood size, or survivals on these generations were recorded,[61] and how methylation changes affect gene expressions remain an unanswered question especially in germ cells. The present study highlights the consistent upregulation of a gene encoding for an enzyme involved in methylation changes in irradiated gonads, but this does not necessarily support the hypothesis of a concrete epigenetic change happening since post transcriptional changes can occur. However, this area of research would deserve future interest since epigenetic changes occur under exposure to radiation and could be involved in the transmission of various effects through generations.

Fish have become resilient with perch able to produce seemingly normal gonads; however, the delay in oocyte development observed in these seemingly developed samples suggests that adaptation to environmental radiation may require a higher energy cost to maintain reproduction. Perch uses a considerable amount of energy, mainly lipids, from endogenous reserves and food for ovarian maturation and spawning[68] and can expand up to 22.8% of its total body energy (3.481 kJ) during the spawning period.[69]

The present study addresses the effects of long-term environmental exposure to radiation on the transcriptome of perch collected in highly contaminated lakes in the 10 km Chernobyl exclusion zone as compared to fish collected in reference lakes close to natural radiation background. The study is the most comprehensive to date of gene responses to radiation in fish in natural ecosystems. It revealed differences of transcripts abundance involved in gonad development and pathways previously found modulated under exposure to radiation. There is good evidence that radiation is the causal factor for the subtle delay observed in oocyte development in developed/irradiated phenotypes.[4] However, the undeveloped phenotype could not be causally linked to radiation, though this is a possible cause. The study highlighted a great number of differentially expressed genes that may point toward additional directions of further research on the encoded proteins (that represent a higher level of biological significance) especially those involved in the delay of the maturation of oocytes in female fish.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02575.

Differential expression analysis results from DESeq2 for comparisons between the phenotypes a) undeveloped gonads vs developed gonads, b) undeveloped gonads vs developed/irradiated gonads, c) developed/irradiated gonads vs developed gonads. Samples from different sampling sites were combined as replicates. BaseMean, log2FoldChange, lfcSE, stat, pvalue and padj represent standard output from the DESeq2 package. FPKM values represent the mean over all sampling sites. Transcripts were classified as being significantly differentially expressed if they showed a fold change greater than 2-fold (upregulated or downregulated), an adjusted p value less than 0.05, and a mean normalized FPKM expression value greater than 1 for at least one of the two conditions. The raw read counts and normalized FPKM values for all 534,099 transcripts are provided (XLSX)

Gene ontology results for the comparisons between the phenotypes for (a) undeveloped gonads vs developed gonads, (b) undeveloped gonads vs developed/irradiated gonads, (c) developed/irradiated gonads vs developed gonads. Gene ontology terms are collected into three classes; biological process, molecular function and cellular component (XLSX)

Assembly and annotation of the European perch; general health condition; functional classification of transcriptome; hydrological parameters of the lakes and the total dose rate to perch; composition of the six different pools used for next generation sequencing analyses; transcriptome assembly statistics from Trinity; genes related to gonad development and pathway previously found responding to radiation exposure; number of unique genes present in each of the top GO classes; heatmap showing the normalized abundance levels (FPKM) across all 33 047 filtered transcripts (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Authors are grateful to Vadislav, Viktor Rizewski, Alexander Kaglyan, Andrey Leshchenko for fish collection and Alexander Zubey for helping with fish processing. This work was completed as part of the Transfer−Exposure−Effects (TREE) consortium under the Radioactivity and the Environment (RATE) program, funded by the Environment Agency and Radioactive Waste Management Ltd (NERC grant no. NE/L000393/1). Dr. S.R. was partially funded through an Expanding Excellence in England (E3) grant from Research England.

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