ABSTRACT

Role of Directly and Indirectly Acting Chemicals on Development and Oxidative Stress in Various Early Life Stages

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Recent studies in our laboratory comparatively explored toxicity responses associated with oxidative stress (OS) pathways in fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) at early life stages. However, discrepancies between responses of these two common fish models have not been fully investigated across developmental ages. The objective of this study was to examine how OS responses vary: 1. throughout development; 2. between directly acting chemicals and metabolized chemicals; 3. between fathead minnow and zebrafish. Fathead minnow and zebrafish were exposed to R-(-)-carvone or bisphenol-A (BPA). Fish were exposed to control and 40% of the 96-h LC_{50} value. At 24, 48, 72, and 96 hours post exposure, mortality, hatching rate, and developmental deformity were assessed. Expression of select OS associated genes was measured using qPCR. Results demonstrate that OS responses varied across development in both species, but were also different between both zebrafish and fathead minnow. Furthermore, these responses differed across development between directly acting compounds and metabolized compounds, but only for acute toxic endpoints. In future studies, it will be important to consider bioavailability and uptake in understanding whether adverse outcomes vary across developmental age and between species.

APPROVED BY DIRECTOR OF HONORS THESIS

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ROLE OF DIRECTLY AND INDIRECTLY ACTING CHEMICALS ON DEVELOPMENT AND OXIDATIVE STRESS IN VARIOUS EARLY LIFE STAGES

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirement for the

Honors Program

By

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Waco, Texas

May 2018

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AWKNOWLEDGMENTS

This research could not have been possible without the support of Baylor University's University Research Committee Small and Mid-Range Grant Programs and the U.S. National Science Foundation (Project #: CHE-1339637) with additional support from the Environmental Protection Agency. The content is solely the responsibility of Yoomin Jo and does not represent the official views of Baylor University. The author would like to give special thanks to Bridgett Hill, Baylor Steele, and Gavin Saari for their assistance with statistical analysis and meaningful conversations. Finally, the author would like to acknowledge Dr. Jone Corrales without whose mentorship and support, this thesis could not have been possible.

CHAPTER ONE

Introduction

Limited to no safety data exist for the 87,000 industrial chemicals currently in commerce. This serious deficit in basic hazard information for public health and the environment results from the continuous introduction of new chemicals to commerce, a backlog of around 62,000 chemical substances grandfathered when US Congress passed the Toxic Substances Control Act (TSCA) in 1976 (http://www.epa.gov/tsca-inventory), and resources associated with animal experiments. Animal experiments are critical toward the accumulation of toxicity data of current chemicals and the research and development of novel compounds. However, traditional *in vivo* models can be time consuming, laborious, and costly, with over $2 billion used in toxicity testing every year worldwide (Hartung 2009). Furthermore, the use of current animal models have experienced backlash from institutional figures, activists, and the general public in terms of ethical experimentation (Friere et al 2017). In 1959, Russel and Burch addressed these issues promoting the *3 Rs Principle* –Replacement, Reduction, and Refinement. The principle has been utilized in the development of alternative toxicological models and the adoption of more ethical practices of animal testing, encouraging the minimization of suffering or distress when animal models are unavoidable. Limited time, money, and ethical questions have increased the importance of alternative toxicological models toward the creation of a more robust toxicological database.

The use of alternative models has been accepted by many in the global scientific community and has decreased cost and labor during toxicity assessments. Vertebrate, invertebrate, and non-animal models have become increasingly popular in scientific publications, increasing 909% from 1990 to 2015 (Friere et al 2017). The fish embryo acute toxicity (FET) test for embryonic zebrafish (*Danio rerio*) is one alternative model that satisfies the 3 Rs. This model initially became popular in the field of developmental biology due to the transparency of the eggs and suitability in mutagenicity screens. In 1990, Gorge and Nagel were one of the first to use zebrafish in toxicity testing, and today, more than 1,000 labs worldwide use zebrafish (Strähle et al. 2012, Scholz 2013). Similarly, the use of fathead minnow (*Pimephales promelas*) as a model for aquatic toxicity testing has increased since its initial use in the 1950s. The fathead minnow model is now the most widely used small fish model for regulatory ecotoxicology in North America (Ankley and Villeneuve 2006). Despite the popularity of both the zebrafish and fathead minnow models, challenges and opportunities remain regarding translatability of produced data and optimization of test strategies for sensitive life-stages (Ankley et al 2005, Ankley and Villeneuve 2006, Braunbeck et al 2015, Jeffries et al 2014, Jeffries et al 2015, Kristofco et al 2018). Therefore, it is important to understand the limitations of such models.

Previous research by Corrales et al. (2017) studied oxidative stress (OS) responses to a broad range of chemical classes in two common *in vivo* fish models, the zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*). OS has been an endpoint of interest due to a range of epidemiological studies and literature associating OS with a range of adverse outcomes such as cancer (Toyokuni et al 1995), diabetes (Damasceno et

al 2002), atherosclerosis (Singh and Jialal 2006), and neurodegenerative disorders (Coyle and Puttfarcken (1993). The study found that OS responses were markedly different between fish models and across chemicals. In an effort to maximize comparability and transferability of the Corrales et al (2017) findings, zebrafish studies followed OECD (Organisation for Economic Co-operation and Development) FET Test NO. 236 while fathead minnow experiments followed US EPA (Environmental Protection Agency) Whole Effluent Toxicity guidelines. However, these standard guidelines dictate initiation of experiments at different developmental ages at the start and throughout chemical exposure for zebrafish and fathead minnows. Observed variations between these OS responses suggest that metabolism of compounds may be influenced by developmental age or species-specific differences, or perhaps both. Therefore, the primary focus of this study was: 1. to explore how OS responses vary throughout development and evaluate if these responses were conserved between zebrafish and fathead minnows; 2. to investigate how these responses varied between directly acting chemicals versus those that are metabolized throughout development. Specifically, I tested the hypothesis that zebrafish and fathead minnow at older developmental ages would have increased sensitivity to metabolized chemicals due to the ability to produce a higher rate of metabolic activity.

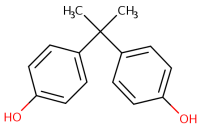
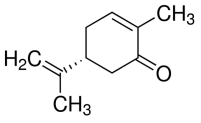
CHAPTER TWO

Methods and Materials

Experimental Chemicals

Eight experiments were conducted using two chemicals based on their mechanistic domain and previous observations Corrales et al (201&). R-Carvone and BPA were purchased from Sigma-Aldrich (St. Louis, MO, USA) in their purest form available (Ask Dr. Corrales). Common stock solution of each compound was used for each *in vivo* toxicity study.

Table 1. Structures, Properties, and Mechanistic Domain Designations of Study Chemicals

Chemical	**CASRN**	**Structure**	**Verhaar class**	**Mechanistic domain**	**Molecular weight (g/mol)**	**Log *P* (Log Kow)**
Bisphenol A	80-05-7		2	PRO-MA	228.29	4.04
R-(-)-Carvone	6485-40-1		3	MA	150.22	1.64

Fish Model Cultures

Zebrafish

Tropical 5D wild type zebrafish (*D. rerio*) were cultured at a density of <4 fish per liter in a z-mod recirculating system (Marine Biotech Systems Beverly, MA, USA) with water (pH 7.0, 260 ppm Instant Ocean, Cincinnati, OH, USA) at 36-28°C under 16:8 light/dark cycle. Larval fish were fed twice a day with brine shrimp (*Artemia* sp. Nauplii) and once a day with Tetramin Tropical Flakes.

Fathead Minnows

Fathead minnows (*P. promelas*) were kept in a flow through system with aged, dechlorinated tap water at 25±1°C under 16:8 light/dark photoperiod. Larval fish were fed twice a day with brine shrimp (*Artemia* sp. Nauplii; Pentair AES, Apopka, FL) and TetraMin Tropical Flakes (Pentair AES, Apopka, FL, USA). Adult breeding fish were at least 120 days old before being placed in the tanks with 1:4-5 male to female ratio. Embryos were collected at 30% Epiboly stages for embryonic exposures. Larvae were collected at 129 hours post fertilization (hpf) for larval exposures. All conditions for zebrafish and fathead minnows followed Institutional Animal Care and Use Committee protocols approved at Baylor University

Experimental Design of Toxicity Studies

All *in vivo* fathead minnow exposures used standardized toxicology experimental designs from the US Environmental Protection Agency (EPA). Zebrafish exposures followed designs from the Organization for Economic Cooperation and Development (FET OECD no. 236). Initial studies were performed for 96 hr LC_{50} values of zebrafish larvae and

fathead minnow embryos for both compounds. Glass beakers were used as experimental units. For embryonic exposure studies, 15-20 embryos (to obtain the necessary amount of RNA for qPCR analysis) of either species were placed in a 100 mL beaker containing 30 mL of water with 4 replicates for each time point. For larval exposure studies, 10 fish of either species were placed in a 500 mL beaker containing 200 mL of water with 6 replicates for each time point. Prior to exposures, all solutions were titrated to 7.5 pH and general water chemistry (alkalinity, hardness, dissolved oxygen, and temperature) was monitored throughout study. In sublethal studies, fish were exposed to concentrations of 40% 96h LC_{50}. An additional solvent control was used for BPA with 0.01% DMSO solution. Experiments were conducted in climate-controlled incubators with backup power and supply. Fish were collected in triplets at four timepoints (24 hrs, 48 hrs, 72 hrs, 96 hrs) after initiating exposure. Each replicate contained 20 zebrafish or fathead minnows in 1-4 dpf (days post fertilization) studies and 20 zebrafish or fathead minnows in 6-9 dpf studies.

Following sublethal studies, observations were made and tissue was collected for gene expression. Observations included mortality, hatching rate (embryos), and developmental deformity assessment. Pericardial edema, yolk sac edema, spinal curvature, bent tail, abnormal pectoral fin, uninflated swim bladder, and other deformities were assessed prior to collection. Gene expression (mRNA) of glutamate-cysteine ligase catalytic subunit (*gclc*), glutathione, *S*-transferase (*gst*), nuclear factor erythroid 2-like 2 (*nrf2*), and superoxide dismutase (*sod)* were measured following tissue collection. Specific isoforms in zebrafish were *gstp1*, *nrf2a*, and *sod1*.

Antioxidant Gene Expression Using qPCR

Following previously reported methods by Corrales et al (2017), RNA collected from sublethal studies was isolated with RNAzol (Molecular Research Center, Cincinnati, OH, USA) and purified with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). 500 ng of RNA was then reverse transcribed to cDNA using TaqMan Reverse Transcription Reagents (Applied Bioysystems by Life Technologies, Carlsbad, CA, USA) for 25 ng/µL cDNA. Target genes were then measured through real-time reverse transcription polymerase chain reaction (quantitative PCR, qPCR) using 1 µL cDNA, 200 nM final concentration of forward and reverse primers for each gene, and 1X Power SYBR Green PCR Master Mix (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Genes were amplified using StepOnePlus Real-Time PCR System (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) in conditions of 95°C for 10 minutes, 40 cycles of 95°C for 10s, and 60°C for 1 min with three replicates of each sample.

Statistical Analyses

Mean lethal concentrations in initial 96 h experiments were calculated in R using the "drc" package (R Core Team 2014). Mortality and sublethal (hatching, deformity, gene expression) toxicity data was analyzed using Sigma Plot (La Jolla, CA, USA). Statistical differences in mortality between controls and treatment (40% 96-h LC_{50}), hatching and deformity, and gene expression between controls and treatment were determined using t-tests. For data that did not meet the assumptions of normality, a Mann Whitney Rank Sum Test was performed. Significant differences in gene expression across development and between treatments were determined using one-way ANOVA followed by Tukey's posthoc test. To determine the interaction of effects between chemical exposure and

organism life stage (1-4 dpf vs 6-9 dpf) after 96 h exposure to either bisphenol-a or R-(-)-carvone, a two-way ANOVA was used. For bisphenol a, DMSO solvent controls were used for statistical analysis. Responses were considered significant at $p \leq 0.05$.

CHAPTER THREE

Results and Discussion

Results for Acute Toxicity Studies

Calculated 96-h LC_{50} values (Table 1) for R-(-)-carvone and bisphenol-a were relatively different between zebrafish and fathead minnow. Zebrafish were less sensitive to R-(-)-carvone than fathead minnow at 4 dpf and 9 dpf, but were more sensitive to bisphenol-a at 4 dpf. Across development, R-(-)-carvone LC_{50} values increased by around 25% in zebrafish, while LC_{50} values for the same compound in fathead minnow increased by around 60%. However, bisphenol-a LC_{50} values decreased across development in both zebrafish and fathead minnow by 50% and 70% respectively.

Table 2: R-(−)-carvone and bisphenol A (BPA) 96 h-LC_{50} (mg/L) values for zebrafish and fathead minnow at 4 and 9 days post fertilization (dpf).

	Zebrafish		**Fathead minnow**	
	4 dpf	9 dpf	4 dpf	9 dpf
R-(-)-carvone	58.2 mg/L	78.3 mg/L	36.5 mg/L	58.6 mg/L
Bisphenol-a	12.8 mg/L	6.3 mg/L	15.1 mg/L	4.2 mg/L

Results for Sublethal Toxicity Studies

Hatching

R-(-)-carvone significantly ($p < 0.05$) affected zebrafish hatching rates at 2 dpf a 3 dpf. All zebrafish hatched by 4 dpf. However, R-(-)-carvone did not significantly ($p > 0.05$) affect hatching rates in fathead minnow. Bisphenol-a only exerted a significant ($p < 0.05$) effect on zebrafish hatching rates at 2 dpf with no significant difference in hatching rates observed between control and treatments for fathead minnow embryos. Although neither R-(-)-carvone or bisphenol-a significantly ($p > 0.05$) affected hatching rate in fathead minnow embryos, the hatching rate of fathead minnow samples was low in both treatment and control, which is not uncommon for this model. In both fathead minnow studies, less than 50% of embryos hatched by 4 dpf.

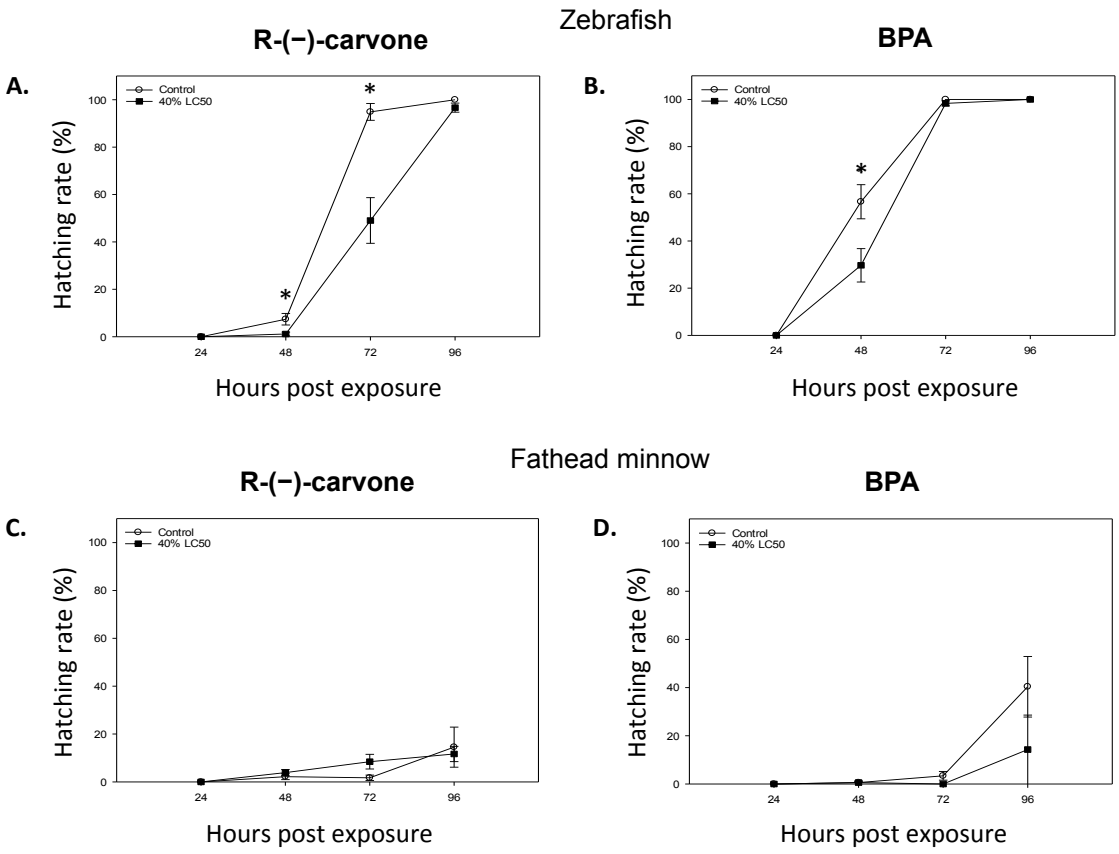


Figure 1: Percent hatching in 1-4 days post fertilization zebrafish (A, B) and fathead minnow (C, D) during a 96 h exposure to either R-(−)-carvone (A, C) or bisphenol A (BPA) (B, D). Embryos ($N = 4$, 15 or 20 embryos per replicate) were exposed to either chemical starting at the 30% epiboly stage. At 24, 48, 72, and 96 h post exposure, hatching was documented and then percent cumulative hatching calculated. The asterisks represent statistical differences between treatment groups at each time point (t-test, $p < 0.05$).

Developmental Deformities

Zebrafish. R-(-)-carvone exposure significantly increased incidence of spinal curvature only at 3 dpf (Table 3). However, exposure to R-(-)-carvone significantly increased the incidence of uninflated swim bladders in zebrafish at 4 dpf. However, in the zebrafish 6-9 dpf studies, R-(-)-carvone treated fish had a significantly ($p < 0.05$) decreased incidence of uninflated swim bladder compared to controls (Table 3).

Additionally, incidence of other deformities was significantly higher in R-(-)-carvone treated fish at 3 dpf and 4 dpf. Zebrafish at these time points were observed to have light gray, opaque cavity from the yolk sac down to the trunk or were stunted (Table 3). Compared to R-(-)-carvone, exposure to bisphenol-a did not cause any significant ($p > 0.05$) increase in spinal curvature at any time points. However, fish treated with bisphenol-a did have a significantly ($p < 0.05$) higher incidence of uninflated swim bladder at 4 dpf (Table 3). Treated fish at 4 dpf also experienced a significant ($p < 0.05$) increase in other deformities. Fish at this time point were either more pigmented than normal, stunted, or had a minimally inflated swim bladder (Table 3).

Fathead Minnow. Fish treated with R-(-)-carvone were observed to have a significantly ($p < 0.05$) higher incidence of uninflated swim bladder at 6 dpf and 8 dpf (Table 4). Additionally, R-(-)-carvone exposure also statistically ($p < 0.05$) increased the incidence of other, noteworthy deformities. Fish experiencing these deformities were either more pigmented than normal, less pigmented than normal, swam slowly, or did not swim at all (Table 4). Some fish experienced more than one of these deformities. There was no statistical ($p > 0.05$) difference between control and bisphenol-a treated fish for any observed deformities (Table 4).

Table 3: Percent incidence of developmental deformities in zebrafish following a 96 h exposure to either R-(−)-carvone or bisphenol A (BPA) starting at the 30% epiboly stage ($N = 4$, 15 or 20 embryos per replicate) or at 5 dpf (days post fertilization) ($N = 6$, 10 larvae per replicate). At 24, 48, 72, and 96 h, fish were observed for the presence or absence of developmental deformities: pericardial edema (PE), yolk sac edema (YSE), spinal curvature (SC), bent tail (BT), abnormal pectoral fins (APF), and uninflated swim bladder (USB); 'Other' refers to an opaque cavity and/or change in pigmentation. N/A indicates unhatched fish that could not be examined. The asterisks represent statistical differences between treatment and control or solvent control (DMSO) for a given chemical at each time point (t-test, $p < 0.05$).

		1 – 4 dpf							**6 – 9 dpf**						
	Time post exposure (h)	PE	YSE	SC	BT	APF	USW	Other	PE	YSE	SC	BT	APF	USW	Other
Control	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	100	3
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0	2
	72	2	0	3	0	0	100	0	0	0	2	0	0	0	2
	96	0	0	8	0	0	32	8	0	0	3	0	0	0	3
R-(—)-carvone 40% 96h-LC_{50}	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	**0***	17
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	2	0	0	0	8
	72	0	0	**30***	0	0	100	**81***	0	0	0	0	0	0	17
	96	0	0	5	0	0	**100***	**86***	0	0	6	0	0	0	33
Solvent Control	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0	0
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0	0
	72	0	0	0	0	0	100	25	0	0	0	0	0	0	0
	96	2	0	2	0	0	3	0	0	0	2	0	0	0	4
BPA 40% 96h-LC_{50}	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	4	7
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	2	7
	72	0	0	7	2	2	100	17	0	0	0	0	0	0	0
	96	0	0	0	0	0	**44***	**18***	0	0	0	0	0	2	2

Table 4: Percent incidence of developmental deformities in fathead minnow following a 96 h exposure to either R-(−)-carvone or bisphenol A (BPA) starting at the 30% epiboly stage (N = 4, 15 or 20 embryos per replicate) or at 5 dpf (days post fertilization) (N = 6, 10 embryos per replicate). At 24, 48, 72, and 96 h, fish were observed for the presence or absence of developmental deformities: pericardial edema (PE), yolk sac edema (YSE), spinal curvature (SC), bent tail (BT), abnormal pectoral fins (APF), and uninflated swim bladder (USB); 'Other' refers to an opaque cavity and/or change in pigmentation. N/A indicates unhatched fish that could not be examined. The asterisks represent statistical differences between treatment and control or solvent control (DMSO) for a given chemical at each time point (t-test, $p < 0.05$).

		1 – 4 dpf							**6 – 9dpf**						
	Time post exposure (h)	PE	YSE	SC	BT	APF	USW	Other	PE	YSE	SC	BT	APF	USW	Other
Control	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0	3
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	3	2
	72	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	0	7	0	0	3	2
	96	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	3	0	0	3	3
R-(—)-carvone 40% 96h-LC_{50}	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	**40***	**20***
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	10	0	0	23	7
	72	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	17	0	0	**17***	**30***
	96	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	14	0	0	18	**54***
Solvent Control	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	3	17	2	0	19	24
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5	2	14	0	0	7	21
	72	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	0	5	0	0	5	10
	96	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	7	0	0	3	5
BPA 40% 96h-LC_{50}	24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	5	33	0	0	20	40
	48	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	2	20	0	0	17	25
	72	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	2	8	0	0	3	13
	96	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	2	5	2	0	2	5

Gene Expression

Zebrafish. Oxidative stress gene responses differed across development between untreated fish in different age groups (1-4 v 6-9 dpf). Control zebrafish 1-4 dpf experienced an overall increase of gene expression, especially in *gstp1*, *nrf2a*, and *sod1* where 1 dpf significantly ($p < 0.05$) differed from 4 dpf (Figure 2A). However, this trend was not apparent in the 6-9 dpf control group (Figure 2B). Zebrafish exposed to R-(-)-carvone had a significant ($p < 0.05$) difference at *gclc* expression at 3 dpf and 4 dpf (Figure 2A), but no such difference was observed in the 6-9 dpf treated group (Figure 2B). R-(-)-carvone caused a significant ($p < 0.05$) upregulation of the *gstp1* gene at all exposure time points in both 1-4 dpf (Figure 2A) and 6-9 dpf (Figure 2B) exposed groups compared to controls. *Nrf2a* expression was significantly ($p < 0.05$) altered at 1 dpf and 3 dpf in the 1-4 dpf developmental group (Figure 2A), but was only significantly affected at 8 dpf in the 6-9 dpf group (Figure 2B). *Sod1* expression did not significantly ($p > 0.05$) change in treated zebrafish at 1-4 dpf (Figure 2A), however, significant ($p < 0.05$) differences were observed at 7, 8, and 9 dpf in exposed zebrafish 6-9 dpf (Figure 2B). Exposure to bisphenol-a caused different gene expression responses than those caused in fish exposed to R-(-)-carvone. Fish exposed to bisphenol-a did not cause a significant ($p > 0.05$) difference in the expression of *gclc* in either developmental age group (1-4 dpf and 6-9 dpf) (Figure 2C and 2D). However, treated fish did experience significant ($p < 0.05$) changes in *gstp1* expression at 1 dpf and 4 dpf (Figure 2C). No such differences were found in the treated 6-9 dpf fish (Figure 2D). Sublethal levels of bisphenol-a also caused a significant ($p < 0.05$) difference in expression of *nrf2a* at 3 dpf and 4 dpf in treated zebrafish 1-4 dpf (Figure 2C). Similarly to *gstp1*, there were no significant ($p >$

0.05) changes in *nrf2a* expression in zebrafish exposed to bisphenol-a at 6-9 dpf (Figure 2D). There were also no statistically ($p > 0.05$) different responses in *sod1* expression between treated and control fish in either 1-4 dpf (Figure 2C) or 6-9 dpf groups (Figure 2D).

Fathead Minnow. As opposed to zebrafish responses, oxidative stress gene expression in untreated fathead minnow 1-4 dpf generally decreased across development (Figure 3A). This trend was similar in 6-9 dpf fish in the control group (Figure 3B). However, similar to zebrafish gene response, fathead minnow exposure to R-(-)-carvone caused significant ($p < 0.05$) upregulation of *gclc* gene expression in 3 dpf and 4 dpf in the 1-4 dpf developmental group (Figure 3A) and at 7 dpf and 8 dpf in the 6-9 dpf developmental group (Figure 3B). These significant differences were similarly observed in *gstp1* expression in exposed fish (Figure 3A and 3B). Fathead minnow treated with R-(-)-carvone did not cause any significant ($p > 0.05$) differences in *nrf2a* expression compared to controls in either developmental age groups (Figure 3A and 3B). This response differed from zebrafish *nrf2a* response to R-(-)-carvone exposure (Figure 2A and 2B). However, fathead minnow exposed to R-(-)-carvone did significantly ($p < 0.05$) alter expression of *sod1* at 3 dpf in the 1-4 dpf age group (Figure 3A), unlike exposed zebrafish at similar developmental ages. However, exposure to R-(-)-carvone at 8 dpf in the 6-9 dpf age group in fathead minnow did significantly ($p < 0.05$) alter expression of *sod1* (Figure 3B), a similar response occurred in exposed zebrafish (Figure 2B). Treatment of sublethal concentrations of bisphenol-a did not significantly ($p > 0.05$) affect the expression of *gclc* or *gstp1* compared to untreated groups in fathead minnows at any developmental time point in 1-4 dpf (Figure 3C) or 6-9 dpf (Figure 3D), although

expression of *gclc* was significantly ($p < 0.05$) altered in exposed zebrafish (Figure 2C and 2D). Furthermore, bisphenol-a exposure in fathead minnows significantly ($p < 0.05$) altered *nrf2a* expression at 8 dpf in the 6-9 dpf developmental group (Figure 3D), a response not observed in exposed zebrafish (Figure 2D). Similarly to zebrafish 1-4 dpf, no significant ($p < 0.05$) differences in *nrf2a* expression were observed in treated 1-4 dpf age group (Figure 3C). Similarly to zebrafish 1-4 dpf and 6-9 dpf, *Sod1* expression in fathead minnows was not significantly affected by bisphenol-a at any time point in either developmental group (Figure 3C and 3D).

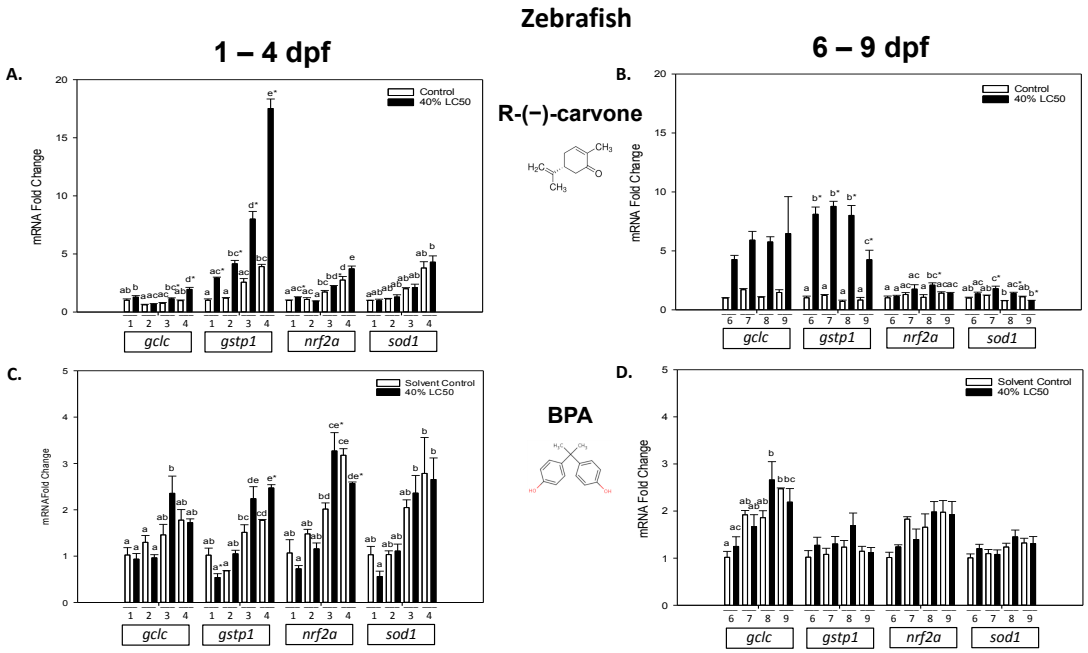


Figure 2: Changes in mRNA expression in zebrafish 1-4 days post fertilization (dpf; A, C) and 6-9 dpf (B, D) following a 96 h exposure to R-(−)-carvone (A, B) or bisphenol A (BPA)(C, D) as measured by qRT/RT-PCR. Fold change was normalized to *actb1* expression and relative to control or solvent control ($N = 3$, pool of 20). Different letters represent changes throughout developmental time points (one-way ANOVA, $p < 0.05$) and asterisks represent changes between control and treatment in a given developmental stage or time point (t-test, $p < 0.05$).

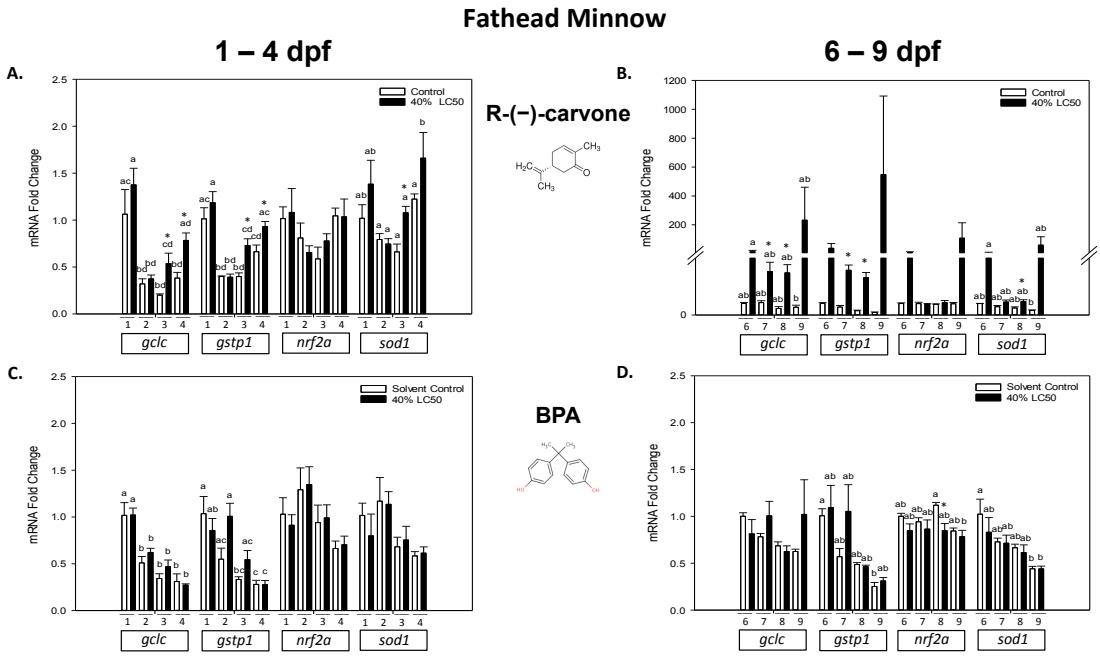


Figure 3: Changes in mRNA expression in fathead minnow 1-4 days post fertilization (dpf; A, C) and 6-9 dpf (B, D) following a 96 h exposure to R-(−)-carvone (A, B) or bisphenol A (BPA)(C, D) as measured by qRT/RT-PCR. Fold change was normalized to *gapdh* expression and relative to control or solvent control ($N = 3$, pool of 20). Different letters represent changes throughout developmental time points (one-way ANOVA, $p < 0.05$) and asterisks represent changes between control and treatment in a given developmental stage or time point (t-test, $p < 0.05$).

Age and Chemical Interaction

Zebrafish Exposure to R-(-)-carvone in zebrafish across development significantly ($p < 0.05$) affected different OS gene expression in various ways. R-(-)-carvone exposure caused significant ($p < 0.05$) upregulation of both *gclc* and *gstp1* in zebrafish 1-4 dpf, compared to control zebrafish 1-4 dpf. However, exposed zebrafish at 6-9 dpf experienced a significant ($p < 0.05$) decrease in *gstp1* and *nrf2a* expression compared to control zebrafish 1-4 dpf. Exposure to bisphenol-a affected the same OS gene differently across development. Zebrafish 1-4 dpf exposed to bisphenol-a exhibited a significant ($p < 0.05$) increase in *gstp1* expression compared to control zebrafish 1-4 dpf, whereas

bisphenol-a exposure caused a significant ($p < 0.05$ decrease) in *gstp1* expression in zebrafish 6-9 dpf compared to control zebrafish 1-4 dpf.

Fathead Minnow As opposed to zebrafish, gene responses of fathead minnow exposed to R-(-)-carvone did not differ across development. Fathead minnow 1-4 dpf and 6-9 dpf experienced a significant ($p < 0.05$) increase in *gstp1* following exposure to R-(-)-carvone when compared to control fathead minnow 1-4 dpf. Similar to R-(-)-carvone responses in fathead minnow, bisphenol-a exposure did not incite different responses across development in fathead minnow. Treated fish 1-4 dpf and 6-9 dpf did not experience any significant ($p < 0.05$) change in OS gene expression compared to control fish 1-4 dpf.

Discussion

Presently, the OECD FET test for zebrafish embryos and US EPA WET guidelines for fathead minnows are a mandatory part of risk and hazard assessments of new industrial compounds. However, concerns remain regarding sensitivity of age groups tested (Embry et al 2010, Jeffries et al 2014, Kristofco et al 2018). Early fish life cycle tests indicated that fish in the embryo-larval and juvenile stages are the most sensitive to chemical exposure (McKim 1977). However, development has been shown to have an effect on sensitivity to different toxicants. A recent study by Kristofco et al. (2016) found that zebrafish in later developmental stages than included in the FET method were more sensitive to diazinon and diphenhydramine exposure. Therefore, the primary objective of this study was to explore how OS responses vary throughout development and evaluate if these responses were conserved between zebrafish and fathead minnow and to investigate

how these responses varied between directly acting chemicals versus those that are metabolized throughout development.

OS occurs as a result of an imbalance between reactive oxygen species (ROS) and antioxidant defenses, which can lead to cellular damage (Limon-Pacheco and Gonsebatt 2009, Lushchak 2011, Hellou et al 2012). The study of OS in aquatic organisms has been a prominent area of research since the emergence of environmental toxicology (Di Giulio et al 1989). Today, biomarkers such as cellular lipid peroxidation, DNA damage, and glutathione depletion are used in aquatic animal models for environmental monitoring (Pandey et al 2003, Valavanidis et al 2011). However, generation of ROS is also a natural part of aerobic life, aiding in the natural functions of cell life and death. In mammals, ROS play an important role in pathways involved in cell proliferation, differentiation, and apoptosis especially during embryonic development (Dennery 2007, Schafer and Buettner 2001). As a result, parameters of oxidative stress during development can be age-related. Similar findings were observed in aquatic organisms (Rudneva et al 1999, Rudneva et al 2010).

In the present study, we observed that OS expression of control zebrafish 1-4 dpf increased (Figure 2A), but stabilized by 6-9 dpf (Figure 2B). Conversely, gene expression in untreated fathead minnow continuously decreased in both 1-4 dpf (Figure 3A) and 6-9 dpf (Figure 3B). Although these trends indicate an age-dependent variation of ROS production, an understanding of ROS production during development in aquatic organisms is severely lacking, with limited literature tracking OS pathways during embryonic-larval stages in even the most popular aquatic models. Additionally, the induction of OS is a frequently debated topic. Depending on the intensity of OS,

biomarkers may increase, decrease, or not change at all (Lushchak 2014). Despite this uncertainty, there is currently no widely accepted system to classify oxidative stress. Further research is required in this area to better understand induced OS from xenobiotics during toxicological testing (Lushchak 2011, Lushchak 2014).

The results presented in this study regarding OS gene expression of zebrafish and fathead minnow across development indicate that normal ROS production through development is species-specific. Understanding baseline ROS production for normal development is important in further understanding OS data gathered from toxicity tests. Previous studies have found that zebrafish and fathead minnow exposed to the same compounds elicited substantially different results for several different endpoints (Corrales et al 2017, Warner et al 2012). Unfortunately, limited literature exists comparing sublethal toxic endpoints between zebrafish and fathead minnow, and literature comparing developmental ROS production between these two models is non-existent. Despite the lack of research in this area, understanding if toxicity responses are conserved between species is of particular importance if we are to use read-across methodologies.

The read-across hypothesis postulates that contaminants in the environment can have similar effects in target and non-target organisms if molecular targets such as receptors and enzymes are conserved (Huggett et al 2003). Genome sequencing projects have demonstrated appreciable similarity among mammalian genes and genes of evolutionarily more primitive organism such as fish. A previous study by Howe et al showed that 70% of human genes have at least one obvious zebrafish orthologue. As a result, the use of alternate animal models in the field of toxicology and biomedicine has gained significant attention. However, limitations to the read-across methodology remain.

Most chemical compounds have multiple mechanisms and modes of actions that can produce a range of effects, ultimately resulting in different toxic responses across organisms (Rand-Weaver et al 2013). By studying how different toxic effects are preserved or not across species, we can develop and implement more appropriate methodologies for future regulatory assessments.

An additional element of my research compared sublethal responses in similar developmental ages across zebrafish and fathead minnow. However, a possible confounding factor not considered in this study is the developmental rate differences exhibited between the two fish models. In this study, 1-4 dpf and 6-9 dpf zebrafish and fathead minnow studies followed OECD FET and US EPA WET standard guidelines, respectively, however, zebrafish and fathead minnow exhibit different developmental rates that were not accounted for when comparing results. This difference is best exhibited by the hatching rates collected. By 3 dpf, all zebrafish had hatched from the chorion, however, fathead minnow usually hatch at 5 dpf, and therefore during the 1-4 dpf studies, minimal fish had hatched (US EPA, 2002). The discrepancy between developmental rates could have further affected the different sensitivity endpoints observed across the test models. A study by Jeffries et al. (2014) comparing sensitivities of the fish embryo acute toxicity (FET) and larval growth and survival (LGS) tests in zebrafish and fathead minnows found that the fathead minnow FET test was more sensitive when exposed to 3,4-dichloroaniline (DCA) than the zebrafish FET test. However, sensitivities were similar across all tests when fish were exposed to ammonia, suggesting that differential sensitivities could be chemical-specific. Further studies are required in order to understand developmental rate differences and how they may affect

sensitivities to ranges of chemicals. Without additional research, it is difficult to directly compare measured OS gene expression across development between zebrafish and fathead minnows.

Another important question proposed in this study is how directly acting chemicals versus chemicals that are metabolized may influence OS gene expression across developmental age. For the purpose of this study, R-(-)-carvone and bisphenol-a were used as the directly acting compound and metabolized compound respectively. R-(-)-carvone is a volatile monoterpene and an enantiomer of the main active component in spearmint oil (Younis et al 2014). As a Michael acceptor (MA), R-(-)-carvone can damage mitochondrial membranes, disrupting the electron transport chain and produce free radicals, causing oxidative stress (Bakkali et al 2008). Bisphenol-a is a pro-Michael acceptor (PRO-MA), which is metabolized to a reactive MA ultimate toxicant, thereby causing oxidative stress. Specifically, bisphenol-a can be metabolized to BPA-glucuronide and to bisphenol *o*-quinone by cytochrome P450, both of which can modify DNA (Atkinson and Roy 1995, Nakagawa and Tayama 2000). Given the metabolic role of bisphenol-a in OS toxicity, I hypothesized that bisphenol-a would have a greater toxic effect on fish at older developmental ages due to higher liver function while R-(-)-carvone would be less toxic across development.

Values for 96-h LC_{50} for zebrafish 1-4 dpf and fathead minnow 6-9 dpf were taken from a previous study (Corrales et al 2018). 96-h LC_{50} values for zebrafish 6-9 dpf and fathead minnow 1-4 dpf were unavailable in literature and therefore, separate acute toxicity tests were performed. As predicted, 96-h LC_{50} values in both fish increased across development, indicating a decreased toxic effect as fish age (Table 2). Conversely,

bisphenol-a 96-h LC_{50} values decreased across development in both species, exhibiting higher toxicity in older organisms (Table 2). The variations in acute toxicity values between R-(-)-carvone and bisphenol-a could be due to the metabolic activity at different developmental stages. In zebrafish, organogenesis begins at around 24 hpf and continue during hatching at 72 hpf (Kimmel et al. 1995, Korzh et al. 2001). During this time, the liver is not metabolically active; therefore, the embryo is more susceptible to directly acting compounds. At later developmental ages, the larva is able to metabolize compounds into different metabolites, which may or may not lead to adverse outcomes depending on the toxicity of the metabolite to the parent compound (Lillicrap et al. 2016). Previous studies of Japanese medaka and zebrafish exposed to organophosphate diazinon exhibited similar trends to bisphenol-a (Hamm and Hinton 2000, Kristofco et al. 2015).

In contrast to our hypothesis, OS gene expression of both zebrafish and fathead minnow across development after exposure to sublethal levels of bisphenol-a did not significantly differ. It is unclear why the sublethal toxicity results differed from the acute toxicity results in this study. The acute toxicity results seem to support the hypothesis that differential metabolic activity between the two developmental age groups can affect sensitivity to different toxicants according to their mechanism of action, but this is not supported by the sublethal results, suggesting that metabolic activity is not a reasonable explanation for observations of increased sublethal toxicity in the present study. Kristofco et al came to similar conclusions when exposure to diazinon and diphenhydramine increased toxicity in 10 dpf zebrafish regardless of mechanism of action. Regardless, further research is needed to understand the relationship among biotransformation capacities of developing and mature fish models.

Based on the results of the toxicity studies in this research, it is possible that bioavailability and uptake could impact the varying sensitivities observed across developmental age. Kristofco et al (2018) observed that differential sensitivity to diphenhydramine across zebrafish developmental stages were related to increased uptake and internal dose. Different structures that develop during embryo-larval development in fish have been shown to influence bioavailability of different contaminants. The chorion during the embryonic stage is one such structure that has been hypothesized to influence bioavailability to selective compounds (Embry et al 2010, Hamm and Hinton 2001). As the fish continues to develop into the larval stage, hatching occurs and the chorion no longer provides the fish with a potential barrier. Furthermore, zebrafish and fathead minnow embryo and larval stages depend on the yolk for nutrition until around 4 to 5 dpf, after which the larvae begin feeding exogenously, introducing another route of exposure that was not explored in this study (Belanger et al 2010). Respiration and gill surface area may also provide an important route of exposure, influencing uptake and bioaccumulation. Gill arches and filaments are the main sites for ion exchange and develop at around 5 dpf in zebrafish and are fully functional by 7 dpf (Jonz and Nurse, 2005, Rombough 1999). However, the gas exchange mainly occurs in the lamellae, which develop between 9 to 12 dpf, outside the developmental ages tested in the present study (Rombough 2007). Although previous laboratory studies have determined that bisphenol-a does not significantly bioaccumulate from water in tissues, this factor may be important for other chemicals (Lee et al 2015, Corrales et al 2015).

Future studies should focus on uptake and internal dose of compounds in the zebrafish and fathead minnow models. Additionally, it is important to explore different

routes of exposure that may play a more important role in toxicological endpoints, and thereby support a more complete understanding of ecological and human OS toxicity. By better understanding how different factors may increase or decrease sensitivity of a model organism, we can more accurately predict toxicity of contaminants.

CHAPTER FOUR

Conclusions

Due to the increasing use of alternative toxicological models, it has become progressively important to understand the limitations associated with their use in order to create a more robust toxicological database. This study addressed the following concerns in alternative toxicological models: whether OS responses vary throughout development, if these responses were conserved between zebrafish and fathead minnows, and whether these responses varied between directly acting chemicals versus those that are metabolized throughout development. My results demonstrate that OS response vary across development in both species, but are also different between both zebrafish and fathead minnow. Furthermore, these responses differed across development between directly acting compounds and metabolized compounds, but only for acute toxic endpoints. In future studies, it is important to consider bioavailability and uptake in understanding how toxicity can vary across developmental age and between species.

BIBLIOGRAPHY

Ankley, G., et al., 2005. Reproductive and Developmental Toxicity and Bioconcentration of Perfluorooctanesulfonate in a Partial Life-Cycle Test with the Fathead Minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry. 24. 2316-2324.

Ankley, G., Villeneuve, D., 2006. The Fathead Minnow in Aquatic Toxicology: Past, Present and Future. Aquatic Toxicology. 78. 91-102.

Atkinson, A., Roy, D., 1995. In Vitro Conversion of Environmental Estrogenic Chemical Bisphenol A to DNA Binding Metabolite(s). Biochemical and Biophysical Research Communications. 210. 424-433.

Bakkali, F., et al., 2008. Biological Effects of Essential Oils –A Review. Food and Chemical Toxicology. 46. 446-475.

Belanger, S., et al., 2010. Saltatory Ontogeny of Fishes and Sensitive Early Life Stages for Ecotoxicology Tests. Aquatic Toxicology. 97. 88-95.

Braunbeck, T., et al., 2015. The Fish Embryo Test (FET): Origin, Applications, and Future. Environmental Science Pollution Research. 22. 16247-16261.

Corrales, J., et al., 2015. Global Assessment of Bisphenol A in the Environment: Review and Analysis of Its Occurrence and Bioaccumulation. Dose-Response: An International Journal. 13. 1-29.

Corrales, J., et al., 2017. Toward the Design of Less Hazardous Chemicals: Exploring Comparative Oxidative Stress in Two Common Animal Models. Chemical Research in Toxicology. 30. 893-904.

Coyle, J., Puttfarcken, P., Oxidative Stress, Glutamate, and Neurodegenerative Disorders. Science. 262. 689-695.

Damasceno, D., et al., 2002. Oxidative Stress and Diabetes in Pregnant Rats. Animal Reproduction Science. 72. 235-244.

Dennery, P., 2007. Effects of Oxidative Stress on Embryonic Development. Birth Defects Research. 81. 155-162.

Di Giulio, R., et al., 1989. Biochemical Responses in Aquatic Animals: A Review of Determinants of Oxidative Stress. Environmental Toxicology and Chemistry. 8. 1103-1123.

Embry, M., et al., 2010. The Fish Embryo Toxicity Test as an Animal Alternative Method in Hazard and Risk Assessment and Scientific Research. Aquatic Toxicology. 97. 79-87.

Freires, I., et al., 2016. Alternative Animal and Non-Animal Models for Drug Discovery and Development: Bonus or Burden?. Pharmaceutical Research. 34. 681-686.

Hamm, J., Hinton, D., 2000. The role of Development and Duration of Exposure to the Embryotoxicity of Diazinon. Aquatic Toxicolgoy. 48. 403-418.

Hartung, T., 2009. Toxicology for the Twenty-First Century. Nature. 460. 208-212.

Hellou, J., et al., 2012. Glutathione, Glutathione *S*-transferase, and Glutathione Conjugates, Complementary Markers of Oxidative Stress in Aquatic Biota. Environmental Science Pollution Research. 18. 2007-2023.

Howe, K., et al., 2013. The Zebrafish Reference Genome Sequence and its Relationship to the Human Genome. Nature. 496. 499-503.

Huggett, D., et al., 2003. A Theoretical Model for Utilizing Mammalian Pharmacology and Safety Data to Prioritize Potential Impacts of Human Pharmaceuticals to Fish. Human and Ecological Risk Assessment. 9. 17789-1799.

Jeffries, M., et al., 2014. Alternative Methods for Toxicity Assessments in Fish: Comparison of the Fish Embryo Toxicity and the Larval Growth and Survival Tests in Zebrafish and Fathead Minnows. Environmental Toxicology and Chemistry. 33. 2584-2594.

Jeffries, M., et al., 2015. The Fish Embryo Toxicity Test as a Replacemet for the Larval Growth and Survival Test: A Comparison of Test Sensitivity and Identification of Alternative Endpoints in Zebrafish and Fathead Minnows. Environmental Toxicology and Chemistry. 24. 1369-1381.

Jonz, M., Nurse, C., 2005. Development of Oxygen Sensing in the Gills of Zebrafish. The Journal of Experimental Biology. 208. 1537-1549.

Kimmel, C., et al., 1995. Stages of Embryonic Development of the Zebrafish. Developmental Dynamics. 203. 253-310.

Korzh, S., et al., 2001. Developmental Analysis of *Ceruloplasmin* Gene and Liver Formation in Zebrafish. Mechanisms of Development. 103. 137-139.

Kristofco, L., et al., 2015. Comparative Pharacology and Toxicology of Pharmaceuticals in the Environment: Diphenhydramine Protection of Diazinon Toxicity in *Danio rerio* but Not *Daphnia magna*. The AAPS Journal. 17. 175-183.

Kristofco, L., et al., 2016. Age Matters: Developmental Stage of *Danio rerio* Larvae Influences Photomotor Response Thresholds to Diazinon or Diphenhydramine. Aquatic Toxicology. 170. 344-354.

Kristofco, L., et al., 2018. Differential Uptake of and Sensitivity to Diphenhydramine in Embryonic and Larval Zebrafish. Environmental Toxicology and Chemistry. 37. 1175-1181.

Lee, C., et al., 2015. Characteristics of Nonylphenol and Bisphenol A Accumulation by Fish and Implications for Ecological and Human Health. Science of the Total Environment. 502. 417-425.

Lillicrap, A., et al., 2016. Alternative Approaches to Vertebrate Ecotoxicity Tests in the 21st Century: A Review of Developments Over the Last 2 Decades and Current Status. Environmental Toxicology and Chemistry. 35. 2637-2646.

Limón-Pacheco, J., Gonsebatt, M., 2009. The Role of Antioxidants and Antioxidant-Related Enzymes in Protective Responses to Environmentally Induced Oxidative Stress. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 674. 137-147.

Lushchak, V., 2011. Environmentally Induced Oxidative Stress in Aquatic Animals. Aquatic Toxicology. 101. 13-30.

Lushchak, V., 2014. Free Radicals, Reactive Oxygen Species, Oxidative Stress and its Classification. Chemico-Biological Interactions. 224. 164-175.

McKim, J., 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-Term Toxicity. Journal of the Fisheries Research Board of Canada. 34. 1148-1154.

Nakagawa, Y., Tayama, S., 2000. Metabolism and Cytotoxicity of Bisphenol A and Other Bisphenols in Isolated Rat Hepatocytes. Organ Toxicity and Mechanisms. 74. 99-105.

OECD. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Publishing.

Pandey, S., et al., 2003. Biomarkers of Oxidative Stress: A Comparative Study of River Yamuna Fish *Wallago attu* (Bl. & Schn.). The Science of the Total Environment. 309. 105-115.

Rand-Weaver, M., et al., The Read-Across Hypothesis and Environmental Risk Assessment of Pharmaceuticals. Environmental Science and Technology. 47. 11384-11395

Rombough, P., 1999. The Gill of Fish Larvae. Is it Primarily a Respiratory or an Ionoregulatory Structure?. Journal of Fish Biology. 55. 186-204.

Rombough P., 2007. The Functional Ontogeny of the Teleost Gill: Which Comes First, Gas or Ion Exchange?. Comparative Biochemistry and Physiology. 148. 732-742.

Rudneva, I., 1999. Antioxidant System of Black Sea Animals in Early Development. Comparative Biochemistry and Physiology. 122. 265-271.

Rudneva, I., et al., 2010. Age Composition and Antioxidant Enzyme Activities in Blood of Black Sea Teleosts. Comparative Biochemistry and Physiology. 151. 229-239.

Russell, W,. Burch, R., 1959. The Principles of Human Experimental Technique. Methuen, London.

Schafer, F., Buettner, G., 2001. Redox Environment of the Cell as Viewed Through the Redox State of the Glutathione Disulfide/Glutathione Couple. Free Radical Biology & Medicine. 30. 1191-1212.

Scholz, S., 2013. Zebrafish Embryos as an Alternative Model for Screening of Drug-Induced Organ Toxicity. Archives of Toxicology. 87. 767-769.

Singh, U., Jialal, I., Oxidative Stress and Atherosclerosis. Pathophysiology. 13. 129-142.

Strähle, U., et al., 2012. Zebrafish Embryos as an Alternative to Animal Experiments –A Commentary on the Definition of the Onset of Protected Life Stages in Animal Welfare Regulations. Reproductive Toxicology. 33. 128-132.

Toyokuni, S., et al., 1995. Persistent Oxidative Stress in Cancer. Federation of European Biochemical Societies. 358. 1-3.

US EPA. 1991. Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures.

US EPA. 2002. Methods for Measuring Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms

Valavanidis, A., et al., 2006. Molecular Biomarkers of Oxidative Stress in Aquatic Organisms in Relation to Toxic Environmental Pollutants. Ecotoxicology and Environmental Safety. 64. 178-189.

Warner, C., et al., 2012. A Systems Toxicology Approach to Elucidate the Mechanisms Involved in RDX Species-Specific Sensitivity. Environmental Science and Technology. 46. 7790-7798.

Younis, Y., Beshir, S., 2004. Carvone-Rich Essential Oils from *Mentha longifolia* (L.) Huds. ssp. *schimperi* Briq. and *Mentha spicata* L. Grown in Sudan. Journal of Essential Oil Research. 16. 539-541.