Characterization of a rho class glutathione-S Transferase in zebrafish (*Danio rerio*)

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Abstract

Characterization of a rho class glutathione-S Transferase in zebrafish (*Danio rerio*)

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Glutathione *S*-transferases (GSTs) are detoxification enzymes important in cellular protection through their metabolism of endogenous and exogenous substrates such as chemotherapeutic agents, insecticides, herbicides, and byproducts of oxidative stress. All higher organisms express multiple GST isoenzymes with various substrate affinities, and rodent GSTs include members of the *alpha*, *mu*, *pi*, *omega*, *theta*, and *zeta* GST classes. A *rho* class GST (*gstr*) has been identified that is unique to fish and other aquatic organisms but has not been well characterized. In this study, we developed zebrafish (*Danio rerio*) *gstr* knockout lines and conducted toxicity studies using tert-butylhydroperoxide (tBHP) and cadmium (Cd) to test the role of *gstr* isoforms in protecting against contaminant-induced oxidative stress. Mutant zebrafish *gstr* knockout lines were developed through genome editing using CRISPR/cas9 targeting the coding sequence of zebrafish *gstr*. Wild-type and knockout zebrafish were exposed to tBHP and Cd at 96 hpf (hours post fertilization) and then analyzed for survival and developmental deformities. Whole embryo lipid peroxidation (LPO), glutathione concentrations (tGSH) and induction of two genes *hsp70*, a biomarker of cell stress, and *gadd45bb*, a biomarker of DNA damage, mRNA was measured to

assess the effects of *gstr* knockout on sublethal embryonic oxidative damage. We found that *gstr* knockout zebrafish were more susceptible than wild-type embryos to the acute toxicity of 110 mg/L tBHP at 72 hours post exposure, and also that surviving *gstr* knockouts showed an increased prevalence of spinal curvature relative to wild-type fish. Exposure to a low dose of (55 μg/L) of Cd caused a loss of whole embryo total glutathione (tGSH) concentrations and an induction in *hsp70* mRNA relative to wild type zebrafish, consistent with sublethal oxidative stress. Transcriptional studies showed that *gstr* mRNA was refractory to induction by either tBHP or Cd. In conclusion, our studies indicate the rho class GST plays a functional role in protecting against chemical induced oxidative stress in zebrafish embryos. However, the lack of induction of *gstr* mRNA despite presence of AREs in the promoter region is consistent with a housekeeping role of the *gstr* gene, potentially in protecting against zebrafish embryos against oxidative damage. Supported by NIEHS Superfund ES04696.

1. Introduction

Oxidative stress (OS) is caused by an imbalance between the generation and neutralization of reactive oxygen species (ROS) by antioxidant mechanisms (Davies, 1996). ROS include molecular and singlet oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical, and some of their derivatives. Most often, ROS are produced naturally as a side product of oxygen metabolism in the mitochondria (Lushchak, 2011). Endogenous production of ROS is part of aerobic life and generates signaling molecules necessary for normal cellular function (Dröge, 2002, Valavanidis et al., 2006). ROS modulate a number of signal transduction pathways such as NF-κB, *p53*, and AP-1 and are co-regulators of cell proliferation, differentiation, and apoptosis. As a result, aquatic organisms have developed systems for the management of free radicals and maintaining redox balance (Zhu et al., 2008). Changes in ROS concentration can lead to disturbances in the organism's redox status, resulting in cellular OS. Specifically, oxidative stress occurs when steady state cellular ROS levels are chronically enhanced, which can lead to DNA, protein, and lipid damage (Hellou et al., 2012).

The antioxidant system in tissues of aquatic organisms mainly consists of low molecular weight scavengers such as glutathione, and large molecule antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and the glutathione *S*-Transferases (GSTs) (Hellou et al., 2012). The GSTs, in particular, are a group of multifunctional phase II detoxification enzymes localized in cytosolic, mitochondrial, and microsomal compartments of cells. The most well-characterized are the cytosolic GSTs, divided into at least six classes: alpha, mu, pi, omega, theta, and zeta (Hayes and Pulford, 1995). Other GST classes have been reported in non-mammalian species, such as the rho class in teleosts and cephalochordates (Glisic et al., 2015). GSTs play an important role in detoxification by catalyzing the nucleophilic attack by reduced glutathione (GSH) on chemicals or the reactive intermediates containing an electrophilic carbon, nitrogen, or sulfur atom (Hayes and Pulford, 1995). This reaction usually results in the reduction of the compound reactivity and increases water solubility, allowing for efficient elimination (Hayes and Pulford, 1995). However, different GST classes exhibit different specificity to electrophilic substrates. Furthermore, different species express different amounts and types of GST in different organs (Glisic et al., 2015). These differences in

GST expression between species and tissues can confer greater protection or susceptibility to chemicals.

GST rho (gstr) was originally identified in plaice, though misclassified as a theta-like GST due to its sequence similarity to mammalian theta class enzymes, despite its unique structural and functional characteristics (Leaver et al., 1993). By 2005, this isoform had been found in several fish species, recognized as distinct in nature, and designated as a rho-class GST (Konishi et al., 2005). Subsequent studies of gstr have shown that it is highly expressed in major tissues (e.g. gills, liver, heart, olfactory system) in various teleosts (Leaver et al., 1993, Konishi et al., 2005, Espinoza et al., 2012). In plaice, gstr conjugates 1-chloro-2,4-dinitrobenzene (CDNB), though not other common GST substrates such as cumene hydroperoxide (CuOOH) and tert-butylhydroperoxide (tBHP) (Leaver et al., 1993). Martinez-Lara et al. (2002) hypothesized that the primary role of gstr was to protect the cells from harmful effects of lipid peroxidation due to its efficiency in detoxifying lipid alkenals and hydroxyalkenals in plaice. A study in the Gallagher laboratory of largemouth bass gstr found that the isoform exhibited high catalytic activity towards 4-hydroxynonenal (4HNE), a highly reactive byproduct generated during lipid peroxidation (Doi et al., 2004). However, in the red sea bream, gstr showed non-detectable activity towards 4HNE, indicating that gstr activity towards byproducts of oxidative stress may differ among species (Konishi et al., 2005). This study only measured activity in one of many gstr isoforms found in the red sea bream.

Studies attempting to characterize the induction of the *gstr* mRNA have provided inconsistent results across aquatic species. Some studies found that exposure to microcystin-LR (MCLR), a cyanobacterial toxin, significantly affected induction of *gstr* in freshwater fishes (Liang et al., 2007, Li et al., 2008). However, Nile tilapia orally exposed to tert-butylhydroquinone (tBHQ) showed little responsiveness of *gstr* isoform mRNAs (He et al., 2013). In carp, *gstr* expression increased by at least 3-fold in the liver following chronic exposure to atrazine (40 days), though *gstr* transcription decreased in brain, kidney, and gill (Xing et al., 2012).

Given the ubiquitous presence of the novel GST rho isoform in aquatic studies, we used zebrafish, a well described model organism, to better characterize the function of this protein and its potential for inducibility by model chemical inducers. Our approach was to develop *gstr*-null zebrafish lines to characterize the role of *gstr* in protecting against chemical induced OS. Zebrafish are widely used in laboratory settings due to their rapid development, optically clear embryos, and

ease of husbandry (Heffern et al., 2018, Wang and Gallagher, 2013). The two chemicals selected for this study, *tert*-Butylhydroperoxide (tBHP) and cadmium (Cd), were identified based on their capabilities to cause OS but also distinctive chemical properties. We hypothesized that *gstr* null zebrafish would be much more sensitive to the acute toxicity and sublethal oxidative stress following exposure these model compounds relative to wild type fish, and that the *gstr* gene would be readily inducible in zebrafish embryos.

2. Materials and methods

2.1 Zebrafish maintenance

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington (PROTO201600948). Adult zebrafish were housed in re-circulating aquaria maintained at 28 ± 0.5 °C on a 14 h light/10 h dark cycle. Fish received 2% of their body weight in flake food per day and were supplemented with live *Artemia* at least once daily. Source water from city municipal water was passed through a reverse osmosis filtration system and adjusted to 1000 ± 100 μS/cm salinity (pH 7.2) using Instant Ocean® salt and $NaHCO_3$. Embryos produced by natural spawning from paired matings were raised in E3 embryo media (EM; 5mM NaCl, 0.17 mM KCL, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$, pH 7.2-7.4) and maintained at a density of 50-60 per 100 mm^2 dish.

2.2 Genomic mutagenesis

Transgenic zebrafish lines were developed using methods described in Mills et al. (2019). Two sites within Exon 1 were targeted separately (site 1: GCACCAGAGCCCGGAGGTGA; site 2: GGCGCTCAATCCCAGAGCTC). Targets were selected using the CRISPRscan algorithm. The corresponding single-guide RNA (sgRNA) templates were created by cloning annealed oligonucleotides into pT7-gRNA (gift from Wenbiao Chen, Addgene plasmid #46759). After sequence confirmation, sgRNAs were transcribed in vitro using MEGAshortsript kit (ThermoFisher) and purified by phenol extraction and ethanol precipitation, then refolded by heating to 98 °C and slow cooling to room temperature.

Fertilized Ekkwill (EKW) strain embryos were collected from spawning tanks within 1 h postfertilization and injected at the one-cell stage using an MPPI-2 pressure injector (ASI). Embryos were injected with sgRNAs in one of two combinations: as ribonucleoprotein complex with cas9 protein (PNA Bio) (300 mM KCL, 500 ng/μL cas9 protein, 125 ng/μL sgRNA#1, 125 ng/μL sgRNA#2); or with cas9 mRNA in vitro transcribed from pCS2-nCas9n (Addgene plasmid #47929) with mMESSAGE mMACHINE kit (ThermoFisher; 100 ng/μL sgRNA#1, 100 ng/μL sgRNA#2, 600 ng/μL cas9 mRNA, 500 mM KCl).

2.3 Effects of chemical exposures on survival and deformities

All *in vivo* zebrafish exposures followed protocols from the Organization for Economic Cooperation and Development (FET OECD no. 236). Exposures were carried out in 100 mm^2 petri dishes. Groups of larvae were added to 25 mL of chemical solution in each dish starting at 4 days old (96 hpf) and were maintained with a daily 60% water change at 28 °C until 7 days old (168 hpf). Exposures to determine survival curves comparisons for *gstr* and wild type controls were determined using 4 days post fertilization (dpf) - 7dpf exposure regime, exposing three replicates of 10 larvae each to four concentrations of each chemical. The chemical concentrations of tBHP (30, 70, 110 mg/L tBHP) and CdCl (70, 210, 410 μg/L $CdCl_2$) used in this study were determined from previous work done in the Gallagher lab (Mills et al., 2019, Wang and Gallagher, 2013). At 24, 48, and 72 hours post exposure (hpe), each replicate was observed for percent survival and two developmental deformity endpoints, including spinal curvatures and swim bladder inflation. Only larvae with clear and distinct curvatures were included as having spinal curvature. Similarly, only larvae with clearly and visibly inflated swim bladders were marked as having inflated swim bladders. The frequency of spinal curvature and inflated swim bladder observed was expressed as percent spinal curvature and percent swim bladder inflation, respectively. At the end of the exposure period, 10 larvae from each exposure group were placed under a light microscope (Nikon, Japan) to measure heart rate. Larvae were mounted on to clean petri dishes using 6% Methyl cellulose while heart rate was measured during a 30 second interval. The heart-rate data recorded was then expressed as heart beats per 30 seconds.

2.4 Lipid peroxidation

Lipid peroxidation was quantified using the thiobarbituric acid (TBA) (Thermo Fisher) assay (Uchiyama and Mihara, 1977). Zebrafish were exposed from 4 dpf to 7 dpf to a sublethal concentration of each chemical and negative control (n = 6 biological replicates, 15 larvae per replicate). Larvae from each replicate were euthanized and then homogenized in 100 μL of Phosphate-buffered saline (PBS) with 0.03% butylated hydroxyanisole (BHA). Homogenized samples were spun down and 50 μL of supernatant was saved for reaction. 300 μL of 1% phosphoric acid and 100 μL of 0.9% TBA were added to the samples and standards. Samples were

then heated in a hot water bath at 95 °C for 45 minutes. The lipids were then extracted by adding 400 µL of n-butanol and vortexed. The resulting samples were centrifuged at 4°C then 200 µL of supernatant was pipetted into a black-walled, black-bottom 96-well plate. Fluorescence was analyzed at an excitation wavelength of 523 nm and an emission wavelength of 550 nm in a spectrophotometer (SpectraMax). The unknown samples were then normalized to the standard curve to obtain µM of MDA (malondialdehyde)/15 larvae.

2.5 Glutathione concentrations

Samples were quantified for total glutathione (tGSH) concentrations as described in Massarsky et al (2017). Samples were homogenized in 200 uL of 5% Salicylic Acid (SA) then centrifuged at 5000 g for 5 min at 4°C. The resulting supernatant was transferred to a fresh 0.5 mL tube and kept at -80°C until needed. To measure tGSH, 10 µL of the sample or standard was pipetted into a clear well, 96-well plate in triplicates. 200 µL of Assay solution (10.5 mg DTNB, 9 mg NADPH, 40 mL KPB-100) were then added to the wells containing the sample or standard and mixed. Finally, 10 µL of Glutathione reductase (2 µL GR, 1 mL KPB-100) was added to each well. The plate was then read at 412 nm for 15-30 min in a spectrophotometer (SpectraMax). To calculate tGSH concentrations, the unknown samples were normalized to the standard curve to obtain values in µM then multiplied by 0.0002 L (corresponding to 200 µL of 5% SA) then divided by 20 (number of larvae in sample). The resulting value was then converted to µM GSH/larvae.

2.6 RNA extraction and quantitative real-time PCR

For analysis of gene expression, larvae were euthanized following chemical exposures and transferred to 2mL centrifuge tubes containing TRIzol and a 5mm steel bead (Qiagen). Samples were homogenized in a TissueLyzer shaker (Qiagen) for 2 minutes at 40 Hz. RNA was extracted from homogenized fish and reverse transcribed into cDNA using iScript RT Supermix (BioRad, Hercules, CA) according to manufacturers' protocols, using 500 ng RNA starting material per cDNA synthesis reaction. qPCR reactions were analyzed on a CFX Connect Real-Time System (BioRad). Samples underwent cycle conditions: 95 °C for 10', followed by 40 cycles of denaturing

(95 °C) for 15” and annealing for 30”. Each 20 µL reaction included 3 µM of each primer, 50 ng cDNA (RNA equivalent), and 1X EvaGreen (Biotium). Efficiency was calculated for each primer pair based on a 7 point standard curve of purified PCR product for that primer pair amplified from a pool of cDNAs using Taq DNA Polymerase (NEB) and purified using the DNA Cleanup Micro kit (Thermo Fisher). Standard curve samples were generated by 100 pg/µL to 0.0001 pg/µL. No-template samples were included as negative controls. Each sample was run in triplicate. The relative expression of each gene was calculated using the efficiency-based method (Pfaffl, 2001). Efficiency was calculated from the average CT (cycle threshold) readings for each point of the standard curve using the formula $E = 10^{(1/slope)}$. Expression of target genes of interest were normalized to the average of two reference genes (*actb1* and *hprt1*), calculated using the equation $(1/E^{CTgene})/(1/E^{CTreference})$ or $E^{CTreference}/E^{CTgene}$.

2.7 Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 6. Survival and deformity data are expressed as the mean ± SEM of n = 9 pools of 10 larvae for each treatment and genotype. Lipid peroxidation and GSH concentration data were expressed as mean ± SEM of n = 6 pools of 15 or 20 larvae respectively. Gene expression data were expressed as mean ± SEM n = 3 pools of 10 larvae. The effects of chemical exposure and genotype on acute and sublethal toxicity were assessed using two-way ANOVA followed by Sidak’s correction for multiple comparisons. The effects of chemical exposure and time post exposure on gene expression were assessed using two-way ANOVA followed by Dunnett’s correction for multiple comparisons. Treatment-related effects were considered significant at $p < 0.05$.

3. Results

3.1 *Gstr*-null larvae exhibit increased sensitivity to acute oxidative stress toxicity

Survival curves for wild-type and *gstr*-null fish after exposure to tBHP and Cd are shown in **Fig. 1A-B**. Percent spinal curvature and percent swim bladder inflation were quantified following tBHP (**Fig 2A-B**) and Cd (**Fig 2C-D**) exposure during the exposure period. *Gstr*-null fish experienced significant declines in survival compared to wild-type fish when exposed to 70 and 110 mg/L tBHP (**Fig 1A**). After 72 hours of exposure to 70 mg/L tBHP, percent survival of *gstr*-null larvae decreased significantly ($p \leq 0.05$) compared to that of wild-types. Similarly, exposure to 110 mg/L tBHP affected both wild-type and *gstr*-null larval survival. However, beginning at post 24 hours of exposure, *gstr*-null larvae experienced a significant ($p \leq 0.001$) decline in survival (30% survival) compared to that of wild-types (67% survival). Survival continued to decrease after 48 hours post exposure (hpe) for both *gstr*-nulls (20% survival) and wild-types (46% survival). After 72 hours of exposure to 110 mg/L tBHP, percent survival of *gstr*-null larvae (11% survival) was significantly ($p \leq 0.001$) lower compared to that of wild-types (39% survival).

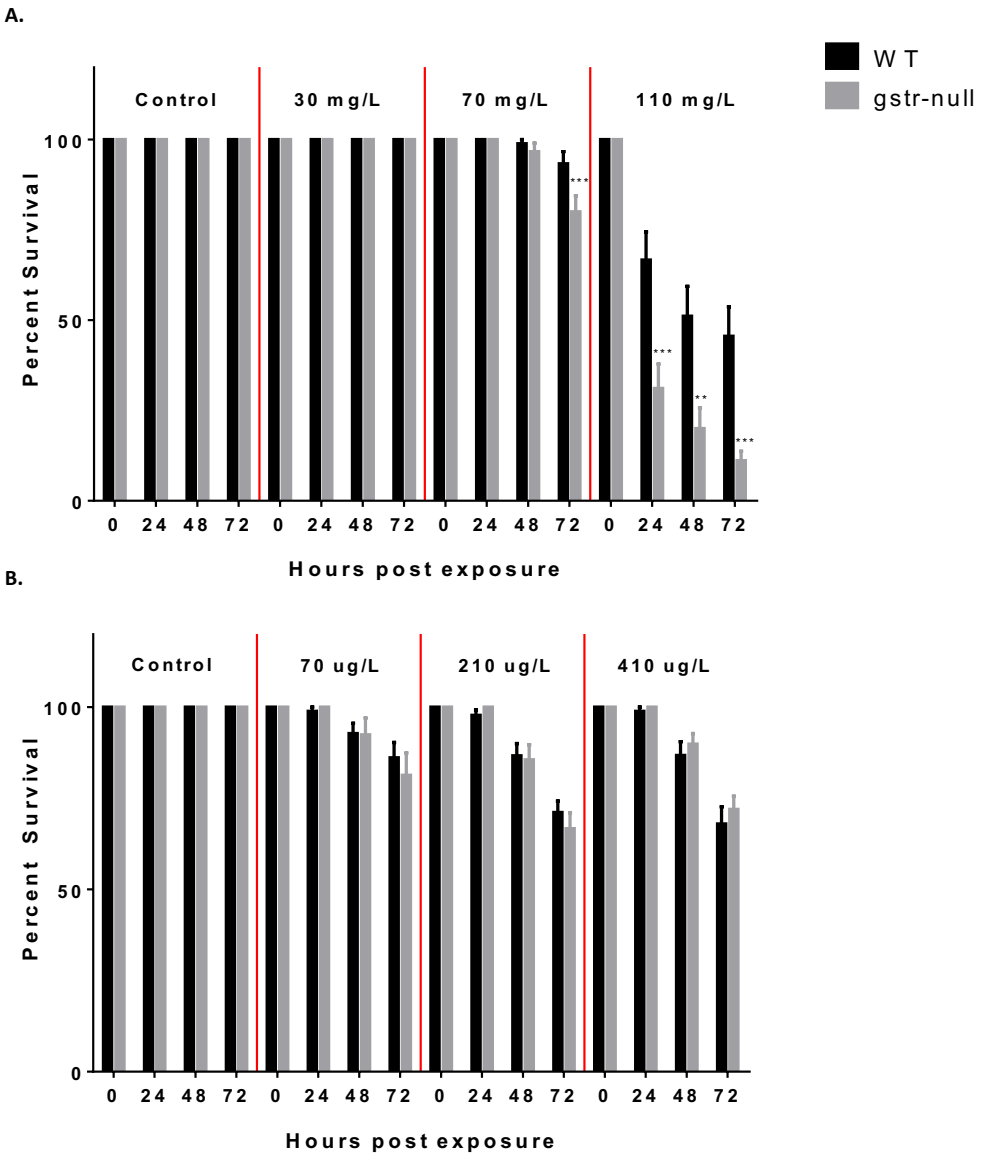

Fig 1. (A) tBHP exposure on survival across genotype and treatment. **(B)** Cd exposure on survival across genotype and treatment. Each bar is an average percent deformity of N=3 experiments, 3 replicates of 10 zebrafish. Error bars represent mean ± SEM. Asterisks represent significant difference from wild-types at each time point. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

When exposed to Cd, there was no difference in survival between *gstr*-null fish and wild-type at any time point or at any concentration. However, null fish exposed to Cd exhibited increased spinal (**Fig 2C**) and swim bladder deformity (**Fig 2D**) compared to wild-type fish. After 48 hpe to 70 µg/L CdCl, 33% of *gstr*-null fish exhibited spinal curvature, which increased to 48% at the end of exposure (72 hpe). At 210 µg/L CdCl, null-fish exhibited significantly ($p \leq 0.0001$)

higher rates of visible spinal curvature (48% deformity) than wild-types (2% deformity) after 24 hours. Percent of larvae with clear and visible spinal curvature continued to increase in null-fish (76% deformity) at 72 hpe, significantly ($p \leq 0.0001$) higher than wild-types (16% deformity). Similar patterns were exhibited at 410 µg/L CdCl, with *gstr*-null fish exhibiting significantly ($p \leq 0.0001$) higher spinal deformity compared to wild-types at 24, 48, and 72 hpe. Furthermore, *gstr* null fish exhibited increased swim bladder deflation following 210 and 410 µg/L CdCl exposure compared to wild-types.

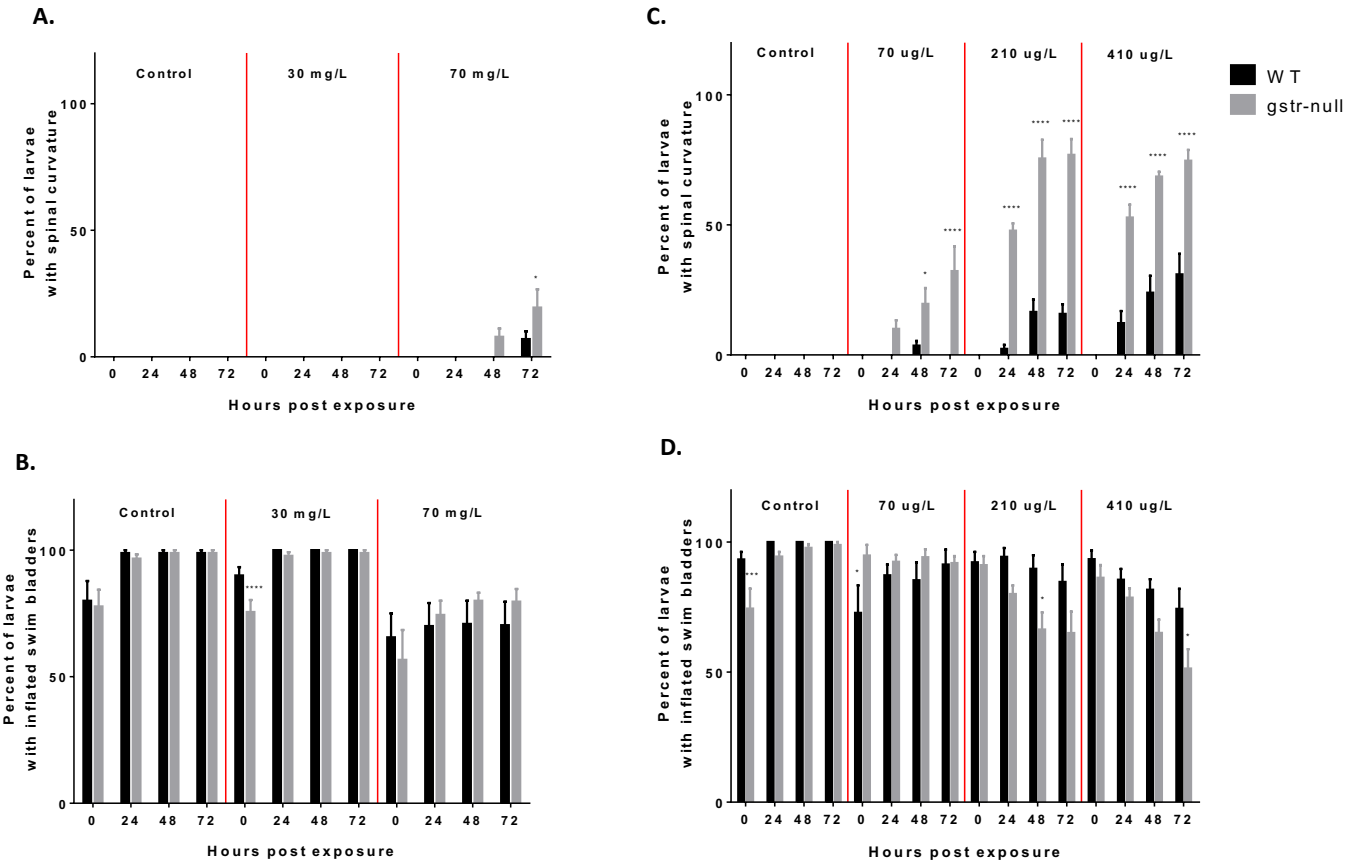

Fig 2. (A) tBHP exposure on spinal curvature across genotype and treatment. **(B)** tBHP exposure on inflated swim bladder across genotype and treatment. **(C)** Cd exposure on spinal curvature across genotype and treatment. **(D)** Cd exposure on inflated swim bladder across genotype and treatment. Each bar is an average percent deformity of N=3 experiments, 3 replicates of 10 zebrafish. Error bars represent mean ± SEM. Asterisks represent significant difference from wild-types at each time point. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, **** = $p < 0.0001$.

At the end of the exposure period, fish were collected from each treatment group and heart rates were measured. Null-fish exhibited a dose-responsive increase in heart-rate following an increase in tBHP concentration (**Fig 3A**). Furthermore, *gstr*-null fish had a significant increase in

heart rate compared to wild-types exposed to 30 ($p \leq 0.05$) and 70 mg/L ($p \leq 0.05$) tBHP. However, Cd exposure resulted in a dose-responsive decrease in heart-rate in null larvae across increasing concentrations. Exposure to 410 µg/L CdCl for 72 hours resulted in a significant ($p \leq 0.05$) decrease in heart rate in *gstr*-null fish compared to wild-types.

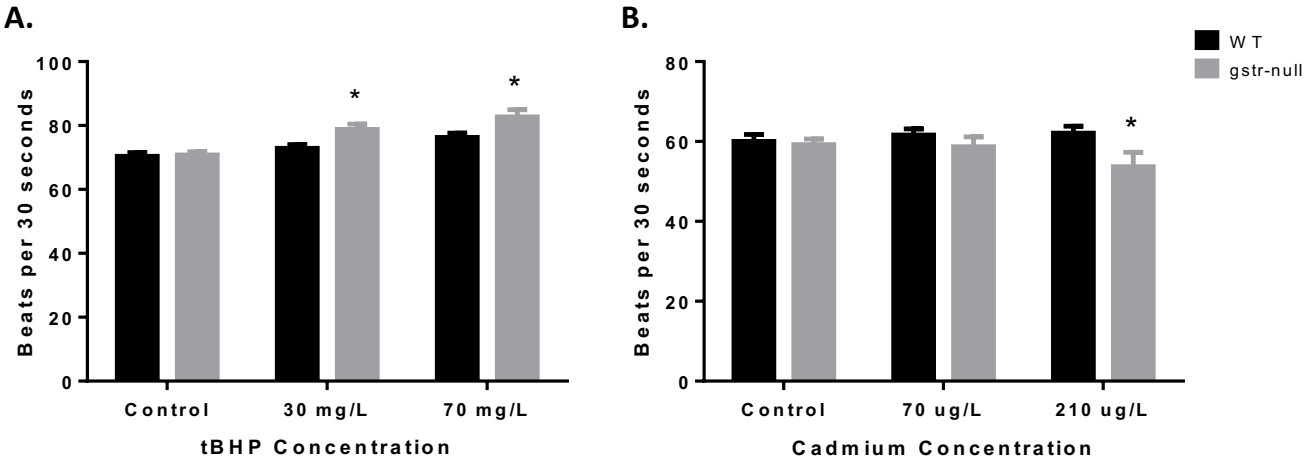

Fig 3. (A) tBHP exposure on heart rate across genotype and treatment. **(B)** Cd exposure on heart rate across genotype and treatment. Each bar is an average heart rate over 30 seconds 25-30 zebrafish each. Error bars represent mean ± SEM. Asterisk represents significant ($p < 0.05$) difference from wild-types at each time point.

3.2 *Gstr*-null larvae exhibit increased sensitivity to sublethal oxidative stress toxicity

In addition to acute toxicity exposures, sublethal exposures were conducted to determine the effects of 30 mg/L tBHP and 55 µg/L Cd on LPO, tGSH concentrations and induction of genes related to cell stress (*hsp70*) and DNA damage (*gadd45bb*). Three separate experiments were conducted for each chemical to quantify LPO, tGSH concentrations, and induction of *hsp70* and *gadd45bb*. Neither tBHP (**Fig 4A**) nor Cd (**Fig 4C**) exposure had statistically significant effects on LPO in either wild-type or *gstr*-null larvae. However, *gstr*-null fish generally exhibited increased LPO levels when compared to wild-types across chemical treatments. Similarly, there was no statistically significant differences in tGSH concentration between wild-type and *gstr*-null larvae when exposed to tBHP (**Fig 4B**) or Cd (**Fig 4D**). However, *gstr* null-fish generally exhibited lower tGSH per fish compared to wild-types. This difference could indicate that *gstr*-null larvae

may have experienced higher baseline oxidative stress levels, which would lead to decreased tGSH compared to wild-types.

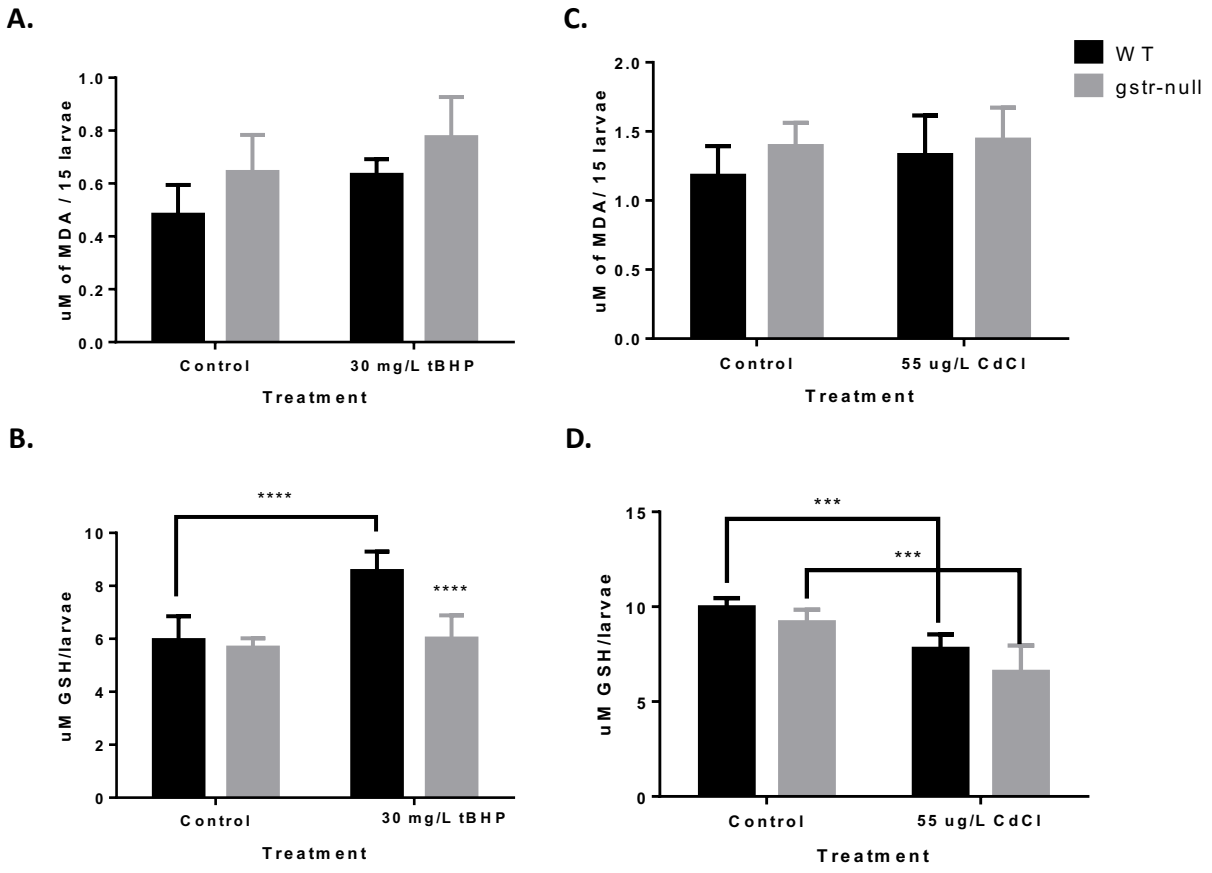

Fig 4. (A) tBHP exposure on LPO across genotype and treatment. **(B)** tBHP exposure on tGSH across genotype and treatment. **(C)** Cd exposure on LPO across genotype and treatment. **(D)** Cd exposure on tGSH across genotype and treatment. Each bar is an average of 6 replicates of 15-20 zebrafish. Error bars represent mean ± SEM. Asterisks represent significant difference from wild-types at each time point. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, **** = $p < 0.0001$.

To better understand the differences in cell stress and DNA damage between wild-type and *gstr*-null larvae, we measured the induction of *hsp70* (**Fig 5A**) and *gadd45bb* (**Fig 5B**) respectively following exposure to tBHP and Cd. *Hsp70* mRNA levels were significantly ($p \leq 0.01$) induced 4.3-fold in *gstr*-null larvae compared to wild-types (2.2-fold) when exposed to Cd, but this effect was not observed following tBHP exposures. Similarly, sublethal Cd exposures, but not tBHP exposures, significantly ($p \leq 0.05$) induced *gadd45bb* mRNA expression in both wild-types (1.9-fold increase) and *gstr*-null larvae (2-fold increase).

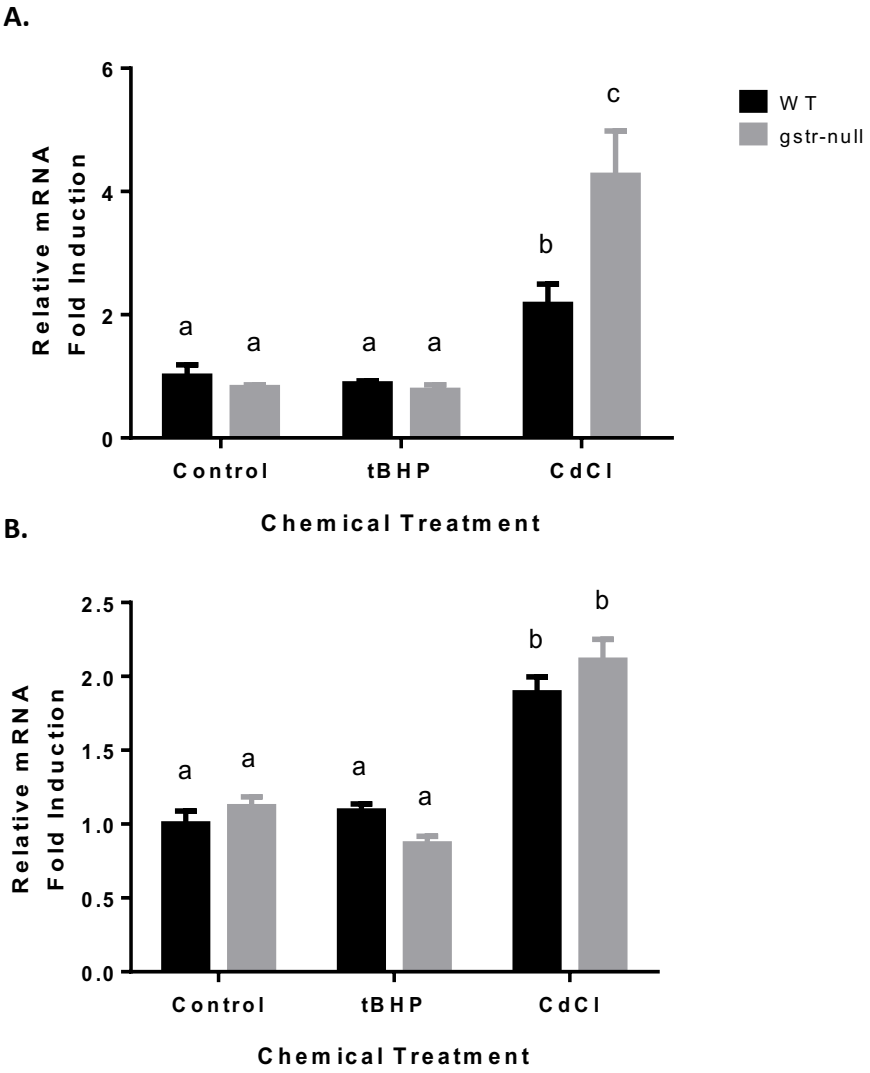

Fig 5. (A) 30 mg/L tBHP and 55 ug/L Cd exposure on *hsp70* induction across genotype and treatment **(B)** 30 mg/L tBHP and 55 ug/L Cd exposure on *gadd45bb* induction across genotype and treatment. Each bar is an average three replicates of 10 zebrafish. Error bars represent mean ± SEM. Letters represent significant difference ($p < 0.05$) across chemical treatment and genotype.

3.3 *Gstr* gene response to model oxidative stress gene inducers

To determine whether *gstr* mRNA was responsive to oxidative stress-mediated gene induction, *gstr* mRNA levels in wild-type larvae were measured in response to sublethal tBHP and Cd using concentrations referenced in our previous studies (Mills et al., 2019, Heffern et al., 2018). *Gstr* mRNA expression was then compared to that of two positive control genes (*gstp1* and *prdx1*). As expected, *gstp1* and *prdx1* were significantly ($p \leq 0.0001$) induced following exposure to tBHP

(**Fig 6A**), peaking at a 4.8-fold increase of *gstp1* and 15.5-fold increase of *prdx1* after 24 hours of exposure. Conversely, *gstr* transcription significantly ($p \leq 0.05$) decreased at 24 hpe by 0.7-fold, and further decreased by 0.5-fold at 48 hpe. Similar patterns were observed when larvae were exposed to Cd (**Fig 6B**). Expression of the positive control genes were significantly elevated ($p \leq 0.001$), peaking at a 1.5-fold increase of *gstp1* and a 5.3-fold increase of *prdx1* at 48 hpe. Conversely, *gstr* mRNA levels were significantly ($p \leq 0.05$) less than controls by 0.37-fold by 72 hpe.

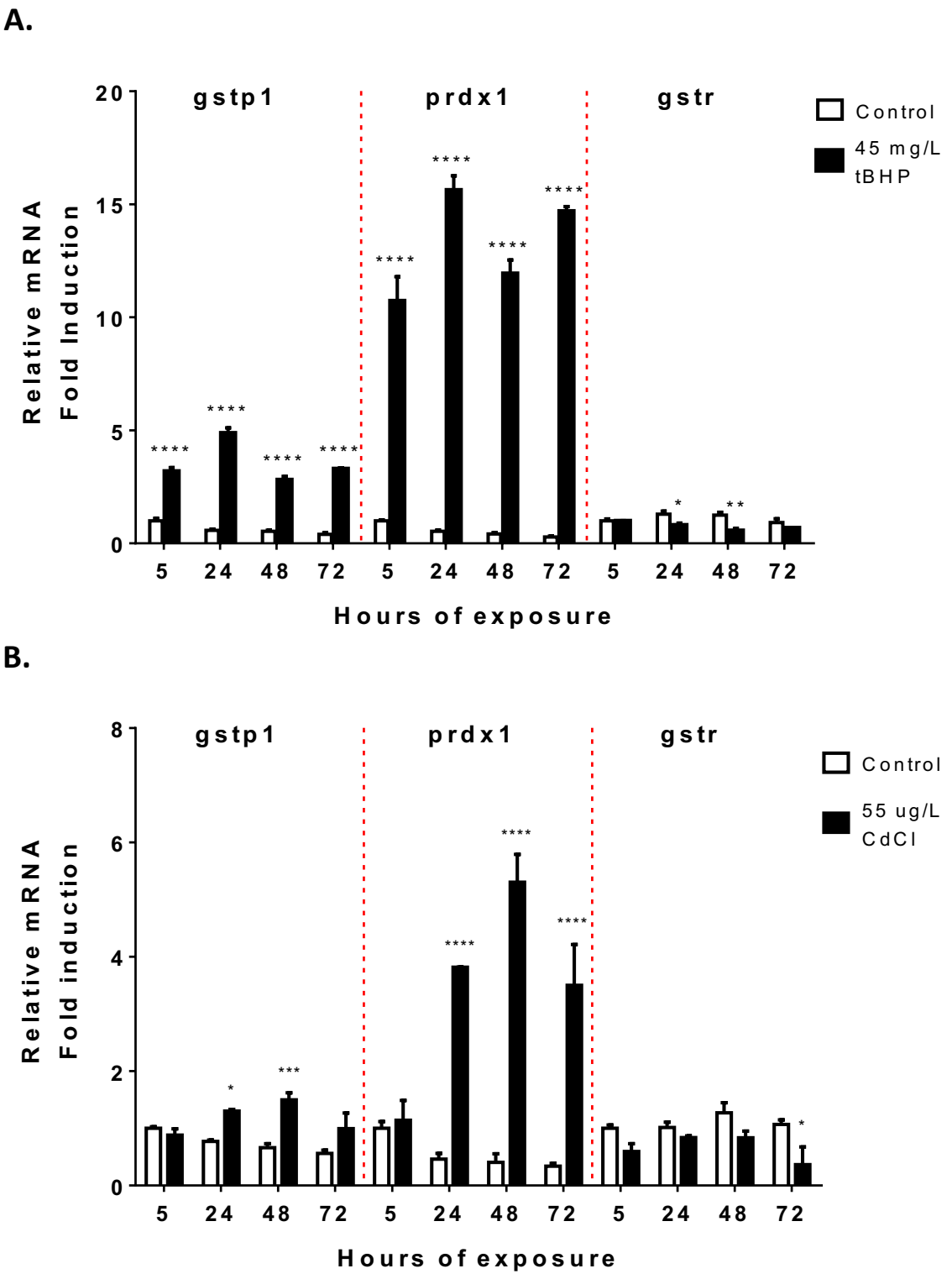

Fig 6. (A) tBHP exposure on gene induction in wild-type larvae **(B)** Cd exposure on gene induction in wild-type larvae. Each bar is an average three replicates of 10 zebrafish. Error bars represent mean ± SEM. Asterisks represent significant difference from wild-types at each time point. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, **** = $p < 0.0001$.

4. Discussion

In our study, we exposed larval zebrafish to the two model OS inducing agents, tBHP and Cd. tBHP is an organic peroxide and can be metabolized into free radical intermediates by cytochrome P450 in hepatocytes or hemoglobin in erythrocytes and initiate LPO, affect cell integrity, and form covalent bonds with cellular molecules resulting in cell injury (Rush et al., 1985, Wang et al., 2000). Previous work in our lab have determined that tBHP significantly decreased survival of *nrf2a* loss of function zebrafish larvae compared to wild-types (Mills et al., 2019). The Nrf2 transcription factor induces a host of mRNAs coding for antioxidant enzymes that protect against oxidative stress. Therefore, loss of Nrf2 leads to increased susceptibility to acute tBHP toxicity. In our study, we found that 110 mg/L tBHP exposure significantly decreased survival rates of *gstr*-null larvae compared to wild-types, but greater percent survival than *nrf2*-nulls (Mills et al., 2019). This supports our hypothesis that gstr has a role in mitigating chemical-induced oxidative stress toxicity. This is further corroborated by our results showing *gstr*-null fish generally exhibit higher baseline levels of LPO. Previous papers have implied a protective role of gstr against LPO, which is supported by results from this study (Martinez-Lara et al., 2002, Doi et al., 2004). Furthermore, 72 hours of sublethal tBHP exposure caused an increase in tGSH concentration in wild-types but not *gstr*-nulls. GSH is an endogenous thiol with a major role in antioxidant defense by contributing to the metabolism of potentially harmful agents and restoring the reducing power of the cell (Meister and Anderson, 1983). A major role of GSH in protecting against oxidative stress is mitigating lipid peroxidation, a major endpoint of tBHP toxicity (Sant et al., 2017). The increased tGSH levels could have contributed to the increased survival of wild-types exposed to tBHP compared to *gstr*-nulls.

Contrary to results obtained with tBHP, exposure to Cd did not significantly affect survival of *gstr*-null larvae compared to wild-type animals. Unlike tBHP, Cd is unable to directly produce ROS as it is less redox-active. However, the mechanisms of acute Cd toxicity involve the depletion of glutathione and protein-bound sulfhydryl groups, resulting in enhanced production of ROS (Cuypers et al., 2010, Liu et al., 2009, Patra et al., 2011). Studies have shown that Cd exposure can result in elevated GSH levels and LPO in the lung, brain, kidney, liver, erythrocytes, and testis (Nzengue et al., 2008, Patra et al., 2011). However, there were no observed significant differences in LPO or tGSH between wild-type and *gstr*-null larvae before or after sublethal Cd exposure in

this study. Although Cd can exert toxicity by inducing OS, unlike tBHP, Cd has additional mechanisms of actions that can lead to toxicity. In addition to OS, Cd disrupts cell proliferation, differentiation, apoptosis, and other cellular activities (Rani et al 2013). These additional mechanisms may have led to the different results observed in this study between tBHP and Cd.

Exposure to Cd at early life stages in organisms has been shown to result in developmental deformities in teleosts (Hallare et al., 2005, Lee et al., 1996, Lizardo-Daudt and Kennedy, 2008). A study in 5 to 28 hpf zebrafish found that embryos exposed to 138 μM Cd were more likely to exhibit head and eye hypoplasia, cardiac edema, hypopigmentation, yok sac deformities, altered axial curvature, and rudimentary tails (Cheng et al 2009). In a study by Blechinger et al. (2002), acute exposure to Cd for 96 hours between 3 and 7 dpf caused increased the incidence of trunk abnormalities such as spinal kyphosis, scoliosis, and lordosis in wild-type zebrafish. Mechanisms responsible for spinal deformations due to Cd have been described in previous studies. In a study by Muramoto (1985), Cd-induced spinal deformity in carp was associated with a depletion in calcium and phosphorus levels. A study in zebrafish found that larvae with Cd-induced spinal deformity was associated with reduced myotome formation necessary for the normal development of a healthy musculo-skeletal system (Cheng et al., 2009). In our study, *gstr*-null larvae exposed to Cd were more likely to develop spinal deformities compared to wild-types at all Cd concentrations post 24, 48, and 72 hours of exposure. These results were somewhat similar in larvae exposed to tBHP, but only *gstr*-null larvae exposed to 70 mg/L tBHP for 48 to 72 hours exhibited higher spinal deformity rates than wild-types. These results imply that *gstr* may have an additional role of protecting larval zebrafish during development. *Gstr* is one of four GST mRNA expressed in zebrafish embryos before 4 dpf (Glisic et al., 2015). Although our study began exposures at 4 dpf, our results imply *gstr* may still be a major antioxidant enzyme in protecting against toxic pollutants during larval development of zebrafish.

To further understand the role of *gstr* in protecting against oxidative stress, we measured induction of *hsp70* and *gadd45bb* mRNAs across treatment and genotype. Hsp70 is a 70 kD stress protein part of a family of heat shock proteins (HSPs) that confer protection against various biotic and abiotic stressors. *Hsp70* is inducible by a myriad of environmental stressors such as OS and exposure to toxic pollutants like metals (Scheil et al., 2008). Similarly, *gadd45* genes have been implicated in stress signaling responses to various physiological and environmental stressors, functioning as stress sensors and resulting in cell cycle arrest, DNA repair, cell survival and

apoptosis (Cretu et al., 2009). In our study, Cd exposure significantly increased *hsp70* and *gadd45bb* mRNA expression in both *gstr*-null and wild-type larvae. Furthermore, *hsp70* induction in Cd exposed *gstr*-null larvae was two-fold higher than observed in wild-types. Several studies have associated Cd exposure to induction of *hsp70* in zebrafish (Blechinger et al., 2002, Blechinger et la., 2007, Hallare et al., 2005). The increased induction of *hsp70* observed in *gstr*-nulls indicate that null larvae experienced an increase in Cd-induced sublethal toxicity. Unlike Cd, exposure to tBHP exposure did not induce *hsp70* or *gadd45bb* in wild-types or *gstr*-null larvae. These results contrast with previous studies from our lab showing strongly induced *hsp70* and *gadd45bb* induction in 96 hpf zebrafish larvae exposed to 800 μM tBHP (Mills and Gallagher, 2017). However, our previous study was conducted using a 3-hour exposure period at a tBHP concentration two times higher than what was used in this study. Therefore, the tBHP concentration used in this study may have been too low to induce *hsp70* and *gadd45bb*.

In addition to the toxicity studies, another aim of our study was to examine the potential for induction of *gstr* mRNA. As discussed, several GST isoforms are inducible by dietary or environmental compounds, and typically these GSTs contain functional regulatory elements in their gene promoters termed *antioxidant regulatory elements* (AREs) that confer induction via Nrf2 (Nguyen et al., 2003). A preliminary *in silico* analysis done in our lab identified 7 potential canonical AREs in the zebrafish *gstr* promoter region. The presence of AREs in the *gstr* promoter indicates a potential for induction of the zebrafish *gstr* gene by model inducers. However, unlike the two positive control *nrf2* pathway-related genes *gstp1* and *prdx1* measured in our study, *gstr* was generally refractory to mRNA induction and was actually down-regulated following exposure to both tBHP and Cd. Similar mRNA modulation patterns have been reported by others and suggest that exposure to tBHP and Cd may have stimulated the degradation of *gstr* mRNA (Castells-Roca et al., 2011, Molina-Navarro et al., 2008). Under homeostatic conditions, mRNA degradation is an important part of protein quality control and can determine protein levels in an organism (Borbolis and Syntichaki, 2015). Initially, it was thought that mRNA degradation required translation inhibition, but studies in yeast have found that some mRNA degradation rates can increase as translation increases (Bicknell and Ricci, 2017). This process can result in a temporary decrease in mRNA stability similar to the down-regulation pattern observed in *gstr* mRNA, which can be used to sharpen the response peaks of induced genes at the expense of higher energy costs (Perez-Ortin et al., 2013).

A previous study in our laboratory reported that the *gstr* mRNA in coho salmon was only minimally elevated in response to Cd (Williams et al., 2016), but the level of mRNA induction in that study was more consistent with transient post-transcriptional mechanisms. Based on our work in zebrafish, it appears that zebrafish *gstr* is not inducible by environmental compounds. This hypothesis is consistent with a study by Ramsden and Gallagher (2016) that demonstrated the lack of induction of the coho salmon *gstr* gene *in vitro*, despite the presence of AREs in the salmon *gstr* promoter. These studies indicate that the presence of AREs in the promoter of certain fish genes may not necessarily indicate that the gene is inducible. The lack of inducibility of *gstr* observed in this study aligns with its potential role of a "housekeeping" gene. Housekeeping genes are genes required for the maintenance of basal cellular functions essential for the existence of a cell and are thus expected to be expressed in all cells of an organism under normal conditions (Eisenberg and Levanon, 2013). Studies conducted in other teleosts have found *gstr* mRNA was induced following exposure to microcystin-LR and atrazine (Liang et al., 2007, Xing et al., 2012), suggesting that *gstr* induction may be species-specific and/or limited to certain environmental pollutants. Interestingly, atrazine is not a prototypical enzyme inducer in aquatic organisms.

5. Conclusions

We provide evidence of the protective role of *gstr* in zebrafish larvae against chemical induced oxidative stress. Our results show that tBHP exposure in larval *gstr*-null zebrafish drastically decreased survival compared to wild-types, whereas Cd exposure significantly increased spinal deformities, providing evidence for a protective capacity of *gstr* during larval development. The fact that expression of GST *rho* mRNA was not induced under a chemical exposure protocol using prototypical Nrf2 inducers, despite the presence of AREs in the promoter, suggests that the antioxidant regulatory elements in the rho promoter are not functional. Furthermore, the fact that tBHP and Cd exposures decreased *gstr* mRNA levels in larvae suggest that steady-state *gstr* mRNA levels may also be sensitive to oxidative stress. Ultimately, these studies suggest that *gstr* may play a role more as a housekeeping gene in protecting zebrafish embryos against unfavorable redox status. Further studies in wild fish are needed to fully understand the potential for evaluating *gstr* mRNA or its functional protein as a biomarker of exposure to pollutants in fish inhabiting polluted field sites.

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