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Roundup®, and other glyphosate-based herbicides, are the most commonly used herbicides in the world, yet their effects on developing fish embryos are not clearly understood. In the present study, medaka embryos were exposed to 0.5 mg/L glyphosate, 0.5 mg/L and 5 mg/L Roundup for 15 days, then allowed to mature. Embryos were examined at 4, 8, 15 and 100 dpf to determine if exposure to environmentally relevant concentrations of glyphosate and Roundup can induce developmental defects in fry after hatching and if exposure induces alterations in gene expression and global epigenetic effects, particularly DNA methylation, during early development resulting in alterations of reproductive success at adulthood. A significant decrease in cumulative hatching success for 0.5 mg/L Roundup and glyphosate exposure groups, and an increase in developmental abnormalities in medaka exposed to 0.5 mg/L glyphosate was observed. A significant downregulation of *Dnmt1* and upregulation of *Tet1, Tet2* and *Tet3* were observed at 15 dpf, suggesting the role of demethylation in the observed phenotype. Furthermore, expression of Gpr54-1 was significantly downregulated in female brain samples exposed to 0.5 mg/L and 5 mg/L Roundup and in Gpr54-2 exposed to 0.5 mg/L Roundup. In testes samples, reproductive genes *Fshr* and *Arα* were significantly downregulated in medaka exposed to 0.5 mg/L Roundup and glyphosate, and in *Dmrt1* and *Dnmt1* exposed to 0.5 mg/L glyphosate. The study demonstrates that Roundup and its active ingredient glyphosate induce direct and long-term developmental, reproductive, and epigenetic effects due to exposure at environmentally relevant concentrations.

EPIGENETIC AND DEVELOPMENTAL EFFECTS FROM EXPOSURE TO GLYPHOSATE AND ROUNDUP ON DEVELOPING MEDAKA FISH (*ORYZIAS LATIPES*)

by

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Approved by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Glyphosate (N-(phosphonomethyl) glycine) was first discovered to possess herbicidal properties in 1970 (Annett *et al.* 2014) and within four years the first commercial formulation was released under the name Roundup® (hereafter Roundup) (Annett *et al.* 2014, Zhang *et al.* 2017). Since the 1970's use of glyphosate-based herbicides (GBHs) has increased 100-fold (Roy *et al.* 2016 (b), Zhang *et al.* 2017), with annual application globally in the range of 0.6 to 1.2 million tons, making GBHs the most widely used herbicides since the 1970's (Annett *et al.* 2014, Mesnage *et al.* 2015, Rodrigues *et al.* 2017). This increase in use can be attributed to the development of genetically modified crops (GMOs) along with glyphosate's action as a broad-spectrum, post emergent, non-selective herbicide (Annett *et al.* 2014, Harayashiki *et al.* 2013, Rodrigues *et al.* 2017, Roy *et al.* 2016 (a), Sulukan *et al.* 2017). The herbicidal action of glyphosate works through the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), leading to a deficiency in protein production and ultimately plant death (Annett *et al.* 2014, Lopes *et al.* 2014, Rodrigues *et al.* 2017, Roy *et al.* 2016 (a), Sulukan *et al.* 2017). Since this mode of action is only present in plants and some microorganisms, it has been thought that glyphosate and Roundup were not harmful to non-target organisms such as animals and humans, yet mounting scientific evidence seems to contradict this idea (Annett *et al.* 2014, 19, Roy *et al.* 2016 (a)).

Non-target organisms, particularly animals and humans, are exposed to glyphosate together with Roundup, as glyphosate is a key component of the Roundup formulation and not used without its additives (Kwiatkowska *et al.* 2017). Sources of exposure include runoff, dermal exposure from residential and commercial use, inhalation through spray drift in high farming areas, and by way of food consumption (Annett *et al.* 2014, Roy *et al.* 2016(b), WHO 2005). Concentrations of glyphosate acid equivalents in natural water bodies have been reported to range between 0.01 and 0.7 mg/L, with direct application levels ranging from 1.7 to 5.2 mg/L (Annett *et al.* 2014, Harayashiki *et al.* 2013, Zhang *et al.* 2017).

The major metabolite of glyphosate is aminomethylphosphonic acid (AMPA), which is further broken down into carbon dioxide and ammonium (Annett *et al.* 2014, WHO 2005). Aminomethylphosphonic acid, while not acutely toxic, has been found to cause DNA damage (Guilherme *et al.* 2014, WHO 2005). Glyphosate is not expected to bioaccumulate in the food web, based on its ionic character and high solubility in water, yet it still may possess a threat to animal and human health due to its detected presence in a variety of aquatic organisms, including fish, and due to the fact that its biotransformation after uptake is not well known (Annett *et al.* 2014, WHO 2005). Previous studies have shown that exposure to glyphosate and/or Roundup have resulted in an increase in developmental abnormalities, decrease in survival rate (Yusof *et al.* 2014), changes in feeding behavior (Giaquinto *et al.* 2017), and changes in reproductive parameters, including a decrease in egg production and sperm motility in different fish species (Lopes *et al.* 2014, Uren Webster *et al.* 2014); yet there is a lack of literature

regarding the toxicity of GBHs, particularly at environmentally relevant doses of exposure, and the epigenetic consequences associated with these exposures (Mesnage *et al.* 2015, Roy *et al.* 2016 (b)).

To better understand the direct toxicity effects of glyphosate and Roundup exposure, the present study utilized medaka fish as a model organism. Medaka fish are excellent vertebrate model species for biomedical and environmental research because they are easy to maintain, adapt to different salinities, only take about 100-120 days to mature and eggs and embryo are transparent making it easy to distinguish abnormal phenotypes (Ishikawa 2000, Koyama *et al.* 2008, Wittbrodt *et al.* 2002). Other advantages of medaka include their well-characterized sex determination system, similarities in germ cell development mechanisms to mammals, fully annotated genome and availability of tools for molecular and genome research (Bhandari *et al.* 2015, Bhandari 2016)

In developing embryos, environmental chemicals induce two types of effects: direct toxic effects and adult onset health effects (Bhandari *et al.* 2015). Direct toxicity can be observed within a short time of exposure while adult onset toxicity is detectable long after the exposure has ceased. It has been believed that higher dose exposure leads to direct toxicity via immediate effects on cellular processes, while low dose exposure leads to adult onset toxicity involving epigenetic mechanisms (Hanson and Skinner 2016), as the period of embryonic development is very sensitive to chemical exposure (Bhandari 2016, Mesnage *et al.* 2015), and this exposure can lead to heritable or reversible modifications that can affect gene expression without changing DNA structure- otherwise

known as epigenetic changes (Patkin and Sofronov 2012). Epigenetic changes can be inherited, causing multigenerational effects (Patkin and Sofronov 2012). The goal of the present study is to investigate the developmental and epigenetic effects of exposure to environmentally relevant doses of glyphosate and its commercial formulation Roundup® (hereafter Roundup) on developing medaka fish.

The most prominent method by which epigenetics regulates gene expression is through DNA methylation (Kim *et al*. 2017), in which a methyl group is added to the 5th position of a cytosine ring at a cytosine-guanine dinucleotide. Areas rich in CpG dinucleotides are referred to as CpG islands. Methylation of CpG islands, particularly those located in the promoter region of genes, have restrictive, silencing effects on genes, while gene body methylation can lead to gene expression (Aluru *et al*. 2015, Dhingra *et al.* 2014, Goll and Bestor 2005, Seritrakul *et al.* 2017).

DNA methlytransferases (DNMTs) enzymes work to catalyze the process of DNA methylation, thereby making them essential for the creation of new DNA methylation patterns and the maintenance of existing patterns (Aluru *et al.* 2015, Yuan *et al*. 2017). *Dnmt1* works as a maintenance methyltransferase, preferably methylating hemi-methylated CpGs, ensuring the same methylation pattern during DNA replication and cell division, while DNMT3 enzymes, known as *de novo* methyltransferases, work to establish new methylation patterns of hypomethylated DNA necessary for tissue-specific differentiation occurring during development (Aluru *et al.* 2015, Diotel *et al.* 2017, Liu *et al*. 2016, Seritrakul *et al.* 2017). Other enzymes active in DNA methylation are ten-eleven translocation (TET) enzymes which oxidize 5-methylcytosine (5mC) to 5-

hydroxymethylcytosine (5hmC), leading to DNA demethylation by removing 5mC from the genome (De la Rica *et al.* 2016, Liu *et al.* 2016, Seritrakul *et al.* 2017).

The reproductive system in animals is the complex result of the endocrine and central nervous system working together to create and release a series of hormones from the hypothalamic-pituitary-gonadal axis (HPG axis) (Cao *et al.* 2018). Kisspeptin, encoded by the gene *Kiss1,* is a neuroendocrine hormone (Song *et al.* 2015) which acts by binding to the G protein-coupled receptor Gpr54 (Usuda *et al.* 2014). In non-mammal vertebrates, such as medaka, there also exists a *Kiss2* gene along with its receptor Gpr54-2 (Nakajo *et al.* 2018, Song *et al.* 2015). The binding of these neuroreceptors play an important role in stimulating the hypothalamic-pituitary-gonadal axis causing the production of gonadotropin-releasing hormone (GnRH) within the hypothalamus (Nakajo *et al.* 2018, Zhang *et al.* 2017). GnRH is important in regulating reproduction because it activates the synthesis and release of gonadotrophins including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary (Gárriz *et al.* 2017; Khor *et al.* 2016, Nakajo *et al.* 2018, Zhang *et al.* 2017). These hormones and their receptors play an important role in regulating the production of sexual steroids and gametogenesis (Gárriz *et al.* 2017).

In females, LH triggers ovulation, the development of the corpus luteum, and prompts theca cells to produce androgens, while FSH works to promote follicular maturation, ultimately leading to ovulation (Cao *et al.* 2018). Steroid hormones in vertebrates are synthesized from cholesterol which must be transported from the cytoplasm to the inner mitochondrial membrane (Rathor *et al.* 2017). This vital step is

facilitated by the lipid transfer protein known as steroidogenic acute regulatory (StAR) protein (Rathor *et al.* 2017). Transcription factor forkhead box L2 (*Foxl2*), which is primarily expressed in the somatic cells of the female gonad, works to suppress the expression of *Dmrt1* making it a vital sex determining gene in females (Herpin *et al.* 2013). Expression of *Foxl2* is closely associated with *Aromatase* (*cyp19a1a*), which is responsible for the synthesis of estrogens from androgen in females (Murozumi *et al.* 2014). Estrogen and androgen are key hormones involved in reproduction, which are activated by binding to their respective receptors (Fergus *et al.* 2013, Oliveira *et al.* 2002). In females' estrogen is essential for ovarian differentiation and plays a key role in regulating gene expression in both males and females (Bertho *et at.* 2016, Fergus *et al.* 2013, Schulz *et al.* 2010).

In males, FSH and LH are thought to function similarly to their counterparts in mammals in which FSH promotes spermatogenesis in the testis through the binding of its receptor in Sertoli cells while LH stimulates Leydig cell production of androgens, including testosterone (Zhang *et al.* 2015). Androgens are involved in a variety of reproduction related processes including spermatogenesis, the expression of behaviors related to reproduction and the development of secondary sex characteristics (Oliveira *et al.* 2002). Lastly, *Dmrt1* gene expression was measured in 100 dpf testes due to its involvement in testicular formation and spermatogenesis, often being referred to as the male sex determining gene (Herpin *et al.* 2013).

Another important gene measured in the study to help confirm phenotype is acetylcholinesterase (AChE), which is responsible for catalyzing the breakdown of the

neurotransmitter acetylcholine (Lopes *et al.* 2017, Menéndez-Helman *et al.* 2012). The inhibition of AChE leads to the buildup of acetylcholine, resulting in cholinergic overactivity which can result in death. (Lopes *et al.* 2017, Menéndez-Helman *et al.* 2012). Due to its importance in the physiological function of fish, acetylcholinesterase is frequently used as a biomarker in studies relating to exposure of a variety of organophosphate chemicals such as glyphosate (Glusczak et al. 2006, Menéndez-Helman *et al.* 2012).

This study has two major aims, the first of which is to test the hypothesis that glyphosate and Roundup exposure induces developmental defects in embryos and fry after hatching. This aim will focus on the effect of exposure to environmentally relevant concentrations of glyphosate and Roundup on developmental endpoints such as hatching success and developmental defects. Aim 2 will test the hypothesis that glyphosate and Roundup exposure induces alterations in gene expression and global epigenetic effects, particularly DNA methylation during early development, which results in alteration of reproductive success at adulthood. In adult medaka, reproductive endpoints such as fecundity (egg laying capacity) and fertilization efficiency (% of fertilized eggs/replicate/day) were examined and associated changes in expression of genes relating to the reproductive axis were determined by quantitative real-time PCR.

CHAPTER II

MATERIALS AND METHODS

2.1 Fish Maintenance

All animal procedures were conducted in accordance with the procedures described by the National Research Council of the US National Academics, “Guide for the care and use of laboratory animals”; and with UNCG guidelines for the humane treatment of test organisms during culture and experimentation. Experimental protocols and study plan were approved by UNCG Institutional Animal Care and Use Committee (protocol #17-006). Hd-rR strain of the wild-type medaka fish (*Oryzias latipes*) were cultured in the Department of Biology at The University of North Carolina at Greensboro from an inbred line. Fish were allowed to mature in 10-gallon tanks with constant aeration and water flow of approximately 20 gallons/day. Water temperature was maintained at 25 ± 1°C, while fish were kept under a light: dark cycle of 14 hr: 10 hr and fed TetraMin Tropical Flakes twice daily. Additional culture conditions include: pH: 6.8-7.2, total alkalinity: 0-40 ppm, nitrate: 0-20 ppm, nitrite: 0 ppm and ammonia: 0- 0.25 ppm.

2.2 Sampling and Chemical Exposure

Upon maturation to adulthood, (approximately 100-120 days) medaka were allowed to spawn and eggs were gently collected by hand from mature females and placed into deionized water (DI) containing 0.05% methylene blue. Methylene blue

treatment is necessary, as medaka embryos develop fungal infections if not treated. Collected eggs were then sorted and fertilized eggs were placed into non-treated, sterile, 48 well plates and exposed to DI water with 0.05% methylene blue for approximately 8 hours. At approximately 8 hours post fertilization (hpf) the water in each well plate was changed to the appropriate exposure solution. Exposure lasted from 8 hpf until 15 days post fertilization (dpf), with daily changes of 50% of exposure media. Upon hatching, fry were transferred to petri dishes containing exposure media without methylene blue. At 15 dpf the exposure media in the petri dishes was changed to control media, with medaka later being allowed to reach sexual maturity in 10-gallon tanks.

Nominal exposure solutions included: control (DI water), 0.5 mg/L glyphosate and two concentrations of Roundup; 0.5 mg/L and 5 mg/L glyphosate acid equivalent. Glyphosate was acquired from Sigma-Aldrich, while Roundup® Ready to Use (hereinafter "Roundup) was purchased from a local garden center. The lower exposure concentration of 0.5 mg/L was chosen to represent an environmentally relevant dose, given that the current maximum contaminant level (MCL) set by the U.S. Environmental Protection Agency (EPA) for glyphosate in drinking water in the United States is 0.7 mg/L (Battaglin *et al.* 2002, Monsanto 2014). The higher exposure concentration of 5 mg/L Roundup was chosen to represent an upper bound of direct application, as concentrations of Roundup (glyphosate acid equivalent) have been reported to range from 1.7 to 5.2 mg/L in areas following direct application (Annett *et al.* 2014, Harayashiki *et al.* 2013).

Medaka samples were collected at 4, 8, 15, and 100 dpf (or upon reaching sexual maturity). Refer to Figure 1 for detail of numbers and timeline for sample collection. Embryo samples collected at 4 dpf were flash frozen, while fry samples collected at 8 dpf and 15 dpf were euthanized using MS-220 (250 mg/L) before being flash frozen in liquid nitrogen. Upon reaching sexual maturity, the remaining medaka fish were allowed to spawn and eggs were collected from females in order to measure fecundity and fertilization efficiency. After eggs were collected, the mature medaka were humanely sacrificed using MS-220 (250 mg/L) and the brain, gonads, liver and gills were subsequently dissected, and flash frozen in liquid nitrogen. Weight and length of each fish was recorded along with the weight of the liver and gonads. All samples were stored at -80°C until genomic analysis was performed.

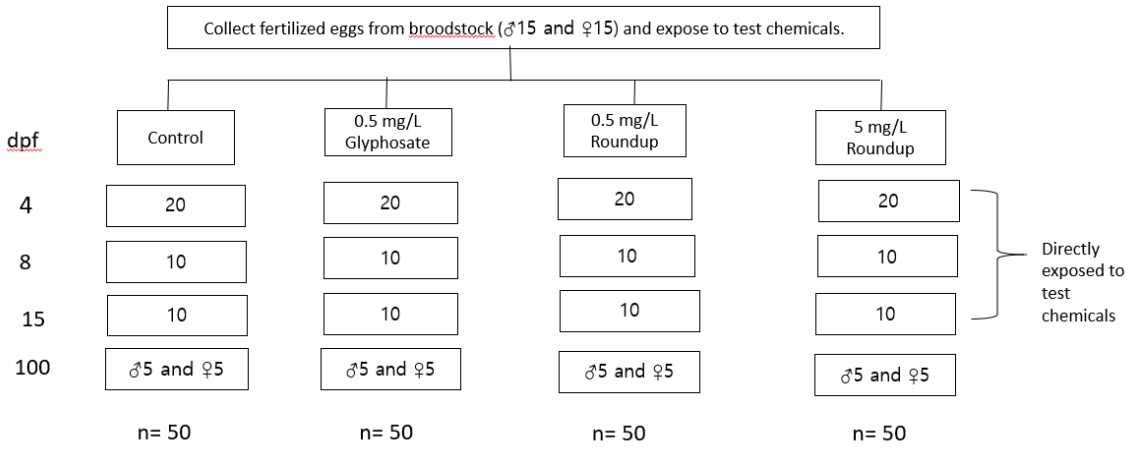


Figure 1. Experimental Design and Sampling Time Points After Embryonic Exposure of Medaka to Glyphosate and Roundup.

Timepoints for sample collection in the experiment were chosen based on four critical timepoints in the medaka lifecycle. Epigenetic gene activity was measured at 4 dpf to gain insight into the epigenetic effects of Roundup and glyphosate during the critical period of development prior to hatching, 8 dpf was chosen as a time point surrounding hatching and 15 dpf was chosen to represent a time point during maturation. Tissue samples were ultimately collected and analyzed at 100 dpf to give insight into the long term reproductive and epigenetic effects of early glyphosate and Roundup exposure.

2.3 Phenotype

Embryos from 0 dpf until hatching were examined daily under a stereomicroscope, and any developmental abnormalities were recorded. Photos were taken of each fry on the day of hatching and of all surviving fry at 15 dpf. Microscopic observations were performed using a Nikon SMZ1000 stereomicroscope equipped with a PlanApo objective; while pictures were captured using a Moticam 2300- 3 megapixel CMOS camera running under the Images Plus capture package. Pictures were later analyzed for changes in phenotype compared to control.

2.4 Fecundity and Fertilization Efficiency

Upon maturation, remaining medaka in each replicate were placed into mating pairs at a ratio of 2 females: 1 male, unless death by treatment was high enough to not enable this ratio. Egg clutches were collected by hand from mating females approximately 1 hour after the lights turned on. Eggs were examined under the stereoscope and the mean number of eggs per female per day (fecundity) was calculated, along with whether the eggs were fertilized or not (fertilization efficiency) for a period of

seven consecutive days. Statistical significance was determined using two-tailed T-test against control with standard error.

2.5 Gene Expression

All embryos, fry, and whole tissue samples were homogenized, and DNA/RNA was extracted using the ZymoResearch Z-R Duet MiniPrep Kit protocol using DNAse and proteinase K treatment. The purity and integrity of RNA for real time qPCR was measured using Thermo Scientific Nano Drop 2000/2000c Spectrophotometer. RNA was then reverse transcribed into cDNA using Applied Biosystems High Capacity cDNA Synthesis kits standard protocol. To assess gene expression, Power-Up SYBR Green Master reagents were used alongside primers that were specifically designed for the medaka genes of interest presented in the study (Table 1). mRNA levels were quantified using real-time qPCR ($\triangle\triangle$CT method) run under the QuantStudio Design and Analysis Software and gene expression data is presented as fold change against control. Statistical significance was determined using two-tailed T-test against control with standard error.

Table 1. Forward and Reverse Primer Sequences Designed for Medaka Genes of Interest.

Primer Name	Forward Primer Sequence	Reverse Primer Sequence
Tet1-5p & Tet1-3p	5’ ACC-TTC-ATC-TTC-ACC-CGG-TT 3’	5’ CTG-AGA-GAC-GAG-GGT-GGT-TT 3’
Tet2-5p & Tet2-3p	5’ AAG-AAG-GAC-AGA-CAG-AGG-GC 3’	5’ GGG-TTG-TAC-TGT-ACT-GGG-CT 3’
Tet3-5p & Tet3-3p	5’ CTG-CTC-GGA-ACA-CCT-TGA-AC 3’	5’ CCC-TAT-GCT-GTT-CTG-TTG-CC 3’
xDnmt1-F & xDnmt1-R	5’ AAC-AGG-TGG-AGA-GCT-ACG-AC 3’	5’ CAG-CTC-TCC-TTT-TCC-CCA-GA 3’
xDnmt3aa-F & xDnmt3aa-R	5’ TTT-GAG-GCT-TTA-CAG-GTG-GC 3’	5’ CTG-TCT-GGT-CTG-AAG-CTC-CA 3’
KISS1F & KISS1R	5’ ATC-TGA-TGG-AGG-GAC-TCC-AAT-G 3’	5’ TGG-CGT-TTC-TTT-ATA-GCC-ACA-G 3’
KISS2F & KISS2R	5’ TGA-AGC-TCC-CTC-TGA-TGT-CC 3’	5’ CCA-CCC-ACA-TGT-CCT-TGA-C 3’
GPR54-1F & GPR54-1R	5’ CTT-CTG-TCC-ATC-CCT-GTG-GT 3’	5’ TCG-CTG-CAG-TAA-ATC-TGT-GG 3’
GPR54-2F & GPR54-2R	5’ ATC-TGG-ACG-AGG-ATG-AGG-AG 3’	5’ CGA-GAA-GAA-CAA-AGG-GAC-CA 3’
xStar-F & xStar-R	5’ GCT-CCC-TTC-TAA-GTT-CTC-GC 3’	5’ CCT-GCT-CGC-TTA-GAA-TGC-TG 3’
xFshr-F & xFshr-R	5’ TGA-GTT-GGT-GGT-GCT-AGA-CA 3’	5’ CAA-CAG-CTT-CTT-CAG-GCC-AG 3’
xLhr-F & xLhr-R	5’ TTT-TGC-CCT-GAA-AAG-CCT-CC 3’	5’ CTC-TAA-AAC-TTT-GTT-TCC-GCC-G 3’
mdERa-F-qPCR-188 & mdERa-R-qPCR-188	5’ATC-GCT-CCC-GGT-TCT-ATA-TCA-G 3’	5’ AAG-CAT-CAC-CTT-GTC-CCA-AC 3’
xDmrt1-F & xDmrt1-R	5’CTT-CTG-CCG-CTG-GAA-AGA-C 3’	5’ CCT-CCT-ATC-GGC-GAC-CTG 3’
mdARa-F & mdARa-R	5’ GGC-ATG-AGG-ATT-TGT-TTC-CA 3’	5’ CAG-GTG-GTT-CTG-CTT-ACC-TG 3’
xCyp19a1a-F & xCyp19a1a-R	5’ TGT-TGA-CGA-GAA-AGA-GCT-GC 3’	5’ GCT-GTC-TTG-TGC-CTC-TGA-TG 3’
xFoxL2-F & xFoxL2-R	5’ CCT-CGT-CCT-ACA-ACC-CCT-AG 3’	5’ GTG-ACC-CAT-GCC-GTT-GTA-AG 3’
mdEF1a-F6 & mdEF1a-R6	5’ AGA-AGG-AAG-CCG-CTG-AGA-TGG 3’	5’ GCT-CAG-CCT-TCA-GTT-TGT-CCA-A 3’

CHAPTER III

RESULTS

3.1 Aim 1

To evaluate the first aim of the study and test whether environmentally relevant concentrations of glyphosate and Roundup exposure can induce developmental defects in medaka, developmental endpoints such as hatching success and developmental abnormalities were evaluated in each treatment group on the day of hatching.

3.2 Hatching Success

Cumulative hatching success (defined as hatching 7-12 dpf) was calculated for each treatment group and is displayed below in Figure 2. The average rate of hatching per treatment group, given all replicates, was 58.7%, 44.5%, 54.9% and 35.2% (for control, 0.5 mg/L Roundup, 5 mg/L Roundup and 0.5 mg/L glyphosate, respectively).

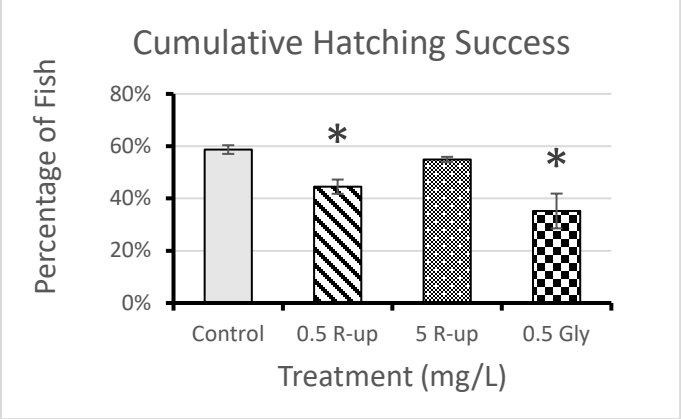


Figure 2. Cumulative Hatching Success. Values expressed as the average percentage of fish that hatched between 7-12 dpf from each treatment group.

Compared to control, cumulative hatching success was significantly decreased (*$p < 0.05$) in medaka exposed to 0.5 mg/L Roundup and 0.5 mg/L glyphosate, indicating that environmentally relevant doses of both treatment groups resulted in an overall decrease in hatching success. The cumulative hatching success was then broken down into normal hatching (defined as hatching 7-10 dpf) and delayed hatching (defined as hatching 11-12 dpf). Statistical significance (*$p < 0.05$) was observed for both normal (Figure 3) and delayed (Figure 4) hatching for 0.5 mg/L Roundup.

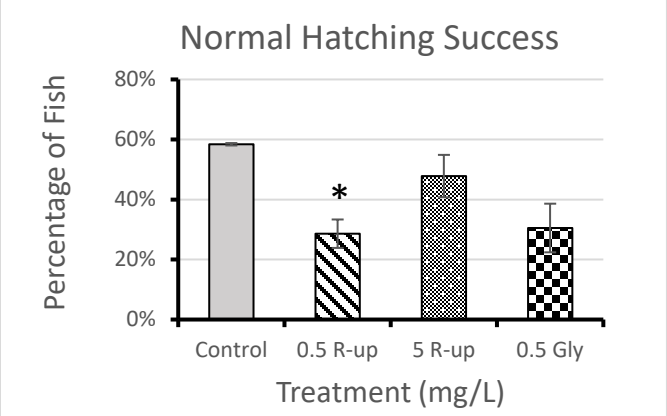


Figure 3. Normal Hatching Success. Values expressed as the average percentage of fish that hatched between 7-10 dpf from each treatment group.

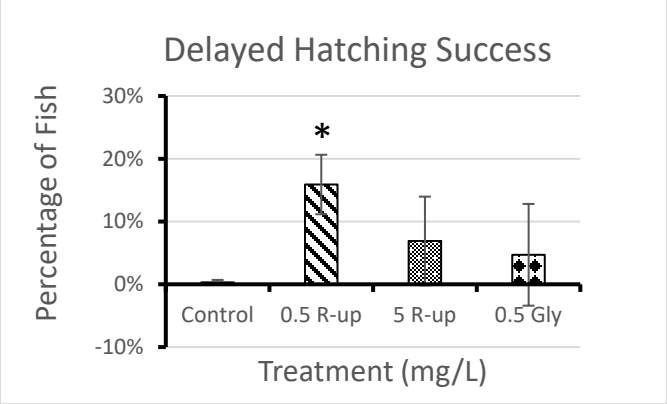
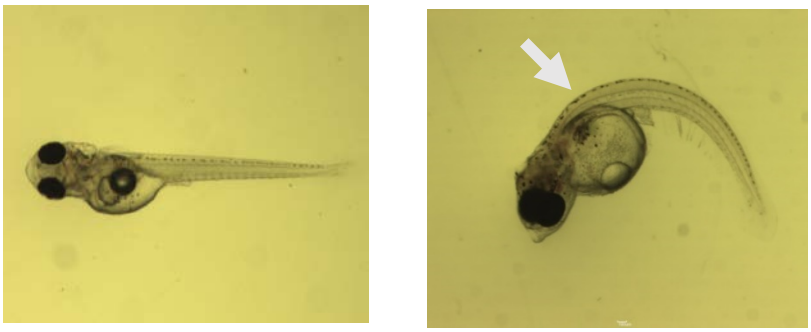


Figure 4. Delayed Hatching Success. Values expressed as the average percentage of fish that hatched between 11-12 dpf from all treatment groups.

3.3 Developmental Abnormalities

Based on photographs of each fry taken on the day of hatching, several developmental abnormalities were observed including: spinal curvature, enlarged yolk sac, uninflated swim bladder and greying of yolk sac.



(A) (B)

Figure 5. Photographic Depiction of Spinal Curvature. 8dpf picture of hatched fry displaying A) Normal spinal curvature- Control B) Severe spinal curvature- 0.5 mg/L glyphosate.

One common phenotypic abnormality observed on the day of hatching was severe spinal curvature (Figure 5). The average rate of severe spinal curvature for each treatment group, given all replicates, was 1.7%, 27.9%, 1.8% and 25% (for control, 0.5 mg/L Roundup, 5 mg/L Roundup and 0.5 mg/L glyphosate, respectively). The average rate of severe spinal curvature increased in medaka exposed to 0.5 mg/L Roundup and glyphosate treatments, with significance observed in medaka exposed to 0.5 mg/L glyphosate compared to control (Figure 6) (*$p < 0.05$).

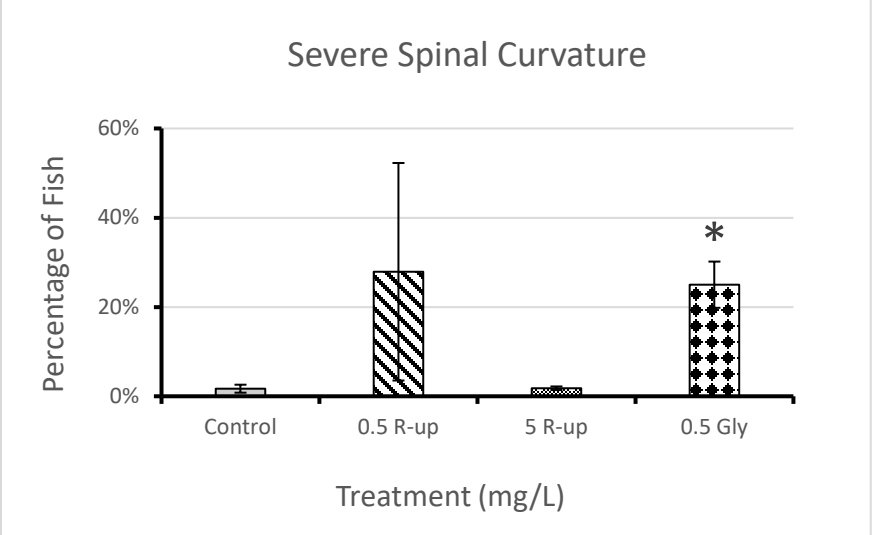
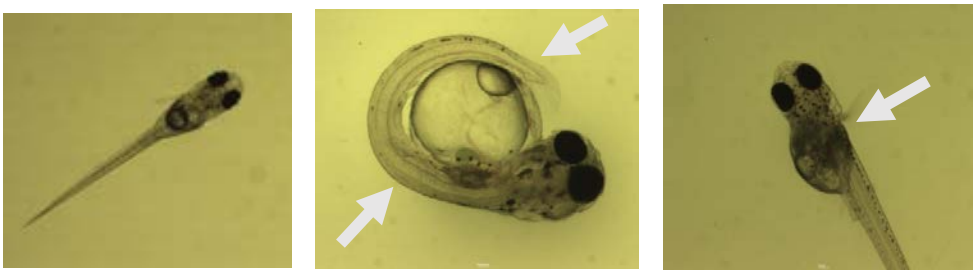


Figure 6. Severe Spinal Curvature. The average rate of fry hatched with severe spinal curvature in each treatment group.

In addition to severe spinal curvature, other developmental abnormalities included enlarged yolk sac, uninflated swim bladder and severe greying of the yolk sac (Figure 7).



(a) (b) (c)

Figure 7. Photographic Depiction of Developmental Abnormalities. Normal fry (a) and developmental abnormalities observed post hatch including enlarged yolk sac and uninflated swim bladder (b) and severe greying of yolk sac (c).

A fry was classified as “poor development” if it possessed three out of four developmental abnormalities. The average rate of fry qualifying as poor development for each treatment group, given all replicates, was 4.3%, 18.2%, 10.10%, and 33.2% (for

control, 0.5 mg/L Roundup, 5 mg/L Roundup and 0.5 mg/L glyphosate, respectively). Significance (*$p < 0.05$) was observed for 0.5 mg/L glyphosate compared to control (Figure 8).

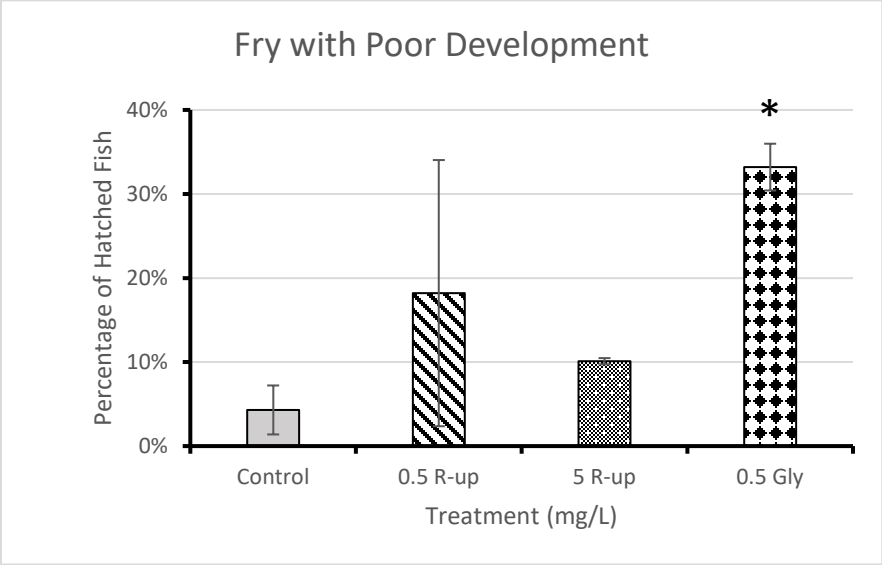


Figure 8. Fry Exhibiting Poor Development. The average rate of fry hatched with 3 out of 4 abnormalities including severe spinal curvature, enlarged yolk sac, greying of yolk sac and uninflated swim bladder.

3.4 Aim 2

To evaluate the hypothesis that environmentally relevant doses of glyphosate and Roundup exposure induces epigenetic effects during early development resulting in alterations in reproductive success at adulthood, epigenetic genes were measured at 4, 8 and 15 dpf, and both epigenetic and reproductive genes were measured upon maturation, as well as fecundity and fertilization efficiency endpoints measured.

3.5 Epigenetic Genes - *Dnmt1* and *Dnmt3aa*- 4, 8 and 15 dpf

Gene expression of *Dnmt1* and *Dnmt3aa* were measured in samples collected at 4, 8 and 15 dpf (Figure 9). While no significance was observed in gene expression levels at 4 and 8dpf, *Dnmt1* expression was downregulated in all treatment groups at 15 dpf, with significance observed for both 0.5 mg/L and 5 mg/L Roundup.

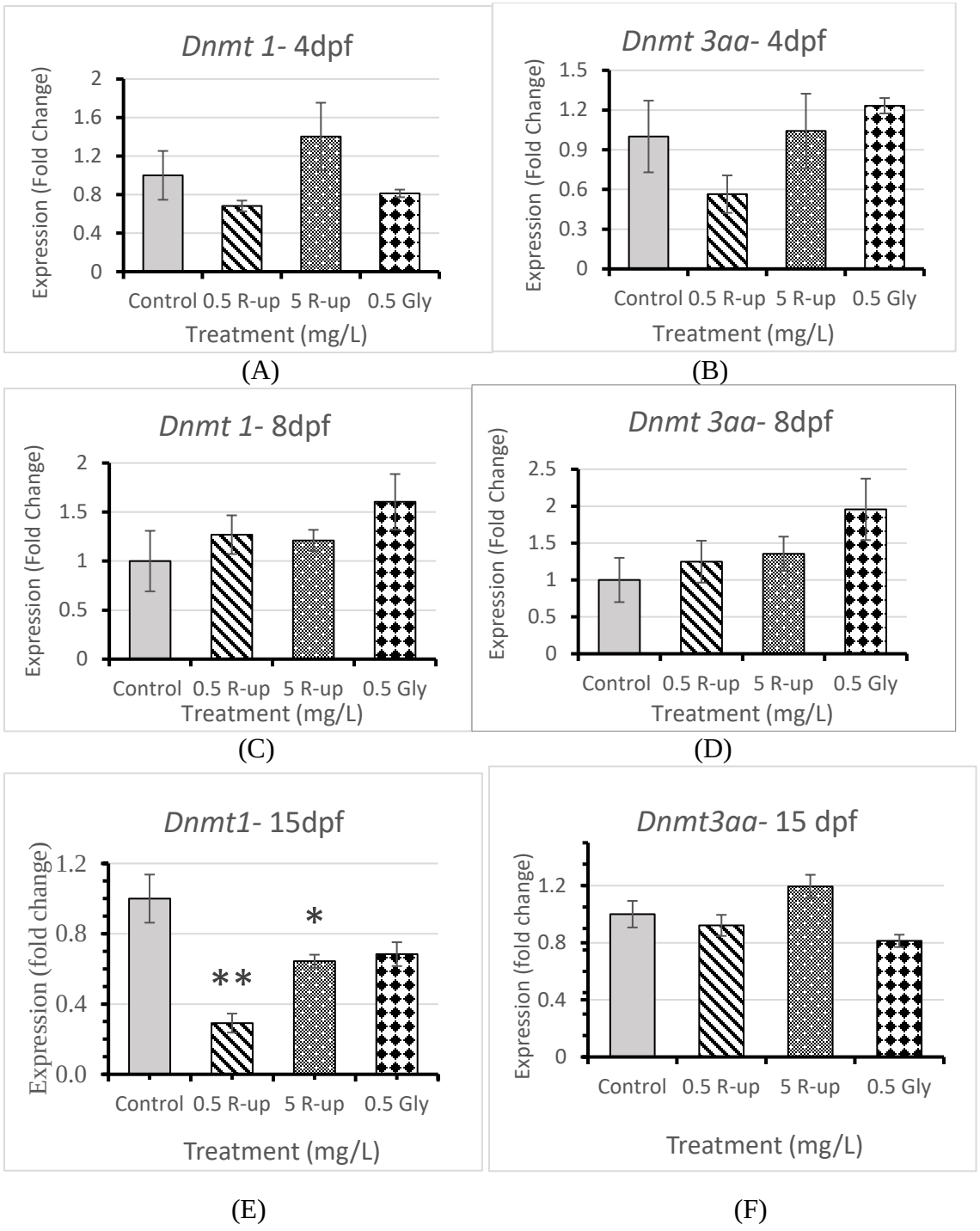
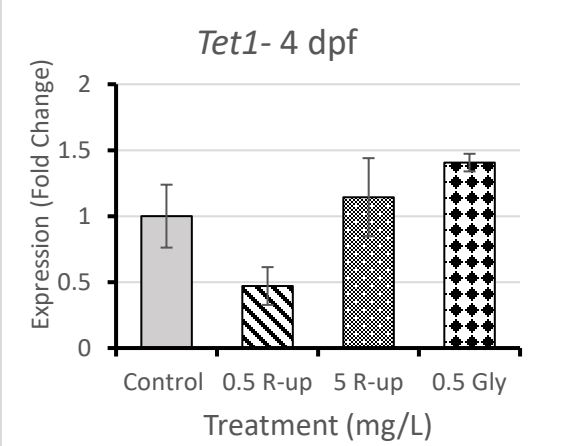


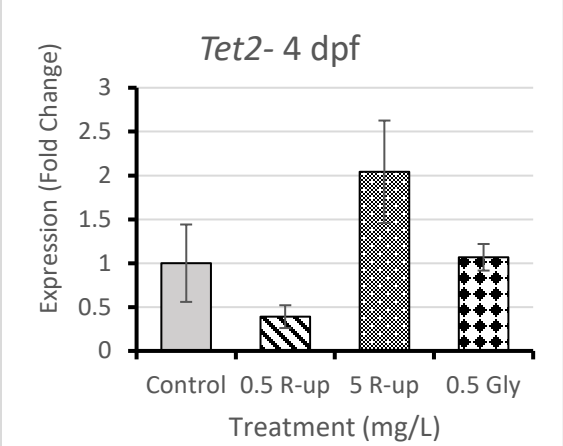
Figure 9. Epigenetic Gene Expression of *Dnmt1* and *Dnmt3aa* Measured at 4, 8 and 15 dpf. Gene expression levels of *Dnmt1* and *Dnmt3aa* at 4 dpf (A, B), 8 dpf (C, D) and 15 dpf (E, F) in medaka embryos/fry, expressed as fold change against control. Significance was observed at 15 dpf with asterisks indicating significance (* $p < 0.05$, ** $p < 0.01$).

3.6 Epigenetic Genes- *Tet1, Tet2, Tet3*- 4, 8, and 15 dpf

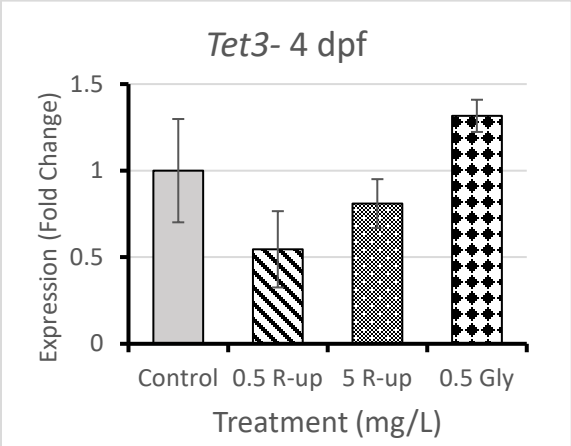
Gene expression levels of *Tet1*, *Tet2* and *Tet3* were measured at 4, 8 and 15 dpf and expression profiles are presented below in Figure 10. Significant upregulation of all *Tet* genes was observed in a dose dependent manner at 15 dpf for the 0.5 mg/L and 5 mg/L Roundup treatment groups, and for *Tet1* and *Tet3* in the 0.5 mg/L glyphosate treatment group. While no significance was observed for any of the *Tet* genes at 4 and 8 dpf, the general trend of expression was downregulation of all *Tet* genes in medaka exposed to 0.5 mg/L Roundup at 4 dpf and upregulation in all *Tet* genes at 8 dpf for the same treatment group. This pattern appears to have the reverse effect in the 5 mg/L Roundup treatment group for the same time points.


Tet1- 4 dpf
Expression (Fold Change)
2
1.5
1
0.5
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

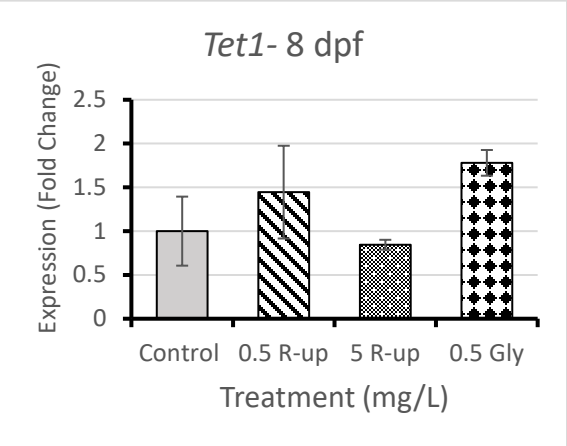
(A)


Tet2- 4 dpf
Expression (Fold Change)
3
2.5
2
1.5
1
0.5
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

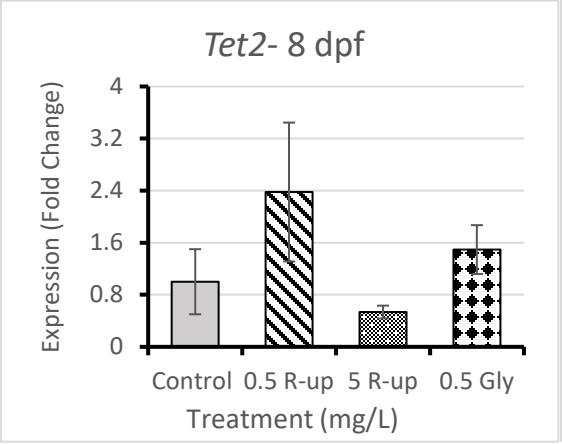
(B)


Tet3- 4 dpf
Expression (Fold Change)
1.5
1
0.5
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

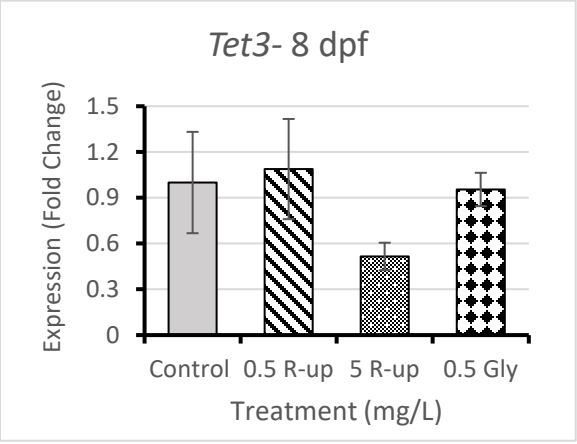
(C)


Tet1- 8 dpf
Expression (Fold Change)
2.5
2
1.5
1
0.5
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

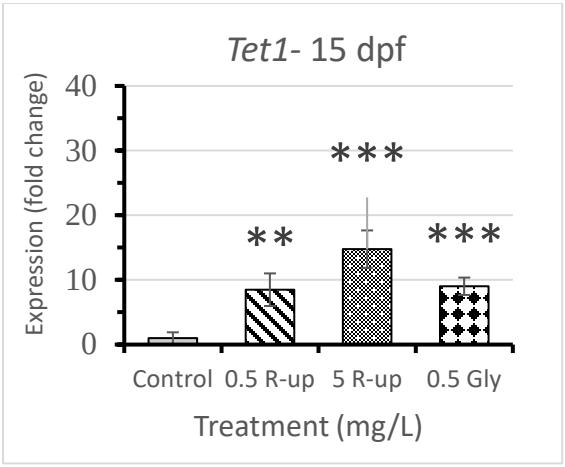
(D)


Tet2- 8 dpf
Expression (Fold Change)
4
3.2
2.4
1.6
0.8
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

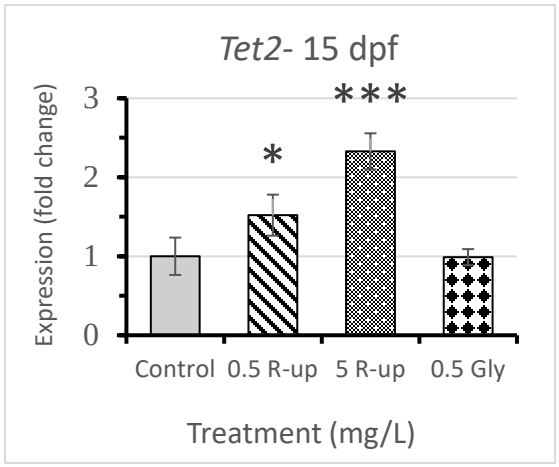
(E)


Tet3- 8 dpf
Expression (Fold Change)
1.5
1.2
0.9
0.6
0.3
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

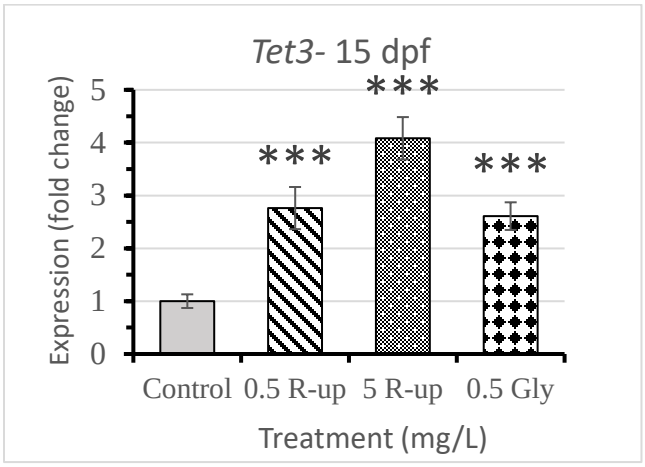
(F)



(G)



(H)



(I)

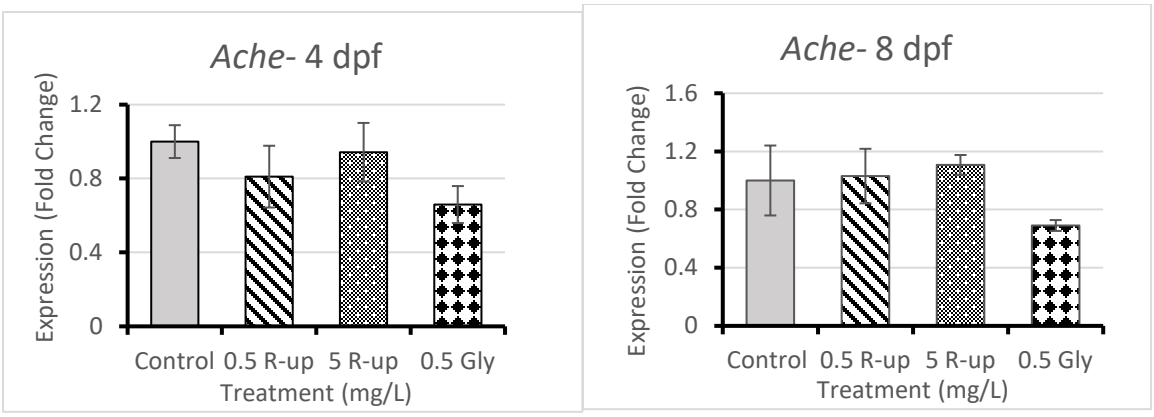
Figure 10. Epigenetic Gene Expression of *Tet1*, *Tet2*, and *Tet3* Measured at 4, 8 and 15 dpf. Gene expression levels of *Tet1*, *Tet2* and *Tet3* at 4 dpf (A-C), 8 dpf (D-F) and 15 dpf (G-I) in medaka embryos/fry, expressed as fold change against control. Significance was observed at 15 dpf with asterisks indicating significance (* $p < 0.05$, ** $p < 0.01$, ***$p<0.001$).

3.7 Acetylcholinesterase- 4, 8 and 15 dpf

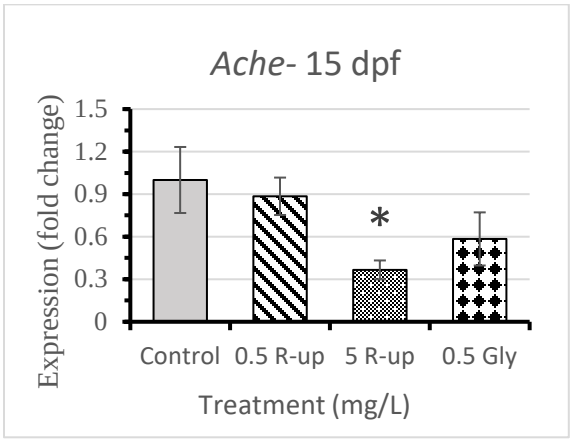
Ache expression profiles for 4, 8 and 15 dpf are displayed below in Figure 11.

Ache was downregulated at all time points for medaka exposed to 0.5 mg/L glyphosate

and in all treatment groups at 15 dpf, with significance observed for the 5 mg/L Roundup treatment group at 15 dpf.



(A) (B)

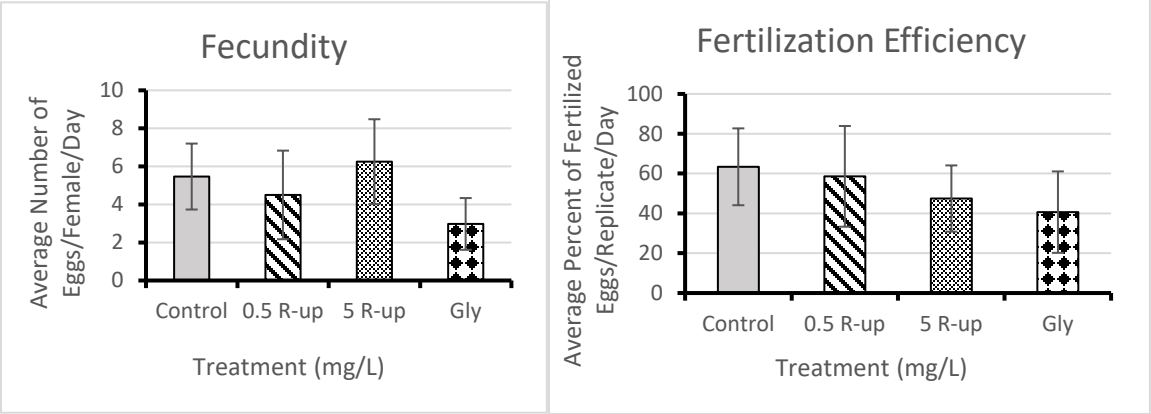


(C)

Figure 11. Acetylcholinesterase Gene Expression Measured at 4, 8 and 15 dpf. Gene expression levels of acetylcholinesterase (*Ache*) at 4 dpf (A), 8 dpf (B) and 15 dpf (C) in medaka embryos/fry, expressed as fold change against control. Significance was observed at 15 dpf with asterisks indicating significance (*$p < 0.05$).

3.8 Fecundity and Fertilization Efficiency

Fertilization efficiency and fecundity were calculated for each treatment group and displayed below in Figure 12. Fecundity, represented as the average number of eggs/female/day (mean ± SE) for each treatment group, given all replicates, was 5.5 ± 1.7, 4.5 ± 2.3, 6.3 ± 2.2 and 3.0 ± 1.4 (for control, 0.5 mg/L Roundup, 5 mg/L Roundup and 0.5 mg/L glyphosate respectively). Fertilization efficiency represented as the average percentage of fertilized eggs/replicate/day (mean ± SE) for each treatment group, given all replicates, was 63.4 ± 19.3, 58.6 ± 25.3, 47.5 ± 16.6 and 40.6 ± 20.5 (for control, 0.5 mg/L Roundup, 5 mg/L Roundup and 0.5 mg/L glyphosate respectively). While not significant, glyphosate appears to have a negative effect on both fertilization efficiency and fecundity compared to control.

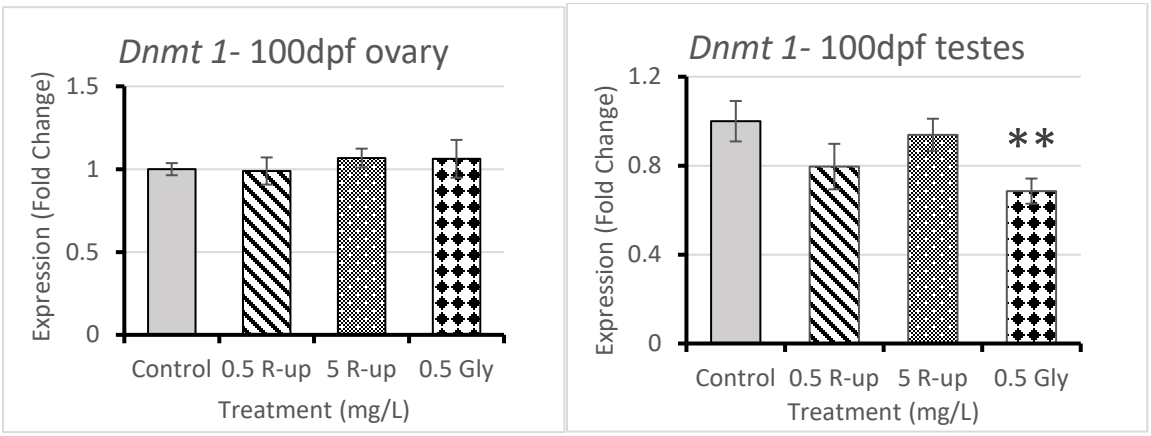


(A) (B)

Figure 12. Fecundity and Fertilization Efficiency of Mature Medaka. Fecundity (A) represented as the average number of eggs per female per day and fertilization efficiency (B) represented as the average percent of fertilized eggs per replicate per day taken from each treatment group upon maturation for a period of seven consecutive days.

3.9 Epigenetic Genes – *Dnmt1*- 100dpf

Dnmt1 expression levels were measured in ovaries and testes from mature female and male medaka respectfully in each treatment group. *Dnmt1* expression was significantly lower ($p < 0.01$) in testes exposed to 0.5 mg/L glyphosate (Figure 13).

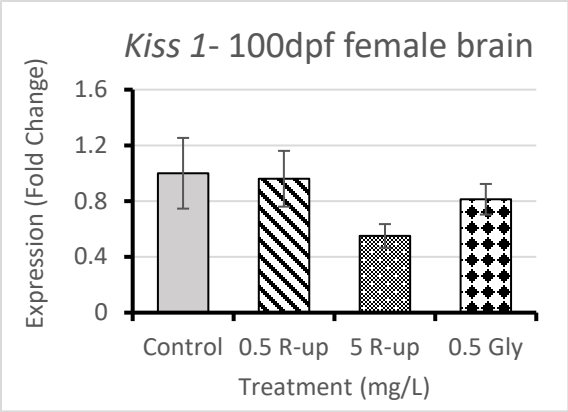


(A) (B)

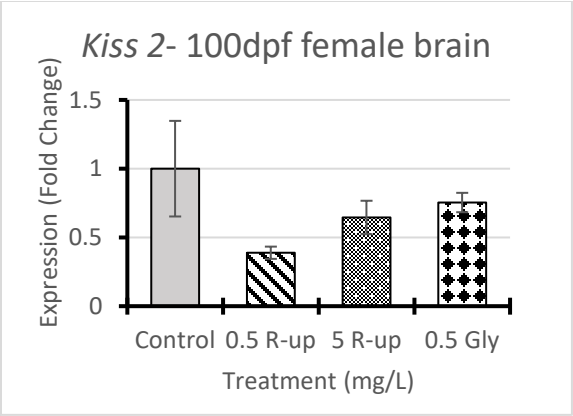
Figure 13. *Dnmt1* Gene Expression in Mature Male Medaka Gonads. Epigenetic gene expression levels of *Dnmt1* in 100 dpf ovaries (A) and testes (B) expressed as fold change against control. Significance was observed in testes with asterisks indicating significance (** $p < 0.01$).

3.10 Reproductive Genes- *Kiss1, Kiss2,* Gpr54-1, Gpr54-2- 100dpf-Brain

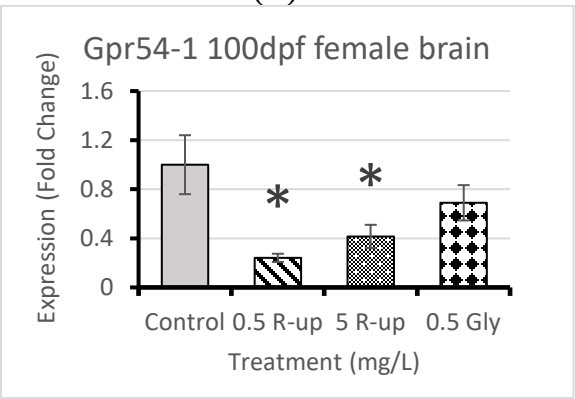
Previous studies have shown that endocrine disrupting chemicals can cause reproductive dysfunction due to their effect on kisspeptin neurons (Usuda *et al.* 2014), thus in the present study, *Kiss1, Kiss2,* Gpr54-1 and Gpr54-2 levels were measured in male and female brain samples upon maturation (Figure 14). In female brain samples, Gpr54-1 was significantly downregulated in medaka exposed to 0.5 and 5 mg/L Roundup while Gpr54-2 was significantly downregulated in medaka exposed to 0.5 mg/L Roundup. No significance was observed for any gene or receptor in the male brain.


Kiss 1- 100dpf female brain
Expression (Fold Change)
1.6
1.2
0.8
0.4
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

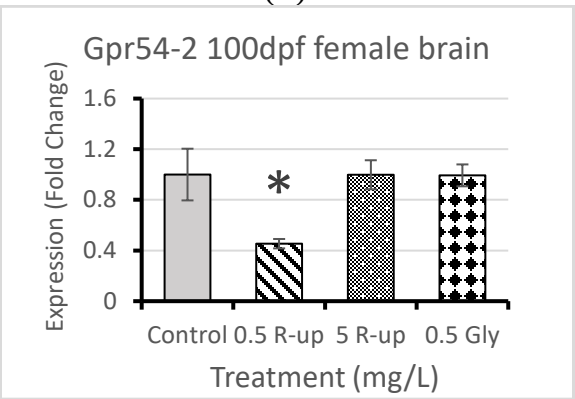
(A)


Kiss 2- 100dpf female brain
Expression (Fold Change)
1.5
1
0.5
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

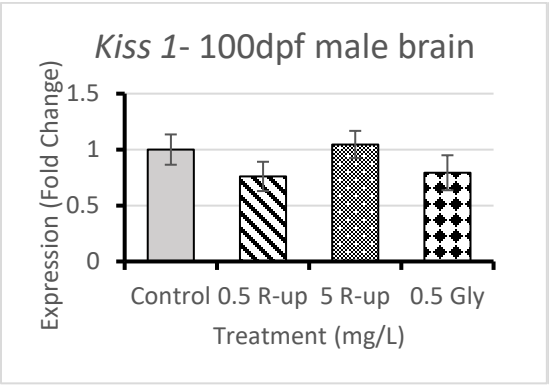
(B)


Gpr54-1 100dpf female brain
Expression (Fold Change)
1.6
1.2
0.8
0.4
0
*
*
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

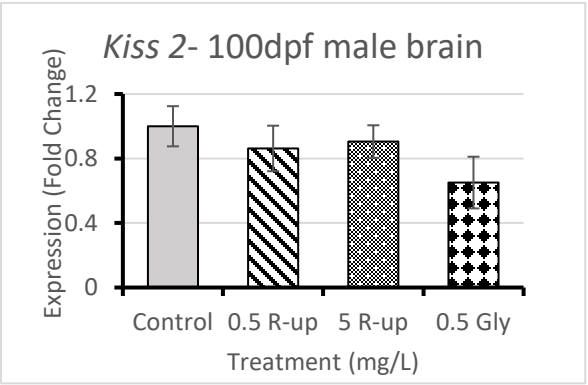
(C)


Gpr54-2 100dpf female brain
Expression (Fold Change)
1.6
1.2
0.8
0.4
0
*
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

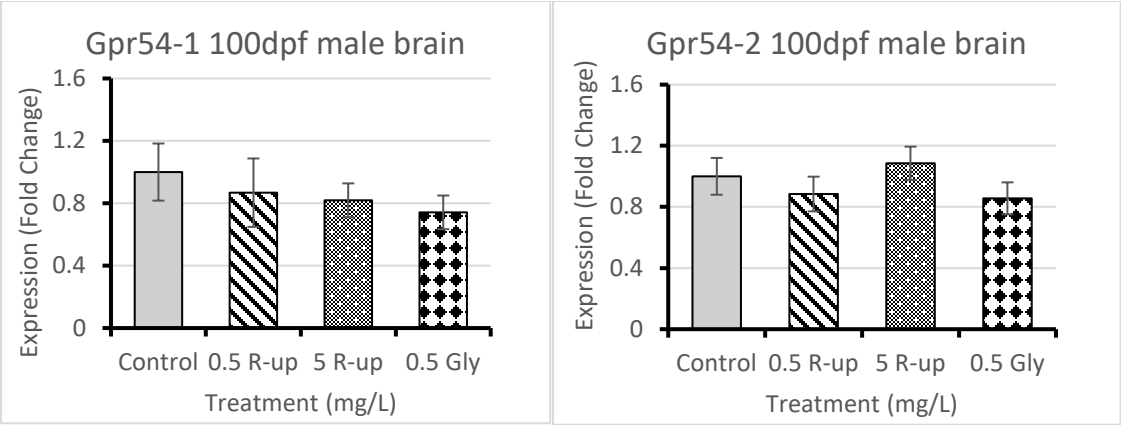
(D)


Kiss 1- 100dpf male brain
Expression (Fold Change)
1.5
1
0.5
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

(E)


Kiss 2- 100dpf male brain
Expression (Fold Change)
1.2
0.8
0.4
0
Control
0.5 R-up
5 R-up
0.5 Gly
Treatment (mg/L)

(F)



(G) (H)

Figure 14. Reproductive Gene Expression of *Kiss1*, *Kiss2*, Gpr54-1 and Gpr54-2 Measured in Mature Medaka Brain Samples. Reproductive gene expression levels of *Kiss1*, *Kiss2*, Gpr54-1 and Gpr54-2 for each treatment group in 100 dpf female brain samples (A-D) and male brain samples (E-H) expressed as fold change against control. Significance was observed in Gpr54-1 and Gpr54-2 in female brain samples with asterisks indicating significance (* $p < 0.05$).

3.11 Reproductive Genes- *Star, Fshr, Lhr, Erα, Cyp19a1a, FoxL2*-100dpf- Ovaries

Gene expression levels of *Star*, *Fshr*, *Lhr*, *Erα, Cyp19a1a* and *FoxL2* were measured in mature medaka ovary samples from each treatment group. Gene expression profiles are displayed below in Figure 15. Gene expression for medaka exposed to 0.5 mg/L glyphosate showed a tendency to decrease in all reproductive genes, while gene expression in medaka exposed to 5 mg/L Roundup showed a tendency to increase in all genes measured in 100 dpf ovaries with the exception of *Cyp19a1a*, compared to control although there was no significance.

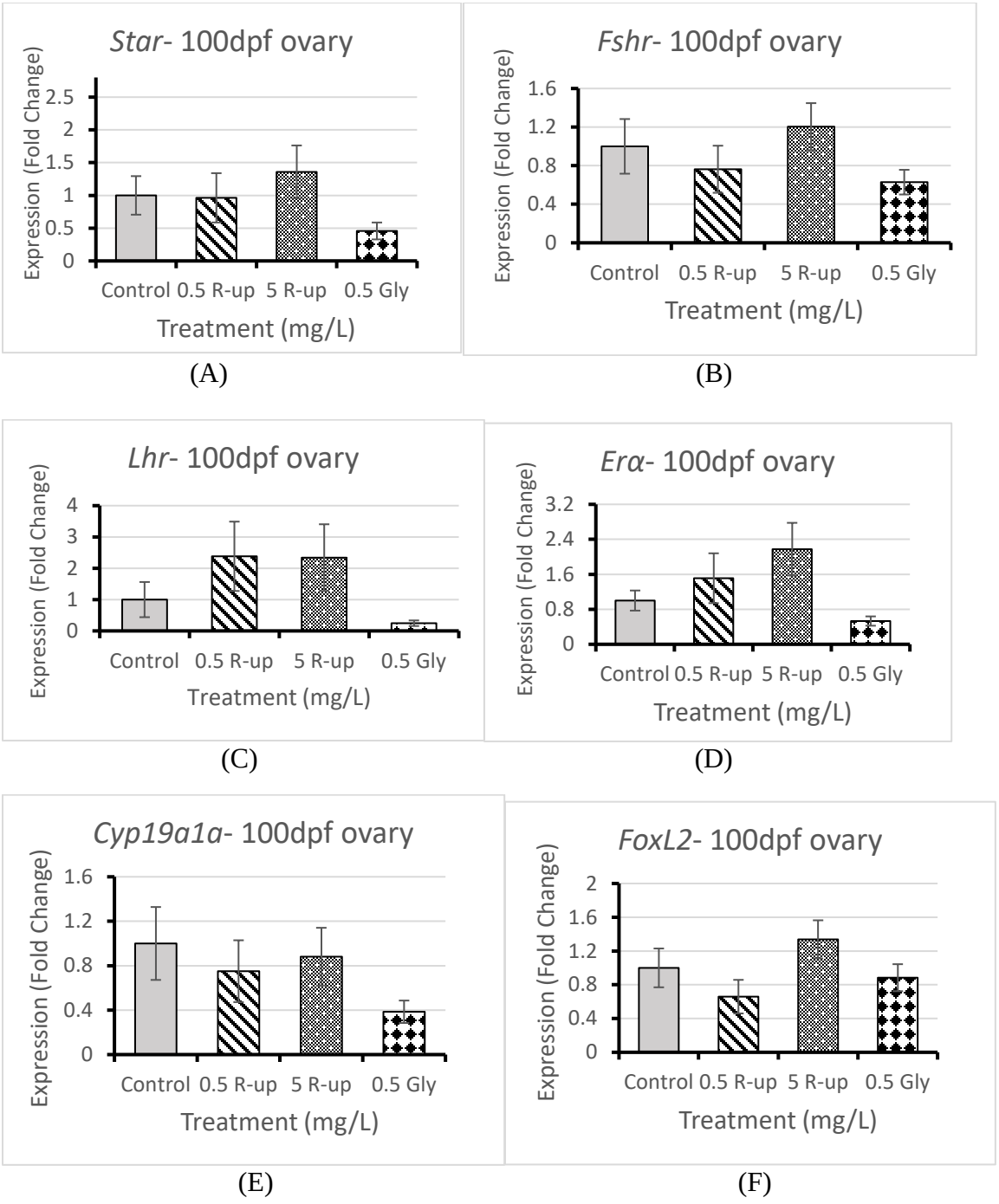


Figure 15. Expression of *Star, Fshr, Lhr, Erα, Cyp19a1a* and *FoxL2* in Mature Female Ovaries. Reproductive gene expression levels for each treatment group of *Star* (A), *Fshr* (B), *Lhr* (C), *Erα* (D), *Cyp19a1a* (E), and *FoxL2* (F) measured in 100 dpf female ovary samples. No significance was observed in any of the genes.

3.12 Reproductive Genes-*Star, Fshr, Lhr, Erα, Arα, Dmrt1*-100dpf- Testes

Gene expression levels of *Star, Fshr, Lhr, Erα, Arα* and *Dmrt1* were measured in mature medaka testes samples from each treatment group. Gene expression profiles are displayed below in Figure 16. Expression levels of *Fshr* and *Arα* were significantly downregulated in 0.5 mg/L Roundup and glyphosate treatment groups. *Dmrt1* expression also showed a tendency to decrease in medaka exposed to 0.5 mg/L Roundup and glyphosate, with significance observed in the 0.5 mg/L glyphosate treatment group.

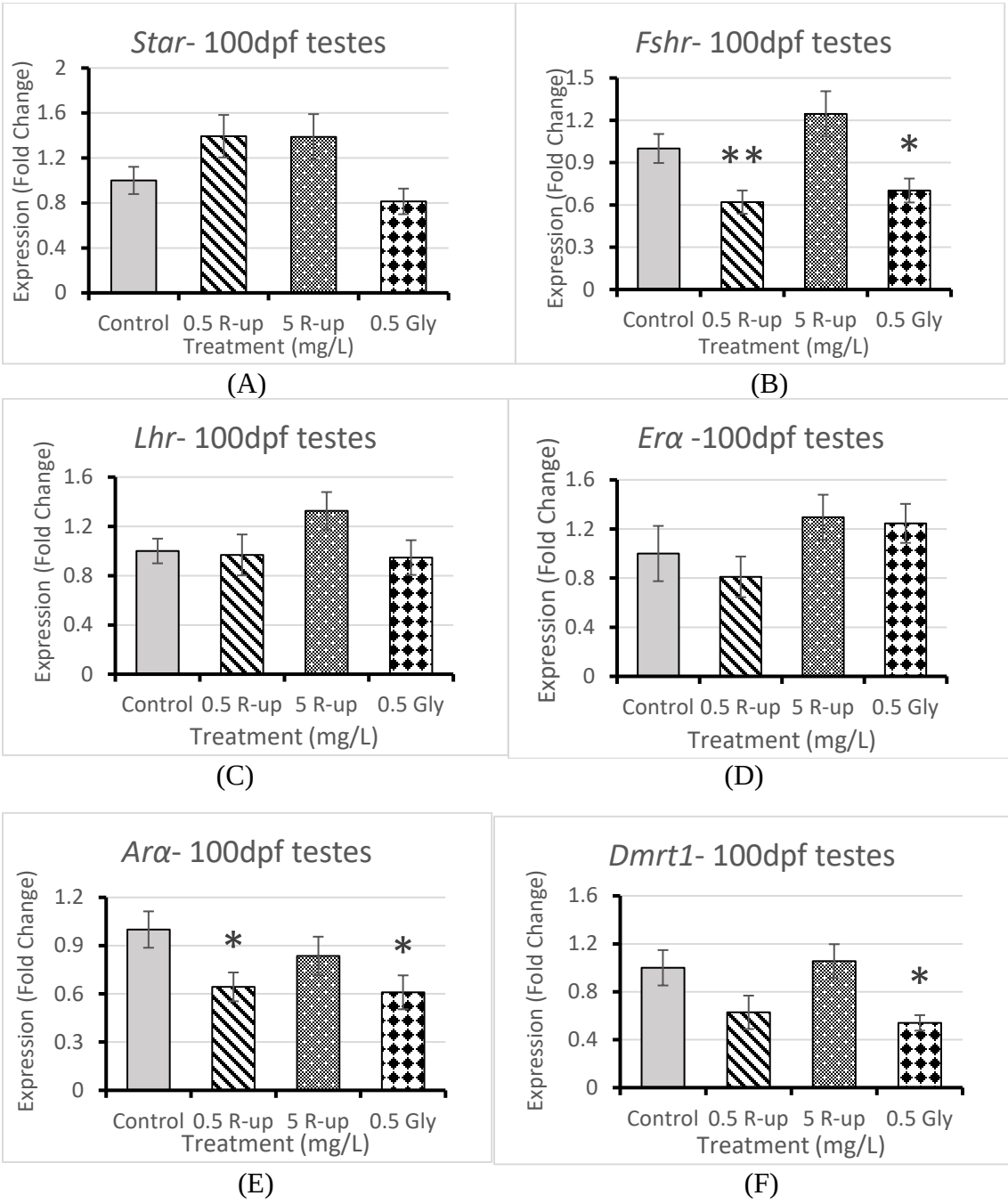


Figure 16. Reproductive Gene Expression of *Star, Fshr, Lhr, Erα, Arα, and Dmrt1* in Mature Male Testes. Reproductive gene expression levels for each treatment group of *Star* (A), *Fshr* (B), *Lhr* (C), *Erα* (D), *Arα* (E), and *Dmrt1* (F) measured in 100 dpf male testes samples. Significant downregulation was observed in *Fshr* and *Arα* for 0.5 mg/L Roundup and in *Fshr*, *Arα* and *Dmrt1* for 0.5 mg/L glyphosate. Significance is indicated by asterisks (*$p<0.05$, ** $p< 0.01$).

CHAPTER IV

DISCUSSION

Due to its increasing use, its prevalence in aquatic environments, and its presence in surface and ground waters, it is vital to evaluate the health effects of glyphosate and Roundup exposure. The focus of this study was to determine whether or not exposure to environmentally relevant concentrations of glyphosate and Roundup during critical periods of development induced developmental defects in the form of changes in hatching success and developmental abnormalities, and whether or not this exposure resulted in changes in gene expression and global epigenetic effects, particularly DNA methylation, which resulted in alterations of reproductive success at adulthood.

Previous studies have reported changes in hatching success and the presence of developmental abnormalities, including tail malformations, yolk sac edema, spinal curvature and pericardial edema in fish species exposed to Roundup and/or glyphosate, yet many of these studies were conducted using environmentally irrelevant concentrations (Sulukan *et al.* 2017, Uren Webster *et al.* 2014, Yusof *et al.* 2014, Zhang *et al.* 2017). Yusof *et al.* (2014) reported a dose-dependent increase in developmental abnormalities in Java medaka exposed to Roundup (glyphosate acid equivalent) including shrunken yolk, abnormal body curvature, slow development and disproportionate head and body size, yet the concentrations used in the study were 20-100 times the highest concentration used in the present study. In the present study, using environmentally

relevant concentrations, an increase in severe spinal curvature and other abnormalities including enlarged yolk sac, greying of yolk sac and uninflated swim bladder were observed in medaka exposed to 0.5 mg/L glyphosate. Sulukan et al. (2017) reported similar findings in which a significant increase in body malformations (including tail malformations, short tail and head malformations) were observed in zebrafish starting at 1 mg/L glyphosate; while other malformations, including pericardial edema, yolk sac edema and spinal curvature were significantly increased as well, but only in exposure groups exceeding 1 mg/L glyphosate including: 5, 10 and 100 mg/L glyphosate, which were concentrations greater than those used in the present study.

Cumulative hatching success was also found to be significantly decreased in medaka exposed to 0.5 mg/L Roundup and 0.5 mg/L glyphosate. Previous studies evaluating the effects of glyphosate on hatching success have reported an increase in hatching rate following glyphosate exposure, contradicting our current findings, yet significance in the previous studies was not observed until exposure concentrations reached 10 mg/L glyphosate and 400 mg/L glyphosate respectfully (Uren Webster *et al.* 2014, Zhang *et al.* 2017), proposing the idea that hatching rate might result in different outcomes based on exposure concentration.

In the present study *Ache* gene expression was significantly decreased at 15 dpf in fry exposed to 5 mg/L Roundup. Exposure to glyphosate and GBHs has previously been shown to cause a decrease in AChE activity and gene expression in a variety of fish species, confirming the results found in the present study (Glusczak *et al.* 2006, Glusczak *et al.* 2007, Lopes *et al.* 2017). Glusczak *et al.* (2006) found that exposing piava

(*Leporinus obtusidens*) to 3, 6, 10 and 20 mg/L Roundup (glyphosate acid equivalent) for 96 hours resulted in a significant decrease in AChE activity in the brain for all exposure groups, while Lopes *et al.* (2017) reported a significant decrease in *Ache* gene expression in brain samples of male zebrafish exposed to 5 and 10 mg/L glyphosate after 24 hours exposure. A decrease in *Ache* gene expression suggests a buildup of acetylcholine in the synapse, leading to an increase in hypercholinergic activity, resulting in negative effects on aquatic species, as proper AChE function is essential for swimming, orientation, predator avoidance and prey capture throughout the life cycle of aquatic species (Glusczak *et al.* 2006).

The present study is unique in that it focused on the effects of early life exposure in both early life and later in adulthood. No significant difference was observed in fecundity and fertilization efficiency in the fish at adulthood. Although no study has been performed focusing on the effects of developmental exposure on adult reproductive health, a recent study reported a significant decrease in fecundity after adult zebrafish were exposed to 10 mg/L glyphosate for 21 days (Uren Webster *et al.* 2014). The same study reported no significant difference in fertilization efficiency given any of the exposure groups (0.01, 0.5 and 10 mg/L Roundup (glyphosate acid equivalent) and 10 mg/L glyphosate) (Uren Webster *et al.* 2014). Using concentrations similar to those in the current study, Folmar *et al.* (1979) reported no change in fecundity 30 days after exposing rainbow trout to 0.02, 0.2 and 2 mg/L Roundup (glyphosate acid equivalent) for 12 hours. It appears that glyphosate and Roundup might affect fecundity at higher

exposure concentrations and have little effect on fertilization efficiency at environmentally relevant concentrations.

Downregulation of *Dnmt1* expression with significance observed for 0.5 mg/L and 5 mg/L Roundup, and a significant upregulation in expression of *Tet1, Tet2* and *Tet3* in almost every exposure group, was observed at 15 dpf. Together the decrease in *Dnmt1* expression and increase in *Tet1, Tet2 and Tet3* expression suggests an overall decrease in DNA methylation. This decrease in overall DNA methylation is the result of active DNA demethylation by TET enzymes through the oxidation of 5mC, and passive DNA methylation due to the decrease in *Dnmt1* expression, resulting in newly incorporated cytosines during cellular replication remaining unmethylated, resulting in an overall decrease in DNA methylation (Aluru *et al.* 2015, De la Rica *et al.* 2016, Diotel *et al.* 2017, Liu *et al*. 2016, Moore *et al.* 2013, Seritrakul *et al.* 2017). The present results suggest epigenetic activation of the genome at the 15 dpf stage in response to developmental exposure to glyphosate and Roundup, corresponding to the free-swimming larvae stage in medaka. These findings provide support for the relevance of epigenetic mechanisms in the observed phenotypic abnormalities. A significant decrease in *Dnmt1* was also observed in testes samples exposed to 0.5 mg/L glyphosate at 100 dpf, suggesting the persistence of passive DNA demethylation mechanisms in males at adulthood.

To date no studies have been conducted analyzing the epigenetic effects of glyphosate and/or Roundup exposure utilizing model organisms. One study exposing human peripheral blood mononuclear cells (PBMCs) to 0.25 mM and 0.5 mM

concentrations of glyphosate reported a significant decrease (p= 0.017) in global DNA methylation at 0.25 mM with borderline significance (p = 0.084) observed at 0.5 mM (Kwiatkowska *et al.* 2017). These results further support the notion that exposure to glyphosate and Roundup result in a decrease in DNA methylation.

The direct consequence of exposure to glyphosate and/or Roundup on *Kiss1*, *Kiss2*, Gpr54-1 and Gpr54-2 gene expression have not been previously reported in any model system. Following developmental exposure to glyphosate and Roundup, female brain samples showed a significant decrease in Gpr54-1 gene expression in medaka exposed to 0.5 mg/L and 5 mg/L Roundup alongside a significant decrease in Gpr54-2 expression for medaka exposed to 0.5 mg/L Roundup. Previous research, primarily focused on the effects of endocrine disrupting chemicals (EDC) on *Kiss1* activity, have shown a decrease in hypothalamic *Kiss1* expression in exposed fetuses following maternal ovine exposure to a mixture of EDC's and other environmental contaminants (sewer sludge) (Bellingham *et al.* 2009, Tena-Sempere 2009). Furthermore, a significant decrease in *Kiss1* hypothalamic mRNA levels was observed in rats at puberty following neonatal exposure to BPA, while neonatal exposure to synthetic oestrogen, estrodial benzoate (EB), caused a significant dose-dependent decrease in hypothalamic *Kiss1* mRNA expression levels in both female and male rats proceeding puberty (Navarro *et al.* 2009, Tena-Sempere 2009). Since 2003, the Kiss/Gpr54 system has been established as a major regulator of the reproductive system, necessary in fertility and the timing of puberty (Page *et al.* 2011, Tena-Sempere 2009). Together the evidence above suggests that exposure to EDC's, including Roundup, during critical periods in development,

cause an effect on the Kiss/Gpr54 system which has the potential to result in reproductive consequences at adulthood due to the effect that the Kiss/Gpr54 system has on stimulating the HPG axis and consequently the production of GnRH, leading to the release of gonadotrophins which play a major role in reproduction (Nakajo *et al.* 2018, Zhang *et al.* 2017). The present results suggest that glyphosate and Roundup are capable of inducing effects on the brain reproductive axis through the downregulation of the Kiss and GnRH signaling system.

While there was no significant difference in gene expression levels measured in mature ovary samples, there was significant downregulation of *Fshr* and *Arα* gene expression in mature testes observed in medaka exposed to 0.5 mg/L Roundup and 0.5 mg/L glyphosate, and a significant downregulation of *Dmrt1* gene expression in mature testes from medaka exposed to 0.5 mg/L glyphosate. These results suggest that glyphosate and Roundup affect the male reproductive system by modulating genes required for spermatogenesis. Several previous studies have evaluated the effects of glyphosate and/or GBHs on male reproductive parameters (Harayashiki *et al.* 2013, Hued *et al.* 2012, Lopes *et al.* 2014, Owagboriaye *et al.* 2017, Romano *et al.* 2010). In studies utilizing fish as a model organism, sperm motility, motility period, DNA integrity, mitochondrial functionality and a reduction in mating success have all been reported as a consequence of glyphosate and/or GBH exposure, with concentrations ranging from 0.13 mg/L to 10 mg/L (Harayashiki *et al.* 2013, Hued *et al.* 2012, Lopes *et al.* 2014). Roundup was also shown to decrease testosterone, FSH and LH levels, as well as cause a significant reduction in sperm count and percent motility in male albino rats exposed to

3.6, 50.4 and 248.4 mg/kg bw/d for 12 weeks (Owagboriaye *et al.* 2017). Another study focusing on male developmental consequences following GBH exposure found reduced testosterone production, a delay in puberty and alterations in the function and structure of testes starting at exposure concentrations of 5 mg/kg bw/d in prepubertal Wistar rats (Romana *et al.* 2010). In summary, the findings in the current and previous studies, support the idea that glyphosate and Roundup exposure result in negative consequences for parameters related to male sexual reproduction.

In the current study many of the significant effects were observed at the exposure level of 0.5 mg/L for both glyphosate and Roundup, while previous literature has observed developmental and reproductive effects of glyphosate and Roundup at much higher concentrations. Glyphosate and GBHs have been characterized as endocrine-disrupting chemicals (EDC) due to their ability to interfere with a variety of endocrine-signaling systems including steroid hormones (Myers *et al.* 2016). One characteristic of EDCs, in addition to their ability to alter gene expression patterns and hormone systems, is their ability to display non-monotonic dose-responses (Myers *et al.* 2016, Vandenberg 2014). A non-monotonic dose response can take the shape of a U-shaped curve in which low (environmentally relevant) and high dose levels exhibit a greater response compared to intermediate dosing levels (Vandenberg 2014). A non-monotonic dose response can help explain the developmental and reproductive results observed for exposure to glyphosate and Roundup in the present, and previous studies, in which the majority of responses have been observed at low (environmentally relevant) and extremely high

concentrations, but a lack of response is often observed at intermediate dose levels, mainly 5 mg/L Roundup in the present study.

This study illustrated that exposure to environmentally relevant doses of glyphosate and Roundup during critical periods of development in medaka fish can lead to developmental abnormalities including a decrease in cumulative hatching success, increase in developmental abnormalities and changes in gene expression of epigenetic and reproductive genes. Studies evaluating the toxic effects of glyphosate and Roundup exposure, particularly long-term studies at environmentally relevant doses that compare glyphosate and Roundup simultaneously, are lacking. The present study is unique in that it is the first to illustrate long-term epigenetic effects of early exposure to glyphosate and Roundup, and to explore the effects of glyphosate and Roundup on Kisspeptin genes and receptors in the brain. Due to the fact that medaka are a biomedical research model organism there use as a model organism in the present study provides significant insight into both ecological and human health risks rendered by glyphosate and Roundup exposure.

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