Supporting Information

Effects of glyphosate and its formulation, Roundup®, on reproduction in zebrafish (*Danio rerio*).

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This Supporting Information contains:

Supplemental Experimental Section

Fish maintenance

Colonies of 4 male and 4 female adult WIK strain zebrafish (20 weeks old; originating form a stock kept at the University of Exeter) were established in individual 15 L glass tanks and allowed to breed naturally during a 7 day acclimation period. Each tank was aerated and supplied with a water flow rate of 48 L /day. The aquarium water supply was reverse-osmosis treated tap water reconstituted with analar-grade salts to produce standardized synthetic freshwater according to OECD guidelines, as described in Paull et al. [1], and maintained at 28 ± 0.5 °C and pH 7-7.5. Fish were kept under a 12h light:dark cycle (with 30 minute dawn/dusk transitional periods) and fed twice daily with live *Artemia nauplii* and flake food (Tetra; Melle, Germany) to satiation.

Water chemistry

Glyphosate quantification was carried out using a 6420B Triple Quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. 20 µl of sample were loaded onto a Zorbax Eclipse Plus C_{18} 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. Glyphosate was eluted from the column using the following gradient: 0-3 min, 0-75% B, 0.15 ml/min; 3-4 min – 100% B, 0.25 ml/min; 4.1 min – 0% B, 0.15 ml/min. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 l min^{-1}, nebuliser pressure 35 psig, capillary voltage +4 kV. All analysis was carried out in positive ion mode with the precursor ion *m/z* of 170.1 and a transition *m/z* of 87.8. Fragmentor and collision energy voltages were optimised to 60 and 5 respectively. Glyphosate was used to generate a standard curve. All samples were diluted accordingly to ensure peaks areas were within the linear range of the curve (0.05 – 1.0 mg/L).

Transcript profiling

Transcripts of genes encoding steroidogenic enzymes, sex steroid receptors and antioxidant enzymes were quantified in the gonads of exposed fish using real time quantitative PCR (RT-QPCR). Primers for each target gene were designed with Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) using zebrafish NCBI refseq sequences, and purchased from MWG-Biotech (Ebersburg, Germany). Primer specificity throughout the range of detection was confirmed by the observation of single amplification products of the expected size and Tm, and optimised by performing a standard curve for each primer pair as described previously [40]. Over the detection range, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was > 0.99 in each case, and efficiencies were between 1.95 and 2.18. The primer sequences, PCR product sizes, annealing temperatures and PCR efficiencies for each primer pair are shown in Table S1.

RNA was extracted from the gonads of eight male and eight female fish from each treatment group using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA concentration and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). cDNA was synthesised according to manufacturer's instructions

from 2 µg of total RNA treated with RQ1 DNase (Promega, Southampton, UK) using random hexamers (MWG-Biotech) and M-MLV reverse transcriptase (Promega). cDNA was diluted (ovary 1:4, testis 1:2) and RT-QPCR was performed in duplicate in an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green chemistry as described [40]. A template-minus negative control was run in duplicate on each plate to verify the absence of cDNA contamination. Efficiency-corrected relative expression levels were determined by normalizing to a control gene, ribosomal protein l8 (*rpl8*), which was previously shown to have consistent expression in ovaries and testis [2].

Histological analysis

Gonads were fixed in Bouin's solution for four hours, then washed and stored in 70 % ethanol prior to histological analysis. Samples were dehydrated in 70-100% industrial methylated spirit (IMS) and ethanol using a Shandon (Citadel 2000) tissue processor, and then embedded in paraffin wax. The embedded samples were cut in 5 µm sections, floated in 30% IMS in a water bath at 45 °C, and then laid on glass slides and dried on a 40 °C heat tray overnight. Sections were stained with Harris' non-acidified Haemoatoxylin and Eosin Y (both Thermoshandon, Pittsburg, U.S.) and mounted with Histomount (National Diagnostics, Hull, UK). Analysis was conducted with a light microscope (Zeiss Axioskop 40, Carl Zeiss, Oberkochen, Germany) connected to an Olympus DP70 camera (Olympus Optical) and using analySIS image processing 3.2 software (Soft Imaging System, Munster, Germany). Two slides, each containing multiple sections, were prepared from portions of ovary 100 µm apart, to provide a representative analysis of the ovarian tissue, while single slides were prepared for each testis.

References

1. Paull, G. C.; Van Look, K. J. W.; Santos, E. M.; Filby, A. L.; Gray, D. M.; Nash, J. P.; Tyler, C. R., Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. *Aquatic Toxicology* **2008,** *87*, 115-126.
2. Filby, A.; Tyler, C., Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. *Bmc Molecular Biology* **2007,** *8*, (1), 10.

Table S1: Target genes, primer sequences and assay details for RT-QPCR analysis.

Gene Name	**Gene Symbol**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Product size (bp)**	**Ta (°C)**	**PCR efficiency**
Ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	1.95
Catalase	*cat*	AGTTCCCTCTGATTCCTGTG	ATGGCGATGTGTGTCTGG	173	61.0	2.00
Superoxide dismutase	*sod1*	TTCACTCTCTCACAACTTCTC	GTCACCTTCACTGGCTTC	142	58.0	2.18
Glutathione peroxidase	*gpx1a*	CTGCGTGTTGCCCTTTGAG	GGTGTAATCCCTGACTGTTGTG	189	58.5	1.98
Glutathione S transferase pi	*gstp1*	AACGACAGTGAGGCTTCC	GCATTTGAGGTGGTTGGG	141	56.0	1.85
Glutathione S transferase alpha	*gsta1*	GGTGGCTCTTGGCTGTTG	TGCGATGTAGTTCAGGATGG	170	61.0	2.03
Steroidogenic acute regulatory protein	*star*	TTCTTGAGGACCAGGATG	GACTTGCTTGACATTGGG	197	58.0	2.03
Cytochrome P450, subfamily XIA, polypeptide 1	*cyp11a1*	TGAGTGCTGTGTTGTATG	AAATGTTGGACCCTATGG	159	57.0	2.12
Aromatase	*cyp19a1a*	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	*hsd3b2*	GCAGCATTGAGGTAGCGTGTC	AGGATAAGAGGAGTAAGGCGTGTC	83	60.0	2.12
Cytochrome P450, subfamily XVIIA, polypeptide 1	*cyp17a1*	CGACAGTAAGATTGGGAAAGAAAG	GATGAGGAGCGGAGAAACAG	118	60.5	1.98
Estrogen receptor 1	*esr1a*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.86
Estrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
bcl2-associated X protein a	*baxa*	CTACTTTGCCTGTCGCCTTG	GTCCCATCCACCCTGTTCC	136	60.0	2.14
Tumour protein p53	*tp53*	GCTTGGTGCTGAATGGAC	GAGTGATGATTGTGAGGATGG	98	56.0	2.09

Table S2: Water chemistry analysis of glyphosate in the exposure tank water. Data presented are the measured concentrations for the three replicate treatment tanks on days 7, 14 and 21 are presented as mean values ± SEM. Analysis was conducted using a 6420B Triple Quadrupole (QQQ) mass spectrometer coupled to a 1200 series Rapid Resolution HPLC system.

Nominal concentration	**control**	**0.01 mg/L Roundup**	**0.5 mg/L Roundup**	**10 mg/L Roundup**	**10 mg/L glyphosate**
Day 7	< 0.05	< 0.05	0.43 ± 0.1	8.8 ± 4.2	14.2 ± 1.9
Day 14	< 0.05	< 0.05	0.40 ± 0.1	13.3 ± 0.6	10.0 ± 0.6
Day 21	< 0.05	< 0.05	0.50 ± 0.1	16.3 ± 2.0	17.7 ± 1.5
Mean	**< 0.05**	**< 0.05**	**0.44**	**12.8**	**13.9**

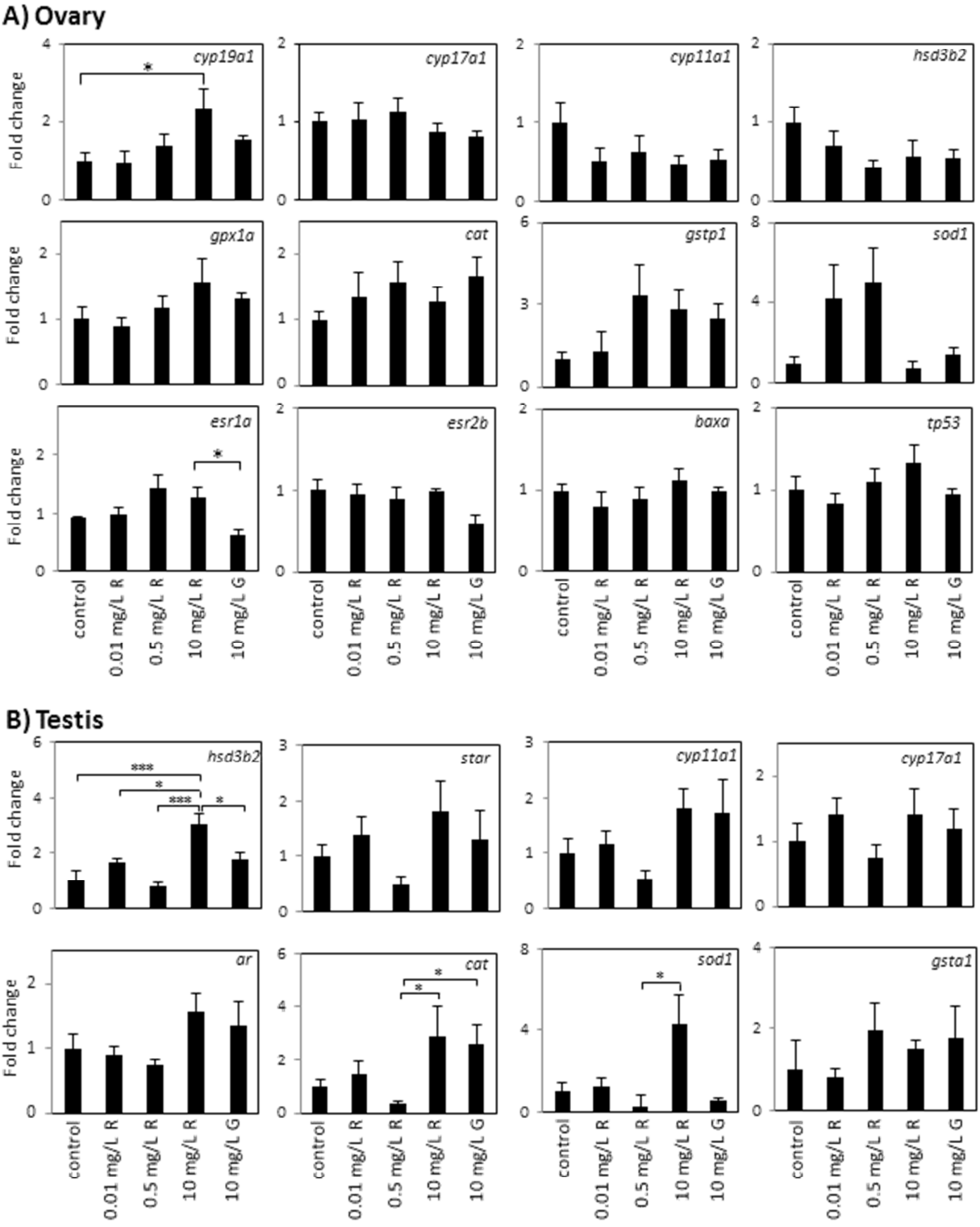

Figure S1. Transcript profiling of target genes in the ovary (A) and testis (B). Data are presented as fold change relative to expression in the control group. Relative expression was calculated as ratio of target gene /rpl8 mRNA concentration. For each treatment, data was collected for 6–8 fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis. Asterisks represent significant differences between treatment groups (*P<0.05 **P<0.01 ***P<0.001).

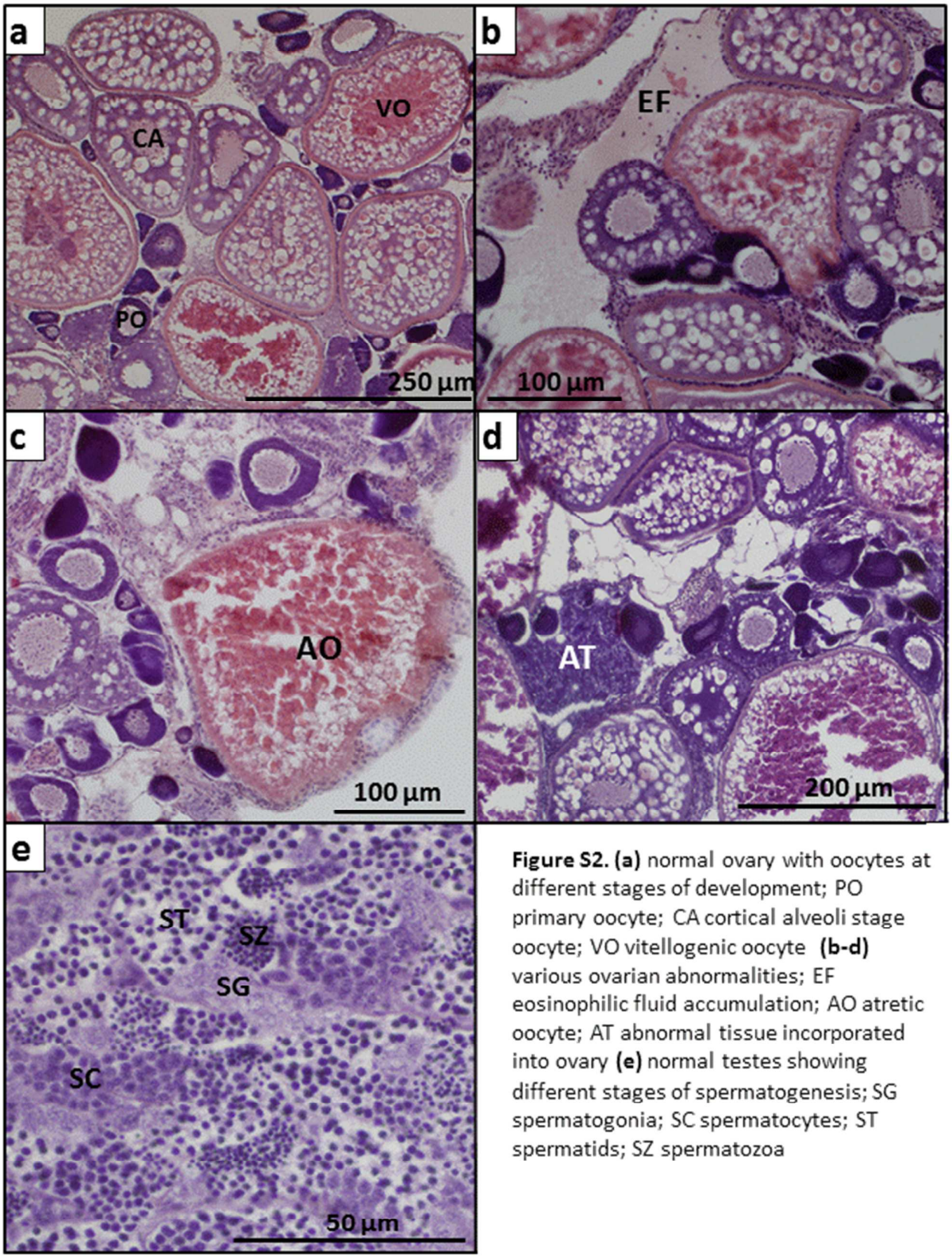

Figure S2. (a) normal ovary with oocytes at different stages of development; PO primary oocyte; CA cortical alveoli stage oocyte; VO vitellogenic oocyte **(b-d)** various ovarian abnormalities; EF eosinophilic fluid accumulation; AO atretic oocyte; AT abnormal tissue incorporated into ovary **(e)** normal testes showing different stages of spermatogenesis; SG spermatogonia; SC spermatocytes; ST spermatids; SZ spermatozoa

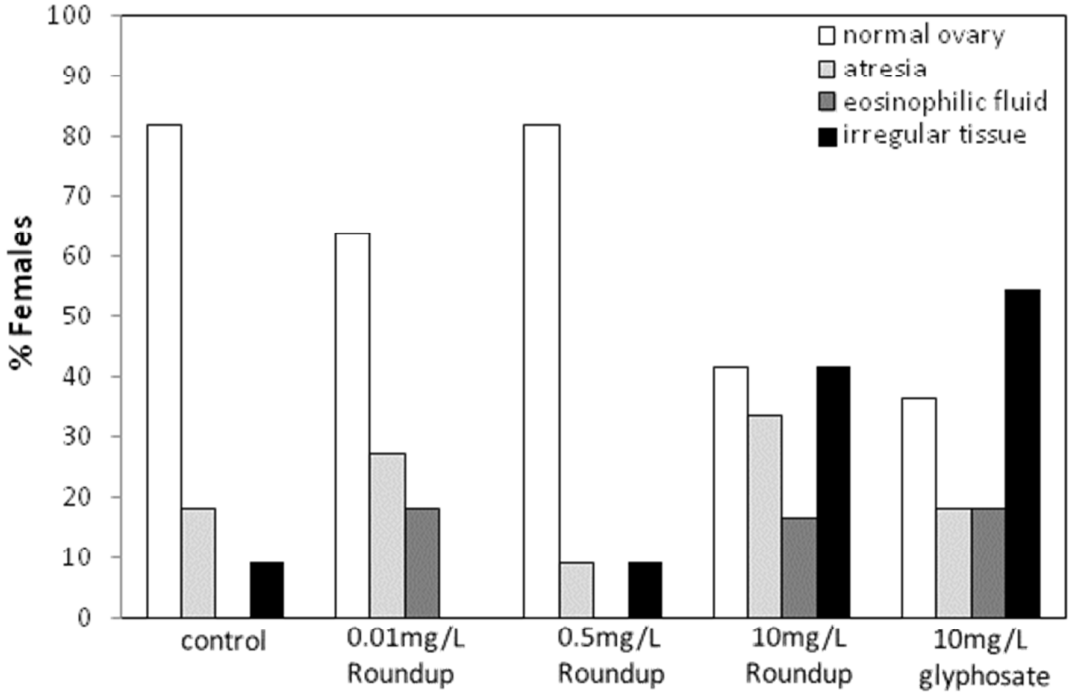

Figure S3. Histological analysis of the ovaries of females exposed to glyphosate and Roundup. Proportion of females in each treatment group showing absence or presence ovarian abnormalities (n=11-12 fish per treatment).

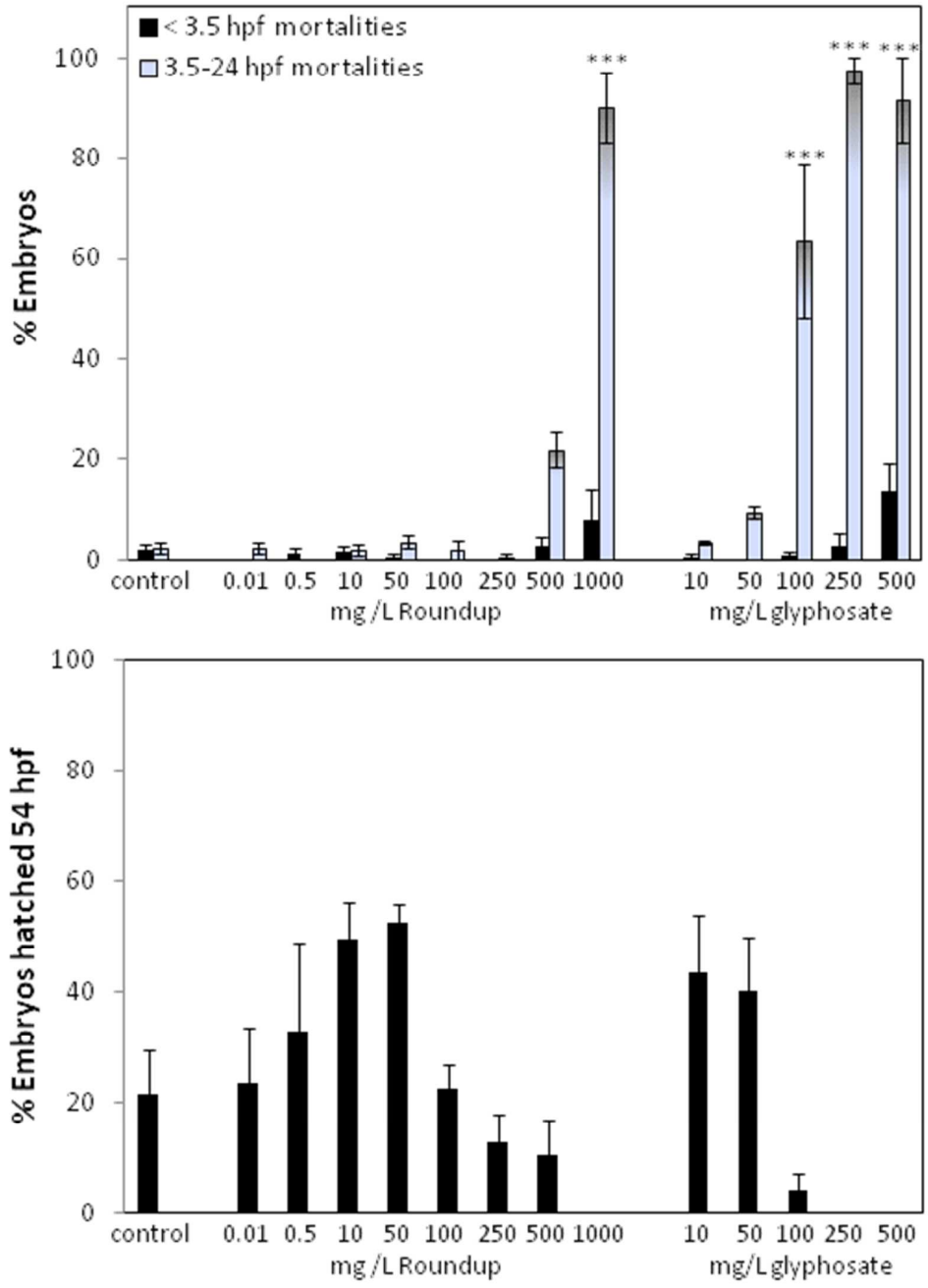

Figure S4. Effects of Roundup and glyphosate on the survival and development of embryos originating from the unexposed control parental population; **(A)** percentage of embryo mortalities that occurred before 3.5 hpf and between 3.5-24 hpf; and **(B)** percentage of embryos that had hatched at 54 hpf. Treatment concentrations include those used during the adult exposure, and higher concentrations (> 10 mg/L) to investigate the thresholds for mortality and development abnormalities to occur. Data presented are mean values ± SEM (n = 3 replicates per treatment concentration, each replicate consisted of 50 embryos). Asterisks indicate significant difference from the control group (*P<0.05 **P<0.01 ***P<0.001).