TOXICITY OF AQUEOUS L-SELENOMETHIONINE EXPOSURE TO EARLY LIFE-STAGES OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) AND ZEBRAFISH (*DANIO RERIO*)

A Thesis Submitted to the College of

Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Toxicology Graduate Program,

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

By

Allyson Kristi Gerhart

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

DISCLAIMER

The thesis was exclusively created to meet the thesis and/or exhibition requirements for the Toxicology Master of Science degree at the University of Saskatchewan. Reference in this thesis to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan, and shall not be used for advertising or product endorsement purposes. Requests for permission to copy or to make other uses of materials in this thesis in whole or part should be addressed to:

Toxicology Graduate Program Chair
University of Saskatchewan
Toxicology Centre, 44 Campus Drive
Saskatoon, Saskatchewan, S7N 5B3, Canada

OR

Dean of the College of Graduate and Postdoctoral Studies
University of Saskatchewan
Thorvaldson Building, 110 Science Place, Rm 116
Saskatoon, Saskatchewan, S7N 5C9, Canada

ABSTRACT

Selenium (Se) is an important micronutrient in all vertebrate organisms due to its involvement in the synthesis of select proteins and enzymes involved in redox metabolism. However, its narrow range between essentiality and toxicity has generated concern as certain industrial activities have increased the loading of Se into the aquatic environment. Oviparous vertebrates such as fishes are particularly susceptible to elevated dietary Se concentrations during early life stages, as Se is readily transferred from mother to embryo. Research has shown that exposure to selenomethionine (SeMet), the predominant form of Se in the diet, during sensitive developmental stages can result in teratogenic (abnormal physiological or developmental) effects in developing fish larvae.

More recent Se research employing aqueous SeMet embryo exposures generated similar effects as those observed in maternal transfer and yolk microinjection studies, potentially offering an alternative to the standard methods of Se exposure. However, these aqueous embryo exposures are a relatively new concept and thus warrant further investigation. As such, the overarching objective of this research was not to replicate an environmentally relevant exposure scenario to produce results with regulatory implications, but to further investigate the mechanism of Se toxicity, as it relates to oxidative stress, through waterborne fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) embryo exposures to SeMet, tert-butyl hydroperoxide (tBOOH), and tert-butyl hydroquinone (tBHQ). In addition, this research aimed to critically evaluate the utility of aqueous exposures as a method for determining the effects of SeMet on developing embryos.

In my first experiment, newly fertilized fathead minnow embryos were exposed for six days to 30, 90, 270, 810, 2430, 7290, 21870, and 65610 µg Se/L (as SeMet). In the second experiment, newly fertilized zebrafish embryos were first exposed for 5 days to 5, 25, 125, and 625µg Se/L (as SeMet). These exposures informed the following experiment in which embryos were exposed to 75 mg/L tBOOH and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a 4h 100 µg/L tBHQ pre-treatment. Survival, hatchability, time to hatch, the frequency and severity of deformities (total and type), and changes in the expression of seven selected antioxidant-associated genes were determined. SeMet exposure reduced hatchability and survival, and increased the incidence and severity of

deformities in both fathead minnow and zebrafish in a concentration-dependent manner. In terms of time to hatch, opposite responses were observed, with a reduced time to hatch in fathead minnow and increased time to hatch in zebrafish. In the second experiment, exposure to tBHQ did not affect any of the endpoints evaluated; however, exposure to tBOOH increased time to hatch and the incidence/severity of deformities, decreased hatchability, and significantly increased expression of glutathione-disulfide reductase (*gsr*) mRNA abundance in the pre-treated tBOOH treatment group. No significant differences were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone.

The results of this study suggest that aqueous embryo exposures to SeMet can impact embryo-larval development, as evidenced by the production of deformities, and thus, the survivability of fish. In terms of the pro-oxidant tBOOH, exposure resulted in significant increases in both the incidence and severity of deformities such as those observed in the SeMet treated embryos, potentially suggesting a similar pathway (i.e. oxidative stress) plays a role in the generation of deformities. Furthermore, my results suggest that early life-stage zebrafish are more sensitive than fathead minnows to aqueous embryo SeMet exposures, inspiring more questions about factors such as developmental pattern and chorion physiology, and how this potentially influences toxicity across fish species.

ACKNOWLEDGEMENTS

I would first like to thank my graduate supervisor Dr. David Janz for his guidance and mentorship during the completion of my degree. He was instrumental in helping me achieve my goals and in my development as a researcher and writer. I would also like to thank my committee members, Dr. Markus Hecker and Dr. Soumya Niyogi for their questions, comments, and advice, which greatly improved the scope and quality of my research. I must also thank my external examiner Dr. Van Ortega for taking the time to review my thesis and attend my defense.

First, a general thank you is due to all the amazing faculty, front office staff (Fiona, Adriana, Tina, and Jordynn), and graduate students I've met in the toxicology program at the U of S. The sense of community/family in this program is extremely unique, and I'm blessed to have had the opportunity to work here and form friendships with all of you. You all made graduate school an amazing experience. I would like to thank Dr. Tim Jardine for encouraging me to pursue an undergraduate honours research project with him and Dr. Markus Hecker during my undergraduate degree, as it ultimately led to my decision to pursue a Masters' degree following graduation. I would also like to thank Lucy (Luciene) Kapronczai for all her encouragement, advice, and support (often in the form of food and pep talks) throughout the course of this degree. I would also like to thank my favorite undergraduate student Emily Kennedy for her assistance in the laboratory and Chamber 15 throughout my exposures. I also want to thank Abigail DeBofsky, James (Alper) Alcaraz, and Kamran Shekh for guiding me through the gene expression (RT-qPCR) portion of my research. I want to thank Chanel Yeung for not only teaching me everything I needed to know about zebrafish and working in Chamber 15, but also for being the best roommate I could have ever asked for. A special thanks to my family for their love, encouragement, advice, and support (in all its varied forms) throughout my entire educational career. Last, but not least, I want to thank my best friend and life partner Kyran Bussiere for loving me unconditionally and supporting me in everything I do.

Finally, I would like to acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding this research and the Toxicology Graduate Program for providing a scholarship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

actb	Actin beta
ahr2	Aryl hydrocarbon receptor 2
ANOVA	Analysis of variance
ARE	Antioxidant response element
AREB	Animal Research Ethics Board
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATRF	Aquatic Toxicology Research Facility
CCME	Canadian Council of Ministers of the Environment
cDNA	Complementary DNA
CI	Confidence interval
C_T	Cycle threshold
CYP1	Cytochrome P450 family 1 enzymes
Cys	Cysteine
d	Day
d.m.	Dry mass
d.w.	Dry weight
DI	Iodothyronine deiodinases
DNA	Deoxyribonucleic acid
dpf	Days post-fertilization
EC_{50}	Effective concentration in 50 percent of a population
EF	Enrichment Function
ef1α	Elongation factor 1 alpha
g	Gram

GCL	Glutamate-cysteine ligase enzyme
gclc	Glutamate-cysteine ligase catalytic subunit
GPx	Glutathione peroxidase enzyme
gpx1a	Glutathione peroxidase 1a
GR	Glutathione reductase enzyme
Grx	Glutaredoxin enzyme
GSH	Reduced glutathione
GSH:GSSG	Reduced:oxidized glutathione
GSI	Graduated Severity Index
gsr	Glutathione-disulfide reductase
GSSG	Oxidized glutathione
GST	Glutathione S-transferase enzyme
gst p1	Glutathione S-transferase piscine 1
gst p2	Glutathione S-transferase piscine 2
h	Hours
H_2O_2	Hydrogen peroxide
hpf	Hours post-fertilization
HPG axis	Hypothalamic-pituitary-gonadal axis
ICP-MS	Inductively coupled plasma-mass spectrometry
Kg	Kilogram
L	Litre
LC_{50}	Concentration causing 50 percent lethality to a population
M	Molar
Met	Methionine

mg	Milligram
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre
min	Minute
mL	Millilitre
mm	Millimetre
mM	millimolar
MOA	Mechanism of action
mRNA	Messenger RNA
MS-222	Tricaine methanesulfonate
n	Number of replicates
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
ng/g	Nanogram per gram
nrf2a	Nuclear factor erythroid 2-related factor 2a
nrf2b	Nuclear factor erythroid 2-related factor 2b
NSERC	Natural Sciences and Engineering Research Council of Canada
p	Probability
PAH	Polycyclic aromatic hydrocarbon
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT qPCR	Real time quantitative polymerase chain reaction
rT3	Reverse triiodothyronine
S	Sulfur

SD	Standard deviation
SE	Standard error of the mean
Se	Selenium
Se^{2-}	Inorganic selenide
SeCys	Selenocysteine
SeMet	Selenomethionine
SePO3-	Selenophosphate
SOD	Superoxide dismutase
sod2	Manganese superoxide dismutase
S-S	Disulfide bond
T3	Triiodothyronine
T4	Thyroxine
tBHQ	Tert-butyl hydroquinone
tBOOH	Tert-butyl hydroperoxide
TR	Thioredoxin reductase
tRNA	Transfer RNA
Trx	Thioredoxin
UCACS	University Committee on Animal Care and Supply
US EPA	United States Environmental Protection Agency
VTG	Vitellogenin
WQG	Water quality guideline
XRE	Xenobiotic response element
°C	Degrees centigrade
µg	Microgram

µg/g	Microgram per gram
µg/L	Microgram per litre
µmol	Micromole
$^{\bullet}OH$	Hydroxyl radical
$O_2^{\bullet-}$	Superoxide anion radical

NOTE TO READERS

This thesis was prepared in a manuscript style and will therefore have some redundancies across sections of research chapters. To reduce these redundancies, descriptions of methods and statistics can be found in their respective chapters. Chapter 1 is a general introduction, and chapters 2-3 are written in the style of publishable manuscripts. Chapter 4 serves as a summary and conclusion to the overall thesis. Chapter 2 of this thesis was published in the *Bulletin of Environmental Contamination and Toxicology* on January 19th, 2019, and chapter 3 was published in *Toxics* - Special Issue "Contaminant Effects on Zebrafish Embryos" on August 29th, 2019. To avoid redundancies in citation lists, all citations have been provided in a combined section at the end of this thesis.

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 Properties and sources of selenium

Regarded as a non-metal or metalloid, Selenium (atomic number 34, atomic mass 78.96) belongs to the group VIA elements (otherwise known as the chalcogens), which includes oxygen, sulfur, tellurium, and polonium (Greenwood & Earnshaw, 1997). Selenium (Se) occurs naturally in geological deposits around the world and is generally associated with marine sedimentary rock formations which include coal or petroleum source rocks, phosphate rocks, and black shale (Presser et al. 2004; Maher et al. 2010). Entry into the environment occurs naturally through processes such as atmospheric deposition, weathering, and other biogeochemical processes (Fernández-Martínez & Charlet, 2009). However, this contribution is considered minimal in comparison to anthropogenic sources. Industrial activities such as crude oil refinement and combustion, coal-fueled generation of electricity, irrigation of seleniferous soils, as well as the mining and smelting of coal, uranium, and phosphate contribute to increased loading of Se into the aquatic environment through the generation of waste by-products (Lemly, 2004; Maher et al. 2010; Janz, 2012).

1.2 Selenium in the aquatic environment

1.2.1 Chemical speciation, uptake, and bioaccumulation

Existing in four main oxidation states, which include selenides (-II), elemental Se (0), selenites (+IV), and selenates (+VI), Se exhibits a wide variety of both organic and inorganic chemical forms (Zhang & Moore, 1996; Meseck & Cutter, 2006). Research has shown that the inorganic oxyanions selenate (SeO_4^{2-}) and selenite (SeO_3^{2}) represent the most abundant forms of dissolved Se in the water column following the discharge of industrial by-products (Fan et al. 2002; Luoma & Presser, 2009). Comparatively, selenite is more bioavailable and toxic than selenate (Niimi & Laham, 1976) however, it is the biotransformation of these oxyanions into organic molecules by bacteria and algae that ultimately raises concern. Organoselenium compounds constitute the largest and perhaps most important species of Se as they are significantly more bioavailable than their inorganic counterparts, with the compound

selenomethionine representing the most toxic form (Niimi & Laham, 1976; Maier, 1990; Riedel et al. 1991).

It is well established in the literature that primary producers such as algae and bacteria are responsible for the initial biotransformation of the oxyanions selenate and selenite into more biologically relevant organic forms (Fan et al. 2002; Neumann et al. 2003; LeBlanc & Wallschläger, 2016). In general, Se is nutritionally required by algae and bacteria for selenoprotein synthesis as well as energy production. In anoxic environments, bacteria often utilize sulfate to obtain energy through reduction, a process where the sulfate ion serves as a terminal electron receptor during energy metabolism (Fernández-Martínez & Charlet, 2009). Due to similarities in their electron shell configurations (Greenwood & Earnshaw, 1997), selenium and sulfur (S) have similar chemical properties and tend to interact or substitute for each other in biological and chemical processes (Young et al. 2010). As such, selenate and selenite, which are chemically analogous to sulfate and sulfite ions, respectively, are also capable of being used for energy production by certain species of bacteria, lending to their accumulation in these primary producers.

In locations contaminated with Se, the largest increase in Se tissue content occurs in biofilms composed of primary producers (Conley et al. 2009; Conley et al. 2013) such as bacteria and algae. However, the degree to which biofilms bioconcentrate Se varies greatly with species composition. As noted by Baines and Fisher (2001), different species of algae under the same exposure conditions can exhibit enrichment functions (EF; the concentration of Se in algae or bacteria divided by the concentration of selenium in the environment) that vary by up to 5 orders of magnitude. In addition to species composition, the dominant form of Se ion in solution has also been shown to influence uptake, with most studies noting that selenite accumulates in algae more readily than selenate (Riedel et al. 1991). Following uptake, aquatic producers readily biotransform selenite and selenite into a range of organoselenium forms including selenoamino acids such as selenomethionine (SeMet) and selenocysteine (SeCys) (Fan et al. 2002), with SeMet representing the predominant form in tissue (Maher et al. 2010). This is concerning as these organic compounds are readily transferred through diet to reach higher trophic levels (Fan et al. 2002). Subsequent incorporation of these seleno-amino acids into proteins results in Se accumulation in tissue, with organisms at the top of the food chain exhibiting the highest concentrations (Hamilton, 2004). Ultimately, Se toxicity is determined by site-specific

characteristics such as water quality, biogeochemistry, and food web structure (Orr et al. 2006). As such, controlled lab studies can contribute to our understanding of Se at certain levels of the food chain, but lab experiments are limited as they cannot address the complexity of entire aquatic ecosystems.

1.2.2 Guidelines for selenium in the aquatic environment

Regulatory guidelines for Se in aquatic systems are continually changing as new information becomes available. The current federal water quality guideline (WQG) for the protection of aquatic life in freshwater enforced by the Canadian Council of Ministers of the Environment (CCME) is 1 μg Se/L (CCME, 2007). However, WQGs vary by province, with British Columbia setting the WQG at 2 μg Se/L (BC MoE, 2014) and Ontario at 100 μg Se/L (MoEE, 1994). In addition, British Columbia has also included guidelines for fish tissue concentrations. The guidelines state that tissue values in the egg/ovary, whole body, and muscle should not exceed 11, 4, and 4 μg Se/g dry weight (d.w.), respectively.

In comparison, the current U.S. Environmental Protection Agency (EPA) water quality criterion for the protection of aquatic life in freshwater include a 30-day average of water concentrations in lentic and lotic systems that should not exceed 1.5 and 3.1 μg Se/L, respectively, more than once in a three-year period (US EPA, 2016). In addition, the US EPA criterion state that fish tissue concentrations in the egg/ovary, whole body, and muscle (skinless, boneless filet) should not exceed 15.1, 8.5, and 11.3 mg Se/kg d.w., respectively (US EPA, 2016).

1.3 The essentiality and toxicity of selenium

1.3.1 Selenium as an essential nutrient to vertebrate health

First identified as an essential nutrient by Schwarz and Foltz in 1957, Se is required for the synthesis of select proteins and enzymes involved primarily in redox metabolism (Fan et al. 2002; Young et al. 2010). Selenoproteins, defined as any protein that contains the SeCys amino acid, are organized based on the location and functionality of SeCys within the molecule. The size of the selenoproteome varies with species (Lobanov et al. 2009; Mariotti et al. 2012). Bony fishes appear to have the largest selenoproteomes, with genes coding for a maximum of 38 selenoproteins in zebrafish, compared to 24 in frogs (Mariotti et al. 2012) and 25 in humans

(Kryukov et al. 2003). While the function of most selenoproteins has not yet been determined (Janz et al. 2012), the iodothyronine deiodinases (DI), thioredoxin reductases (TR), and glutathione peroxidases (GPx) are among those that have been characterized (Lobanov et al. 2009).

In humans, there are three types of iodothyronine deiodinases: Type I (D1 or 5' deiodinase), Type II (D2), and Type III (D3 or 5-deiodinase). Expressed primarily in the liver, kidney, thyroid, and pituitary, these enzymes are vital to metabolic processes such as thermogenesis, growth, and development as they directly influence thyroid hormone metabolism. Specifically, they are responsible for the conversion of the thyroid prohormone thyroxine (T4) into the active triiodothyronine (T3) or inactive reverse triiodothyronine (rT3) forms through the selective removal of iodine atoms from the T4 molecule. Type I and II deiodinase catalyze the removal of an iodine atom from the outer ring of the T4 molecule, converting the inactive hormone into the active form T3. Type III deiodinase catalyzes the removal of an iodine atom from the inner ring, resulting in the unreactive rT3 hormone (Stadtman, 1996; Papp et al. 2007; Marsili et al. 2011).

The thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (TR), and nicotinamide adenine dinucleotide phosphate (NADPH). Trx is a widely distributed redox protein that regulates intracellular redox-dependent processes, enzymes, and transcription factors by controlling the oxidation and reduction of thiol groups, whereas the TR enzymes catalyze the reduction of Trx (Tapiero et al. 2003; Pedrero & Madrid, 2009). Trx, in concert with NADPH and TR, regulates redox signalling by acting as an antioxidant and facilitating the reduction of disulfide bonds in proteins. Trx is kept in a reduced state by an NADPH-dependent reaction involving the TR enzymes, where electrons are taken from NADPH by TR and transferred into the active site of Trx. The reduced state of Trx allows it to serve as an electron donor to other proteins/enzymes (Arnér & Holmgren, 2000; Zhong et al. 2000) such as ribonucleotide reductase, a protein involved in repair mechanisms essential for deoxyribonucleic acid (DNA) synthesis (Tapiero et al. 2003).

Operating in a manner very similar to the thioredoxin system, the glutaredoxin system consists of two main enzymes: glutaredoxin (Grx) and the NADPH-dependent glutathione reductase (GR). Grx, like Trx, is involved with the reduction of disulfide bonds formed in

oxidized proteins, as well as the reduction of protein-glutathione mixed disulphides (deglutathionylation) (Holmgren et al. 2005). These reduction reactions cause Grx to become oxidized, and unlike the thioredoxin system where Trx is directly reduced by TR, Grx is reduced through the oxidization of a cofactor, the oxidization of reduced glutathione (GSH) to oxidized glutathione (GSSG). Once oxidized, GSSG is reduced back to GSH by GR (Michiels et al. 1994), with subsequent reduction/regeneration of GR by NADPH through the acceptance of an electron (Friguet, 2006). It has been noted that the thioredoxin and glutaredoxin systems also serve as electron donors in the reduction of GPx (Björnstedt et al. 1994), an important enzyme that specifically catalyses hydrogen peroxide (H_2O_2) reduction (Michiels et al. 1994; Handy et al. 2009). GPx thus plays a protective role and tries to prevent or minimize the extent of oxidative damage in cells.

In order to produce and maintain the catalytic functions of these selenoenzymes, organisms must obtain sufficient amounts of Se through their diet. Dietary Se requirements for normal physiological function in fish range from 0.1 - 0.5 μg Se/g d.w. (Lemly, 1997), while aquatic birds and amphibians require 0.3 to 1.1 μg Se/g (Stewart et al. 2010) and 0.3 μg Se/g d.w. (Ferrie et al. 2014) respectively. Overall, the uptake and metabolism of Se generally leads to the replacement of thiol groups by Se in sulfur-containing amino acids such as methionine (Met) or cysteine (Cys), with subsequent insertion of these amino acids into proteins (Young et al. 2010). Whether dietary Se is present as an organic or inorganic form, metabolism to the common intermediate selenide (Se^{2-}) must occur before Se can be incorporated into seleno-amino acids and selenoproteins (Ganther, 1986). This is accomplished by β-lyase, which cleaves the organic forms, and GSH, which reduces the inorganic forms (Suzuki & Ogra, 2002).

In mammals, production of SeCys is a natural occurrence that follows a defined process directed by the genes SelA, SelB, SelC, and SelD (Böck et al. 1991). Using selenide, SeCys synthesis follows one of two pathways: nonspecific isoteric substitution of Se for sulfur in an amino acid, or co-translational conjugation of selenophosphate to serine (Young et al. 2010). In the latter case, selenide is phosphorylated to selenophosphate ($SePO^{3-}$) (Suzuki & Ogra, 2002) by selenophosphate synthetase (Veres et al. 1994) and then undergoes conjugation to serine by selenocysteine transfer RNA and the enzyme selenocysteine synthase (Young et al. 2010). SeCys is then incorporated into proteins (via selenocysteinyl-tRNA) that are genetically encoded as selenoproteins, with an encoding DNA sequence that contains the UGA codon and the

selenocysteine insertion sequence (Stadtman, 1996; Young et al. 2010). SeMet, in contrast to SeCys, does not have a specific codon or insertion sequence. Therefore, proteins containing Se in the form of SeMet are referred to as Se-containing proteins as opposed to selenoproteins (Suzuki & Ogra, 2002).

While both Cys and SeCys are naturally produced by the body, Met is one of the eight amino acids that higher animals (ex. rats) and humans are unable to produce due to a lack of the necessary enzymes (Schrauzer, 2000). As such, there is a reliance on dietary sources to obtain Met. Following ingestion of SeMet, metabolization can follow two pathways. The first pathway involves the conversion of SeMet to SeCys via selenohomocysteine and selenocystathionine (Kajander et al. 1991), followed by further degradation to serine or selenide via β-lyase in the liver (Schrauzer, 2000; Suzuki & Ogra, 2002). Selenide can then be utilized for selenoprotein synthesis or undergo methylation and excretion (Schrauzer, 2000). The second proposed pathway suggests that excess SeMet can be directly transformed to methylselenol via γ-lyase (Okuno et al. 2001). Of greater concern however, is the fact that SeMet can also be directly incorporated into body proteins (Schrauzer, 2000; Suzuki & Ogra, 2002). This is due to a fault in the enzyme responsible for charging methionyl-tRNA, methionyl-tRNA synthetase, which is unable to differentiate between SeMet and Met (Moroder, 2005). This is concerning as organisms residing in Se-contaminated environments are consistently exposed to SeMet in their food (Fan et al. 2002). Therefore, any SeMet that is not metabolized to a usable or excretable form will be readily stored in body proteins in place of Met, leading to increasingly higher Se tissue concentrations. As was noted by Schrauzer (2000), organs with high rates of protein synthesis such as the liver, kidney, skeletal muscles, and erythrocytes are particularly susceptible to Se accumulation.

1.3.2 The toxicological effects of selenium in oviparous vertebrates

Numerous studies have shown that oviparous vertebrates such as amphibians, reptiles, birds, and fishes are highly susceptible to elevated dietary Se concentrations during early life stages (Janz et al. 2010). Overall, this is attributed to maternal transfer of Se to the embryo; however, major differences in reproductive physiology and biochemical/physical properties of eggs across species corresponds to different physiological pathways for maternal Se transfer across species (Janz et al. 2010). In fish, dietary Se is maternally transferred into developing

oocytes via yolk proteins, resulting in a substantial Se exposure to the larvae during yolk resorption (Janz et al. 2010). This process is presumably very similar in amphibians and squamate reptiles; however, little is known about the mechanisms of maternal transfer in these organisms (Unrine et al. 2007; Janz et al. 2010). In contrast to fish and amphibians, *in ovo* Se exposure in aquatic birds also involves maternal transfer, however; the majority of the Se is transferred into the albumin as opposed to the yolk sac. As a result, uptake and exposure occurs prior to hatch or yolk sac resorption (Janz et al. 2010).

Overall, developmental abnormalities tend to be the predominant toxicological effect of Se exhibited in oviparous species, and perhaps the most important. In fish, exposure during these sensitive developmental stages can result in spinal deformities (lordosis, kyphosis, scoliosis), missing or misshapen fins, craniofacial malformations, and edema (craniofacial, yolk sac, pericardial) (Lemly, 1997; Janz, 2012). In aquatic birds, exposure is characterized by a reduction or absence of eyes, reduction or malformation of the upper/lower bills as well as the lower limbs, and in rare instances protrusion of the brain from the eye sockets (i.e. exencephaly) (Hoffman & Heinz, 1988; Janz et al. 2010). In amphibians, anatomical alterations include spinal curvatures, craniofacial malformations, abnormal structure of the gut or eye lens, and edema (Massé et al. 2015).

1.3.3 The toxicological effects of selenium in fishes

In fish, production of the yolk protein precursor vitellogenin (VTG) occurs in the liver (Janz et al. 2010) and is regulated by the hypothalamic-pituitary-gonadal axis (HPG axis). (Arukwe & Goksøyr, 2003). VTG is then exported from the liver, transported in the blood, and incorporated into the developing ovarian follicles via receptor-mediated endocytosis (Tyler & Lancaster, 1993). In the follicle, VTG is cleaved enzymatically to form the major yolk lipoproteins (Brooks et al. 1997; Arukwe & Goksøyr, 2003). During production of VTG in the liver, Se is incorporated into the proteins in a dose-dependent manner (Janz, 2012). Therefore, the greater the dietary exposure to Se, the larger the proportion of SeMet that will be incorporated into VTG rather than Met. The amount of Se-enriched VTG transported from the liver to the developing oocytes ultimately determines what dose larvae will be exposed to (Janz et al. 2010). Following fertilization, the developing larvae assimilate and metabolize the Se-contaminated yolk proteins which serve as an initial energy and protein source (Lemly, 1997;

Janz, 2012). Exposure during these sensitive developmental stages can result in edema (craniofacial, yolk sac, pericardial), spinal deformities (lordosis, kyphosis, scoliosis), missing or misshapen fins, and craniofacial malformations (Lemly, 1997; Janz, 2012). Although elevated Se levels in aquatic systems have relatively little impact on adult fish, larval deformities are ecotoxicologically relevant as they can impair recruitment, diminishing population size and community diversity over time (Lemly, 2002).

1.3.3.1 Mechanisms of selenium toxicity

Classically, two main theories regarding the mechanism of Se toxicity in higher order vertebrates were circulating. The first theory was related to the ability of Se to readily substitute for sulfur in sulfur-containing biomolecules during protein synthesis, due to their chemical similarities (Janz et al. 2010; Young et al. 2010). It was originally proposed that this incorrect substitution interfered with the tertiary structure of proteins through disruption of disulfide (S-S) bond formation. The location of sulfur molecules in the linear chain of amino acids determines where disulfide linkages are formed which subsequently dictates protein folding, structure, and function (Diplock & Hoekstra, 1976; Lemly, 2002). Therefore, improper folding of proteins due to uncontrolled Se insertion will likely result in toxicity through the functional impairment of important enzymes (Sunde, 1984). For a period of time, the protein misfolding theory lost support due to the structural characteristics of both Met and SeMet. It was suggested that the terminal methyl group of Met and SeMet would obstruct the Se molecule and prevent the formation of covalent bonds, thus, protein structure would be largely unaffected despite the incorrect insertion (Janz et al. 2010). However, more recent research suggests that the focus should be shifted away from incorrect SeMet insertions towards the metabolization of SeMet into SeCys, and subsequent random replacement of cysteine by SeCys during protein synthesis. In a similar fashion, it is hypothesized that this replacement alters protein structure and induces misfolding, resulting in the formation of insoluble protein adducts (i.e. protein aggregation) (Lazard et al. 2017; Plateau et al. 2017). In addition to protein misfolding, Plateau et al. (2017) suggested that the production of reactive oxygen species (ROS), namely superoxide anion radicals ($O_2^{\bullet-}$), may also contribute to protein misfolding/aggregation as well. Thus, oxidative stress is also theorized to play a role.

Within the last few decades, research investigating the links between SeMet exposure and oxidative stress has grown considerably, with oxidative stress suggested to be the major contributor to teratogenic effects and mortality in fish (Palace et al. 2004; Janz et al. 2010). As was mentioned previously, GSH is an intracellular antioxidant and cofactor utilized by various antioxidant enzymes to return to a reduced state. Enzymes such as GPx, which rely on GSH for reduction, are responsible for maintaining the redox environment within cells. Numerous studies have shown that exposure to elevated levels of Se reduces the ratio of GSH:GSSG (reduced:oxidized glutathione) in animal systems (Misra et al. 2012; Arnold et al. 2016; Mohammad et al. 2017), suggesting a limited capacity to maintain redox homeostasis. Additional interactions between Se metabolites and GSH can also lead to the formation of ROS such as hydrogen peroxide, hydroxyl (˙OH) or superoxide radicals, which can diminish the protective strength of the antioxidant defenses and induce cellular apoptosis and oxidative damage to DNA, lipids, and proteins (Spallholz, 1994; Spallholz et al. 2001; Palace et al. 2004). Overall, the mechanistic story behind Se toxicity is becoming increasingly complicated, as recent studies are suggesting that the mode of action differs by seleno-compound, chemical form, and speciation (Lazard et al. 2017). Regarding SeMet toxicity, there appears to be both oxidative stress and protein misfolding/aggregation involvement, rather than one pathway alone (Lazard et al. 2017; Plateau et al. 2017).

1.3.4 Se contamination case studies

The threat Se poses to the aquatic environment is no longer up for debate, as the consequences associated with industry-driven increases in Se concentrations are well characterized in the literature. One of the most well-known cases associated with Se contamination is that of Kesterson National Wildlife Refuge, located in the San Joaquin Valley of California, USA. Intensive irrigation of arid agricultural soils in the San Joaquin Valley generated large volumes of runoff contaminated with salts and other trace elements such as Se. This led to the abandonment of farm acreages in the San Joaquin Valley due to salt buildup and poor drainage. As a result, drainage systems with a series of flow-regulating reservoirs were implemented, which directed the contaminated runoff into the Sacramento-San Joaquin River Delta and eventually out into the San Francisco Bay and sea. These flow-regulating reservoirs, which were intended to double as wetland habitat for wildlife, quickly became highly contaminated with Se, resulting in a massive fish kill and the death or deformation of thousands

of aquatic birds (Lemly, 1993). In addition to Kesterson National Wildlife Refuge, Belews Lake of North Carolina, USA, represented another highly publicized example of environmental Se contamination. From 1974 up until 1986, selenium-laden wastewater was discharged from a coal-fired power plant to an ash basin (to allow settling), and then flowed back into the lake via canals. Within the first four years of discharge, reproductive failure and increased rates of post-hatch mortality eliminated 19 of the 20 fish species originally present in the lake. Meanwhile, individuals that survived displayed teratogenic deformities of the spine, head, mouth, and fins (Lemly, 2002).

Following these disasters, it became readily apparent that oviparous vertebrates (i.e. amphibians, reptiles, birds, and fishes) were highly susceptible to elevated Se concentrations during early life stages, as Se is readily transferred from mother to embryo through egg yolk proteins (Janz et al. 2010). Exposure to SeMet, the predominant form of Se in the diet, during these sensitive developmental stages often results in developmental abnormalities. In developing fish larvae, these often manifest as spinal/skeletal deformities, missing/misshapen fins, craniofacial malformations, and edema (Lemly, 1997; Janz, 2012). As such, deformities have come to be known as one of the most sensitive and ecotoxicologically relevant endpoints in assessments of Se exposure (in fish), as they can impair recruitment, diminishing population size and diversity over time (Lemly, 2002). However, as far as effects/endpoints go, identifying the presence of deformities in a population indicates toxicity has already taken place and it is likely too late to take action. As such, research focusing on the relationship between Se exposure and the generation of deformities (namely using small fish models) has become a popular topic in the literature.

1.4 Fishes as indicator species

Fish have become important test organisms in aquatic toxicological research namely due to their ecological relevance in aquatic foodwebs. However, they have also been found to serve as a surrogate for other vertebrate models, as much of the basic underlying molecular processes or mechanisms of action (MOA) involved with toxicity are highly conserved across vertebrates (Ankley & Johnson, 2004). As such, chemical assessment research conducted in fish models can be used to make risk predictions in other fish or vertebrate species of concern.

1.4.1 *Pimephales promelas* as a model organism – experiment # 1

Pimephales promelas, a member of the Cyprinidae family commonly known as the fathead minnow, has become one of the most popular and widely used small fish models in toxicological research in North America (Ankley & Villeneuve, 2006). They are ubiquitous in distribution, found in both lentic and lotic freshwater environments across North America (Ankley & Johnson, 2004; Ankley & Villeneuve, 2006). They are olive-yellow in colour with a yellow to white underbelly. Adult males are generally larger than females, and when reproductively active, males develop secondary sex characteristics including dark banding, a dark spot at the anterior side of the dorsal fin, tough tubercles on the snout, and a grey pad of spongy tubercles on the nape (US EPA, 1987; Ankley & Villeneuve, 2006). Adult females remain relatively plain when reproductively active but begin to exhibit an ovipositor a month prior to spawning (US EPA, 1987).

The use of *P. promelas* as a model organism in laboratory research offers numerous advantages. Their extensive use for monitoring and regulatory purposes (Ankley & Villeneuve, 2006) has led to the development of detailed protocols for maintenance and breeding in a controlled laboratory setting (US EPA, 1987). Compared to other fish species, the husbandry of *P. promelas* is relatively easy as they can be housed in aquaria and fed commercially purchased *chironomid* larvae (blood worms). In addition to ease of culture, they also exhibit a short life cycle, with a generation time of only four to five months. When maintained under ideal breeding conditions, reproductively active females are capable of producing large clutches of about 200 eggs in one spawning event at 3-4 day intervals (Ankley et al. 2001). As an added benefit, the transparent appearance of the embryo chorion allows for easy determination of fertilization status, as well as morphological analysis throughout the entire developmental process using a microscope. Due to their extensive use in regulatory research, a large amount of toxicity data exists from both laboratory and field settings (Ankley & Villeneuve, 2006). Furthermore, the embryonic development of *P. promelas* has also been thoroughly studied (US EPA, 1996), thus providing a substantial knowledge base for studying embryo development.

1.4.2 *Danio rerio* as a model organism – experiment # 2

Danio rerio, another member of the Cyprinidae family commonly known as zebrafish, also represents a highly popular and widely used small fish model in toxicological research

(Ankley & Johnson, 2004). Native to the floodplain regions of the Himalayas, *D. rerio* typically inhabit shallow and slow moving or stagnant water bodies. They are distinct in appearance, exhibiting several longitudinal, blue, pigmented stripes along the sides of their body which extend to the caudal fin. The degree of sexual dimorphism is minimal, with slight variations in body shape and colouration providing distinction. Males tend to have a torpedo shaped body, larger anal fins, and yellow-red coloration between the stripes. Conversely, females display a larger, lighter belly, and silver coloration between stripes (Nasiadka & Clark, 2012).

D. rerio possess many traits that lend to their popularity as a model organism in research. For example, extensive use has led to the development of detailed protocols for culturing and maintenance in a controlled lab setting (Nasiadka & Clark, 2012). Compared to other fish species, *D. rerio* husbandry is very easy, as they can be housed in aquaria and fed commercially purchased flake food as well as *Artemia* (brine shrimp). They are also broadcast spawners, releasing eggs that settle to the bottom of the tank (Ankley & Johnson, 2004) as opposed to depositing clutches on the surface of substrates like fathead minnows, which greatly aids in the collection of eggs during breeding. In addition to ease of culture, they also exhibit a short life cycle, with a generation time of only 2-3 months (Ankley & Johnson, 2004). When maintained under ideal breeding conditions, reproductively active females can exhibit some degree of spawning activity almost every day, with larger clutches of about 150 eggs produced every 5-10 days (Ankley & Johnson, 2004). Similar to *P. promelas*, the transparent embryo chorion allows for the determination of fertilization status as well as morphological analysis throughout development. Furthermore, the embryonic development of *D. rerio* has been extensively studied and detailed (Kimmel et al. 1995), thus providing a substantial knowledge base for studying embryo development. Aside from the advantageous biological characteristics of zebrafish, the volume of molecular information available in the literature is extremely useful. For example, the complete zebrafish genome has been sequenced, making genetic work with this species much more efficient than others.

1.4.3 Development of *Pimephales promelas* and *Danio rerio*

A comprehensive characterization of the embryonic stages of *Pimephales promelas* has been outlined by the US EPA (US EPA, 1996), providing a substantial knowledge base for studying embryo development. This document details each step of development extending up

until the point of hatch, with a more general classification of developmental changes following hatch. The entire pre-hatch process is divided into 32 stages of development, ranging from an unfertilized ovum in stage 1 to when the embryos begin hatching in stages 31-32 (approximately 4 days post fertilization [dpf]). Following hatch, development is divided into 9 stages which include milestones such as yolk-sac disappearance at stage 3 (approximately 32h post hatch) and exogenous feeding at stage 4 (approximately 56.6h post hatch), concluding with achievement of the juvenile phase at stage 9 (approximately 443h post hatch) (US EPA, 1996). Under ideal conditions, sexual maturity can be reached within 4-5 months of hatch (Ankley & Villeneuve, 2006).

A detailed account of the embryonic stages of *Danio rerio* has also been outlined by Kimmel et al. (1995). In this document, the entire developmental process is divided into stages of development organized by name as opposed to numbering them, and ranges from the 1-cell stage at 0.2h, the hatching period at 48-72h, to the early larval period at 72 hours post fertilization (hpf). It is during the larval period that events such as yolk-sac disappearance and the commencement of exogenous feeding occurs (Kimmel et al. 1995). Under ideal conditions, the juvenile phase can be reached within 45 dpf, and sexual maturity within 2-3 months of hatch (Ankley & Johnson, 2004; Singleman & Holtzman, 2014).

1.5 Endpoints and biomarkers

Exposure to contaminants can disrupt several key physiological processes in living organisms. By studying alterations in these physiological parameters, one can further ascertain the mechanisms that ultimately lead to adverse effects on organismal health**.**

1.5.1 Selenium water and tissue concentrations

Total Se can be measured in both water and tissue samples, using inductively coupled plasma-mass spectrometry (ICP-MS). In the case of aqueous exposures, it is imperative that water samples are collected and tested to confirm exposure concentrations. In addition to water, the collection of larval/tissue samples is critical in demonstrating that SeMet is in fact taken up by the developing embryos during aqueous exposure, as passage of Se through the chorion is currently a subject of debate.

1.5.2 Hatchability and time to hatch

Although criticized for their lack of specificity to Se (Janz et al. 2010), hatchability parameters and mortality are sensitive indicators/endpoints used to evaluate Se exposed fishes. While previous Se research employing other methods of exposure such as spiked diets (Thomas & Janz, 2014, 2015) or yolk microinjections (Thomas & Janz, 2016) have reported mixed results in terms of hatchability, previous embryo aqueous Se exposure work has generally reported reduced hatchability as well as reductions in days to hatch (Lavado et al. 2012; Kupsco & Schlenk, 2016b).

1.5.3 Mortality

In contrast to hatchability, mortality results have been more consistent, with previous Se research generally reporting reduced survival regardless of the exposure method (Thomas & Janz, 2015, 2016; Arnold et al. 2016; Kupsco & Schlenk, 2016a,b).

1.5.4 Teratogenesis and edema

In addition to hatchability and mortality, larval deformities represent the most sensitive and useful endpoint in assessments of Se exposed fish (Janz et al. 2010). In general, previous Se studies have reported an increased frequency of deformities in fish exposed to Se during early development (Thomas & Janz, 2014, 2015, 2016), with spinal deformities (kyphosis, lordosis, scoliosis) occurring at a greater frequency than other types of malformations (Muscatello et al. 2006). The generation of deformities has also been observed in Japanese medaka embryos exposed aqueously to Se (Kupsco & Schlenk, 2016a,b).

Since the majority of previous Se research has employed methods of exposure such as spiked diets and yolk microinjections, reproductive endpoints such as hatchability, time to hatch, survival, and the frequency and severity of larval deformities (total and type) should be evaluated to determine if aqueously exposing embryos to Se also interferes with these types of reproductive endpoints.

1.5.5 Gene expression biomarkers

As was previously mentioned, the primary mechanism suggested for Se-induced toxicity is oxidative stress. Interactions between Se metabolites and GSH can lead to the formation of ROS such as hydrogen peroxide, and hydroxyl or superoxide radicals (Spallholz, 1994; Spallholz

et al. 2001). Overproduction of these reactive species can overwhelm the antioxidant defense system, leading to an oxidized cellular environment and oxidation of important biomolecules such as DNA, lipids, and proteins. Changes in the expression of genes that code for enzymes relevant to the antioxidant system further reinforce the relationship between oxidative stress and Se induced toxicity. As such, investigating changes in the expression of genes via real time quantitative polymerase chain reaction (RT qPCR) can be used as a biomarker of oxidative stress. In the present study, seven genes relevant to the antioxidant defense system were evaluated, including: nuclear factor erythroid 2-related factor 2 (*nrf2a*), glutathione peroxidase 1a (*gpx1a*), glutathione s-transferase piscine 1 (*gst p1*), glutathione-disulfide reductase (*gsr*), manganese superoxide dismutase (*sod2*), glutamate-cysteine ligase catalytic subunit (*gclc*), and aryl hydrocarbon receptor 2 (*ahr2*).

Previous work has shown that exposure to elevated levels of Se is associated with changes in the expression of oxidant-responsive transcription factors and enzymes (Arnold et al. 2016; Thomas & Janz, 2016). For example, a study involving zebrafish embryos exposed aqueously to different forms of selenium (selenate, selenite, and SeMet) reported a significant increase in glutathione s-transferase piscine 2 (*gst p2)* expression in embryos exposed aqueously to 30 µg/L selenite, while embryos exposed aqueously to 50 and 100 µg/L SeMet showed a significant decrease in *sod2* expression and a significant increase in *gclc* expression, respectively (Arnold et al. 2016). Another study involving zebrafish embryos microinjected with 10 µg Se/g dry mass (d.m.) (as SeMet) reported up-regulation of *nrf2a* at 96 hpf, up-regulation of *gst p1* at 48 hpf, and significantly increased transcript abundance of *ahr2* at 72 hpf. Previous work has suggested that *gpx 1a* is potentially unresponsive to Se exposure, as transcript abundance was not altered in embryos exposed aqueously to selenite or in those receiving SeMet yolk microinjections (Arnold et al. 2016; Thomas & Janz, 2016). In terms of *gsr* expression, limited previous data exists for Se exposures in general; however, one study involving *Caenorhabditis elegans* cultured with 0, 0.05, 0.1, 0.2, and 0.4 mM Se (as selenite) reported no significant changes (< 2-fold changes) in *gsr-1* gene expression (Boehler et al. 2014).

1.5.5.1 Nuclear factor erythroid 2-related factor 2a (*nrf2a*)

Nuclear factor erythroid 2-related factor 2 (Nrf2a), which can be further divided into the genes *nrf2a* and *nrf2b* in fishes, are oxidant-responsive transcription factors with regulatory roles

in the expression of antioxidant genes. Although each gene has been observed to regulate similar sets of genes, *nrf2a* has shown to be primarily an activator of transcription, while *nrf2b* acts primarily as a repressor of gene transcription (Timme-Laragy et al. 2012). Two pathways for ARE activation by Nrf2a have been proposed. The first suggests that, under normal conditions, cytoplasmic Nrf2a is bound to a repressor protein referred to as KEAP1 which contains redox-sensitive cysteines (Zhang & Hannink, 2003; Timme-Laragy et al. 2012). In the presence of oxidative stress, KEAP1 releases from Nrf2a, allowing Nrf2a to enter the nucleus (Timme-Laragy et al. 2012). A subsequent interaction between Nrf2a and the antioxidant response element (ARE) triggers the transcription of ARE-regulated antioxidant proteins such as glutathione S-transferase (GST) (Rushmore et al. 1991). The second pathway is very similar but suggests that the basal/consistent expression of these antioxidant genes by Nrf2a (regardless of redox status) suggests that KEAP1 does not remain bound to Nrf2a in the cytoplasm. Instead, Nrf2a exists alone and only interacts with KEAP1 when it has been targeted for degradation (Nguyen et al. 2009). Nonetheless, transcript abundance of *nrf2a* is a useful biomarker as it should reflect the expression of the genes it activates, with higher levels (as compared to basal levels) indicative of redox imbalance/oxidative stress in the cellular environment.

1.5.5.2 Glutathione peroxidase 1a (*gpx 1a*)

As was mentioned previously, the mammalian glutathione peroxidase (GPx) enzymes serve a protective role in cells as they catalyze the GSH-dependent reduction/degradation of various hydroperoxides (Tapiero et al. 2003). Multiple isoforms of these enzymes have been identified and distinguished based on location of expression in humans (Lobanov et al. 2009). GPx1, which can be further subdivided to GPx1a and GPx1b (genes *gpx 1a* and *gpx 1b*), is thought to be one of the main antioxidant proteins in mammals (Tapiero et al. 2003; Malandrakis et al. 2014). Expressed primarily in the cytoplasm (as well as mitochondria) of every cell type, GPx1 catalyses hydrogen peroxide reduction (Tapiero et al. 2003). As such, altered transcript abundance of *gpx 1a* is a useful biomarker as it is indicative of redox imbalance/oxidative stress in the cellular environment.

1.5.5.3 Glutathione s-transferase piscine 1 (*gst p1*)

Glutathione s-transferase (GST) refers to a family of phase II detoxification enzymes (Kobayashi et al. 2002) that catalyze the conjugation of GSH to harmful/electrophilic

compounds, thereby increasing the solubility and ease of removal by transport proteins (Oakley, 2011). The piscine class of glutathione transferases, encoded by the genes *gst p1* and *gst p2*, are well established downstream products of ARE activation via Nrf2 in zebrafish (Kobayashi et al. 2002; Suzuki et al. 2005). Therefore, altered transcript abundance of *gst p1* is useful not only as another biomarker of redox imbalance/oxidative stress, but a way to confirm if changes in transcript abundance upstream at *nrf2* led to downstream changes in *gst p1* transcript abundance.

1.5.5.4 Glutathione-disulfide reductase (*gsr*)

As discussed previously in section 1.3.1, glutathione reductase is an enzyme in the glutaredoxin system which directly catalyzes the reduction of oxidized glutathione back to reduced glutathione (Michiels et al. 1994). GSH levels, specifically the ratio of reduced glutathione to oxidized glutathione (GSH:GSSG), represents a key component of the antioxidant defense system as well as a useful biomarker for assessing oxidative stress in a system. Consequently, changes in the transcript abundance of *gsr*, which codes for an enzyme that is intimately linked with maintaining GSH ratios, represents another useful biomarker of redox imbalance/oxidative stress.

1.5.5.5 Manganese superoxide dismutase (*sod2*)

The superoxide dismutases (SODs) are a group of enzymes that catalyze the reduction of the superoxide anion radical into hydrogen peroxide and oxygen (Fridovich, 1989). Three versions/isoforms of this enzyme exist in eukaryotes. The first SOD, encoded by the gene *sod1,* contains copper and zinc in its active site (Cu/ZnSOD). It is the predominant SOD as it is expressed in most cells and tissues and accounts for the majority of the total cellular SOD activity. The second SOD, encoded by the gene *sod2*, contains manganese in its active site (MnSOD) and is a key antioxidant enzyme expressed in the mitochondria (Fridovich, 1989; Michiels et al. 1994; Fukui & Zhu, 2010). The third SOD, encoded by the gene *sod3,* also contains copper and zinc in its active site but is expressed extracellularly and is thus termed the extracellular Cu/ZnSOD (EC-SOD). This enzyme is expressed in several tissues, with markedly higher expression in the lungs, liver, and white fat (Ookawara et al. 1998). As with the other radical scavenging enzymes, altered transcript abundance of *sod2* is a useful biomarker of redox imbalance/oxidative stress in the cellular environment.

1.5.5.6 Glutamate-cysteine ligase catalytic subunit (*gclc*)

Glutamate-cysteine ligase (GCL) is a rate-limiting enzyme that catalyzes the first step in GSH synthesis from l-cysteine and glutamate. It is a heterodimeric complex composed of two distinct subunits, the glycine–cysteine ligase catalytic subunit (GCLC) encoded by the gene *gclc*, and the modulatory subunit (GCLM) encoded by the gene *gclm* (Franklin et al. 2009; Jain & Micinski, 2013). As was discussed previously, GSH levels are not only a key component of the antioxidant defense system, but a useful biomarker for redox status. Therefore, the direct relationship between GCL and GSH levels, and thus transcript abundance of GCL's subunit GCLC (*gclc*), represents another useful biomarker of redox imbalance/oxidative stress.

1.5.5.7 Aryl hydrocarbon receptor 2 (*ahr2*)

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor involved with the biotransformation of polycyclic aromatic hydrocarbons (PAHs) as well as a range of other contaminants. Following ligand binding in the cytoplasm, the complex translocates into the nucleus and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This dimer then interacts with xenobiotic response elements (XREs) of numerous genes, resulting in the production of xenobiotic-metabolizing enzymes such as the CYP1 (Cytochrome P450, family 1) and GST enzymes (Timme-Laragy et. al 2012; Kawajiri & Fujii-Kuriyama, 2017). It has been suggested previously that activation of ahr2 might represent another mechanism of Se-induced toxicity, as SeMet yolk microinjections in zebrafish embryos resulted in the up-regulation of ahr2 (Thomas & Janz, 2016). Previous work has also demonstrated that Nrf2a and Nrf2b participate in cross-talk with Ahr2 (Timme-Laragy et. al 2012), and therefore might be related to the changes observed in *ahr2* expression (Thomas & Janz, 2016). Therefore, transcript abundance of *ahr2* should also be evaluated to see if aqueous Se exposures produce similar changes in expression, as well as to see if transcript abundance of *ahr2* does indeed reflect changes in *nrf2a* expression.

1.5.5.8 Actin beta (*actb*) and elongation factor 1 alpha (*ef1a*)

During assessments of gene expression, parallel assessments of control or housekeeping genes serve as experimental procedure controls to catch differences in sample loading or reaction efficiency (Stürzenbaum & Kille, 2001). Housekeeping genes are selected based on two main criteria: first, they serve a pivotal role in cellular maintenance/function and are therefore

expressed ubiquitously in all tissues; and secondly, their expression should be unaffected by the experimental conditions/treatments being investigated and remain consistent across tissues, developmental stages, or test organisms (Stürzenbaum & Kille, 2001; Ruan & Lai, 2007). Although there are many genes that meet these specifications, some of the most commonly used include β-actin (*actb*), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin, cyclophilin, elongation factor 1-α (*ef1α*), ubiquitin, and 18 Svedberg Units (S) rRNA (18S rRNA) (Stürzenbaum & Kille, 2001). In the present study, β-actin (*actb*) and *ef1α* were selected as housekeeping genes due to their ubiquity and key roles in cell function. More specifically, β-actin is one of the major cytoplasmic microfilaments/cytoskeletal components in eukaryotic cells and is involved with cell growth, motility/migration, division, and gene expression (Bunnell et al. 2011), while *ef1α* is directly involved with protein synthesis as it binds aminoacyl-transfer RNA to ribosomes (Stürzenbaum & Kille, 2001).

1.6 Purpose of Research

As the human population continues to grow, so does the need for food, consumer products, electricity, and fuel for transportation. Anthropogenic activities will continue to increase to meet these demands, thus increasing the loading of Se into the environment. The ease with which Se is transferred through diet to reach higher trophic levels is concerning for highly sensitive organisms like oviparous vertebrates; thus, the need to further understand the mechanism behind the observed developmental effects of Se still exists.

Therefore, the purpose of my research is to further investigate the mechanisms of Se toxicity as it relates to the manifestation of larval deformities in fathead minnow and zebrafish embryos exposed aqueously to SeMet during early life stages, as well as critically evaluate the utility of aqueous exposures as a method for determining the effects of SeMet on developing embryos.

1.6.1 Objectives

1. To evaluate the effects of elevated SeMet, in solution, on the hatchability, time to hatch, mortality, and incidence/severity of larval deformities in fathead minnow embryos exposed during early life stages.
2. To further investigate the mechanism of Se toxicity as it relates to oxidative stress by characterizing the effects of waterborne SeMet and tert-butyl hydroperoxide (tBOOH)

exposure, following tert-butyl hydroquinone (tBHQ) pre-treatment, on hatchability, time to hatch, mortality, and the incidence/severity of larval deformities in zebrafish embryos exposed during early life stages.

3. To further investigate the mechanism of Se toxicity as it relates to oxidative stress by characterizing the effects of waterborne SeMet and tert-butyl hydroperoxide (tBOOH) exposure, following tert-butyl hydroquinone (tBHQ) pre-treatment, on the expression of seven antioxidant system-related genes (*nrf2a*, *gpx 1a*, *gst p1*, *gsr*, *sod2*, *gclc*, and *ahr2*) in zebrafish embryos exposed during early life stages.

1.6.2 Hypotheses

1. H_0: Exposure to elevated SeMet, in solution, will have no statistically significant effect on hatchability, time to hatch, mortality, or the incidence/severity of larval deformities in fathead minnow embryos exposed during early life stages.
2. H_0: Exposure to waterborne SeMet and tert-butyl hydroperoxide (tBOOH), following tert-butyl hydroquinone (tBHQ) pre-treatment, will have no statistically significant effect on hatchability, time to hatch, mortality, or the incidence/severity of larval deformities in zebrafish embryos exposed during early life stages.
3. H_0: Exposure to waterborne SeMet and tert-butyl hydroperoxide (tBOOH), following tert-butyl hydroquinone (tBHQ) pre-treatment, will have no statistically significant effect on the expression of *nrf2a*, *gpx 1a*, *gst p1*, *gsr*, *sod2*, *gclc*, and *ahr2* in zebrafish embryos exposed during early life stages.

CHAPTER 2

2.0 TOXICITY OF AQUEOUS L-SELENOMETHIONINE EXPOSURE TO EARLY LIFE-STAGES OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

Preface

The purpose of this research was to characterize the effects of aqueous L-selenomethionine exposures to early life-stages of the fathead minnow (*Pimephales promelas*). Endpoints such as mortality, hatchability, and time to hatch were quantified over six days of exposure, with an extensive deformity analysis of 6-day post fertilization larvae carried out afterwards. This experiment allowed for the generation of an LC_{50} value and data related to the incidence and severity of physical malformations induced by aqueous L-selenomethionine exposures in *Pimephales promelas.*

 The proper citation for this article is:

Gerhart AK, Hecker M, and Janz DM. 2019. Toxicity of Aqueous L -Selenomethionine Exposure to Early Life-Stages of the Fathead Minnow (*Pimephales promelas*). *Bulletin of Environmental Contamination and Toxicology*. 102(3), 323-328. DOI: 10.1007/s00128-018-02537-2

The author contributions to chapter 2 of this thesis were as follows:

Allyson K. Gerhart (University of Saskatchewan) collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript.

David M. Janz (University of Saskatchewan) provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

Markus Hecker (University of Saskatchewan) provided scientific input; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

2.1 Abstract

Aqueous exposures to selenomethionine (SeMet), the major form of selenium (Se) in the diet, represent a rapid and simplified method for determining the embryotoxic effects of SeMet. Using fathead minnows (*Pimephales promelas*) as a model test organism, the objective of this study was to evaluate the effects of waterborne exposure to elevated SeMet on embryos from fertilization to swim-up. Newly fertilized embryos were exposed for 6 days to 30, 90, 270, 810, 2430, 7290, 21870, and 65610 µg Se/L (as SeMet). Survival, hatchability, time to hatch, and the frequency and severity of deformities (total and type) were quantified. SeMet exposure reduced hatchability and time to hatch at concentrations ≥ 21870 µg/L. Significant decreases in survival and significant increases in the incidence and severity of deformities were observed at concentrations ≥ 810 µg/L. The results suggest that early life-stage fathead minnows are more tolerant to aqueous exposure to SeMet compared to medaka and zebrafish.

2.2 Introduction

Selenium (Se) is an important micronutrient in all vertebrate organisms due to its involvement in the synthesis of select proteins and enzymes involved in redox metabolism (Young et al. 2010). However, its narrow range between essentiality and toxicity has generated concern as certain industrial activities have exacerbated the loading of Se into the aquatic environment (Lemly, 2004). Oviparous vertebrates such as amphibians, reptiles, birds, and fishes are particularly susceptible to elevated dietary Se concentrations during early life stages, as Se is readily transferred from mother to embryo through egg yolk proteins (Janz et al. 2010). Exposure to selenomethionine (SeMet), the predominant form of Se in the diet, during sensitive developmental stages can result in teratogenic effects such as spinal deformities (lordosis, kyphosis, scoliosis), missing or misshapen fins, craniofacial malformations, as well as an increase in the incidence of edema (craniofacial, yolk sac, pericardial) and mortality in developing fish larvae (Lemly, 1997; Janz, 2012). Deformities are ecotoxicologically relevant as they can impair recruitment, diminishing population size and diversity over time (Lemly, 2002).

Previous studies addressing the embryotoxic effects of Se have largely consisted of maternal dietary exposures (Janz et al. 2010), with more recent studies using yolk microinjections (Thomas & Janz, 2016) and aqueous embryo exposures (Lavado et al. 2012; Arnold et al. 2016; Kupsco & Schlenk, 2016a,b). Since previous research involving aqueous

embryo exposures to Se utilized Japanese medaka (*Oryzias latipes)* and zebrafish (*Danio rerio)* as model test organisms, similar work on fish species native to North America has received little attention. Using fathead minnows (*Pimephales promelas*) as a model test organism, the purpose of this study was to evaluate the effects of elevated SeMet, in solution, on the hatchability, mortality, and incidence/severity of deformities in developing fathead minnow embryos exposed during early life stages.

2.3 Materials and methods

2.3.1 Test compounds

Seleno-L-methionine (SeMet; ≥ 98% purity) was purchased from Sigma-Aldrich (Oakville, ON, Canada)

2.3.2 Test species

Newly fertilized fathead minnow embryos were collected from an in house adult breeding colony that was maintained in an environmental chamber under controlled temperature (25 +/- 1°C) and photoperiod (16:8 light:dark) in the Aquatic Toxicology Research Facility (ATRF) at the Toxicology Centre, University of Saskatchewan (Saskatoon, Canada).

2.3.3 Aqueous selenomethionine embryo exposures

Healthy embryos between stage 5-10 (within 4.5h of fertilization) of embryonic development (US EPA, 1996) were selected and randomly distributed into glass petri dishes containing 40 mL of test solution. Each individual petri dish contained 20-30 embryos, and there were 3-6 replicate petri dishes in each exposure group. Exposure proceeded for 6 days in E3 Embryo Medium (Cold Spring Harbor Protocols, 2012) alone (control embryos) and E3 Embryo Medium spiked with nominal SeMet concentrations of 30, 90, 270, 810, 2430, 7290, 21870, and 65610 µg Se/L. Dead embryos were removed, and 75% solution changes were performed daily. Following the 6-day exposure, remaining live embryos in each petri dish were euthanized with an overdose of buffered tricaine methanesulfonate (MS-222; 250 mg/L, pH 7.4) (Sigma-Aldrich, Oakville, ON, Canada), fixed in buffered 10% formalin for 18-24h, and stored in 70% ethanol. The preserved larvae were then examined for malformations in a blind fashion using an Olympus model S261 dissecting microscope (Olympus, Melville, NY, USA).

2.3.4 Deformities analysis

In the present study, frequency analysis and the Graduated Severity Index (GSI) were used to quantify deformities in fry (Holm et al. 2003) (Figure C2.S1). During frequency analysis, larvae in each replicate were individually assessed and categorized as either deformed or not deformed. An overall incidence of deformities was thus calculated by dividing the number of individuals classified as deformed by the total number of individuals in that replicate. This process was then repeated for abnormalities in four distinct categories: skeletal curvatures (kyphosis, lordosis, and scoliosis), craniofacial, finfold, and edema.

The severity of abnormalities was also assessed using severity scores adapted from a graduated severity index system described previously (Holm et al. 2003). Severity scores ranged from 0-2, with a score of 0 representing normal; 1 - moderate; and 2 – severe. Total GSI, which describes the overall severity of deformities in each treatment, was calculated by summing the severity score assigned to each category (kyphosis, lordosis, scoliosis, craniofacial, finfold, and edema) for each individual fry in the replicate. The sums calculated for each fry were then averaged to obtain an overall severity for each replicate. In addition to total GSI, the severity of each distinct type of deformity was calculated by averaging the severity scores assigned to each fry in the category of interest in each replicate.

2.3.5 Total selenium analysis

Stock solution samples were filtered and acidified with 2% high purity nitric acid (Fisher Scientific, Hampton, NH, USA) for total Se analysis. Pooled larvae collected for total Se analysis were euthanized with MS-222, collected in microcentrifuge tubes, and freeze dried. Dried samples were then digested using high purity nitric acid and 30% hydrogen peroxide. Inductively coupled plasma-mass spectrometry (ICP-MS) was used to quantify total Se in both water and tissue samples as described previously (Thomas & Janz, 2016). Instrument performance was verified using the standard reference material 1640a solution (National Institute of Standards and Technology), with recoveries of 102% (SD; ± 2.65%) and 104% (SD; ± 3.46%) for water samples and 92.5% (SD; ± 1.92%) for tissue samples. The tissue digestion process was verified using a certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada) for which a recovery of 97.8% (SD; ± 2.05%) was obtained. Instrument detection

limit differed with each run, with a detection limit of 0.029 – 0.164 µg/L for water samples and 0.026 mg/kg for tissue.

2.3.6 Statistical analysis

All results were reported as mean ± standard error, and all analyses were performed using GraphPad Prism Version 7.03 (GraphPad Software: La Jolla, CA, USA). All data were tested for normality and homogeneity of variance using the Shapiro-Wilk normality test and either the Browne-Forsythe test or Bartlett's test as appropriate. When data distribution was normal and exhibited homogeneity of variance, one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to test for significant differences between treatments and the control group. When data distribution was non-normal, or data could not be satisfactorily transformed, a non-parametric Kruskal–Wallis one-way ANOVA by ranks was used followed by Dunn's multiple comparisons post hoc test. Alpha values were two-tailed and set at 0.05. A nonlinear regression, dose-response analysis without constraints was performed on the percent cumulative mortality data to determine the LC_{50} for SeMet exposure.

2.4 Results

2.4.1 Total selenium in stock solutions and tissue

Total Se was quantified in pooled embryos following 6 days of aqueous exposure at concentrations of 810, 2430, 7290, and 21870 µg/L. Total Se in control embryos was 2.26 µg Se/g d.m., while embryos treated aqueously with 810, 2430, 7290, and 21870 µg Se/L exhibited tissue concentrations of 90.68, 53.40, 63.68, and 88.66 µg/g d.m., respectively, indicating bioaccumulation in larvae, although not in a clear exposure concentration-dependent manner. To verify exposure concentrations, test solutions were analyzed for total Se using ICP-MS. Measured concentrations were all in agreement with nominal concentrations (Table 2.1).

Table 2.1 Total Se concentration in test solutions (mean ± SE, n = 2-6) and pooled (51-141 larvae; n = 1) *Pimephales promelas* larvae (6 days post fertilization).

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Tissue Concentration (µg/g dry mass)
Control	0.14 ± 0.02	2.26
30	27.9 ± 0.76	-
90	85.6 ± 5.1	-
270	250.1 ± 5.5	-

810	807.3 ± 25	90.68
2430	2394 ± 83	53.40
7290	7297 ± 406	63.68
21870	23013 ± 980	88.66
65610	63741 ± 8100	-

"-" indicates either missing data or insufficient tissue weight for total Se analysis.

2.4.2 Mortality

SeMet exposures ≥ 810 µg/L significantly decreased embryo survival, with percentages of 22.3 ± 7.6%, 28.7 ± 9.9%, 47.3 ± 9.0%, and 95.8 ± 2.2% (p = 0.0141, 0.0178, < 0.0001, and < 0.0001, respectively) compared to 2.50 ± 1.5% in the controls (Figure C2.S2). An LC_{50} value of 17763 µg/L (95% confidence interval of 9761 – 42503 µg/L) was calculated for SeMet-induced mortality (Figure 2.1).

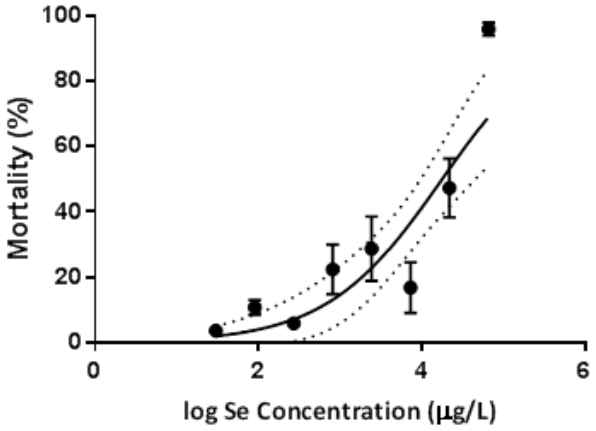


Figure 2.1 Dose-response curve illustrating mean (±SE) percent cumulative mortality (1–6 dpf) of fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Dotted lines represent the 95% confidence interval (CI), and error bars are standard error. Calculated LC_{50} = 17763 µg/L (95% CI = 9761 – 42503 µg/L).

2.4.3 Frequency of deformities

The percentage of total deformities increased significantly at concentrations ≥ 810 µg SeMet/L, with percentages of 67.7 ± 12% and 79.9 ± 3.4% (p = 0.0277 and 0.0102, respectively) compared to 34.1 ± 4.3% in the controls (Figure C2.S3).

In addition to total deformities, the percentage of edema, craniofacial and fin malformations, as well as spinal curvatures in the form of kyphosis, lordosis, and scoliosis were

also calculated (Figure 2.2). The incidence of edema increased significantly, and in a concentration-dependent manner, at concentrations ≥ 810 µg/L, with percentage malformations ranging between 50.9 ± 14% and 73.0 ± 2.9% ($p < 0.0209$, 0.0152, 0.0294, and 0.0020, respectively) compared to 14.4 ± 4.6% in the controls. Fin malformations increased significantly at 810 and 21870 µg/L, with percentages of 27.8 ± 6.2% and 38.7 ± 0.89% ($p = 0.0214$ and 0.0073, respectively) compared to 6.32 ± 3.7% in the controls. For spinal deformities, the percentage of scoliosis increased significantly at 810 and 2430 µg/L, with percentages of 18.8 ± 3.6% and 20.5 ± 7.1% ($p = 0.0224$ and 0.0137, respectively) compared to 2.82 ± 1.3% in the controls.

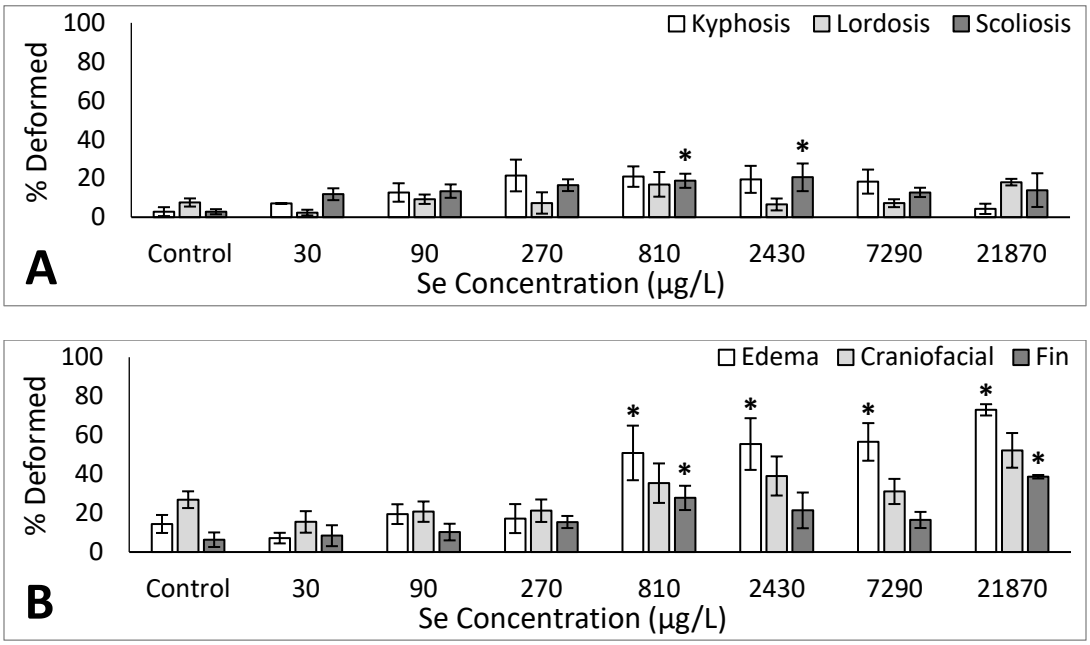


Figure 2.2 Mean (±SE) percentage of (A) skeletal deformities (kyphosis, lordosis, and scoliosis) and (B) other deformities (craniofacial, finfold, and edema) in larval fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via aqueous exposure. Asterisks represent significant differences compared to the control using either a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test, or a one-way ANOVA followed by a Dunnett's multiple comparisons test ($p < 0.05$); n = 3-6 replicates of 20-30 embryos.

2.4.4 Severity of deformities

In addition to frequency, the severity of deformities was also evaluated. Although relatively uncommon in the literature, a total GSI score was calculated to reflect the overall severity of deformities in each treatment. At 810, 2430, and 21870 µg/L a significant increase in the severity of deformities was observed, with total GSI scores of 2.40 ± 0.51, 2.27 ± 0.73, and 2.85 ± 0.06 (p = 0.0150, 0.0378, and 0.0094, respectively) compared to 0.77 ± 0.14 in the control larvae (Figure C2.S4).

In addition to total GSI, the severity of individual categories was also assessed (Figure 2.3). At 810, 2430, and 21870 µg/L, the edema index increased significantly, with GSI scores of 0.63 ± 0.17, 0.69 ± 0.19, and 0.95 ± 0.07 (p = 0.0431, 0.0229, and 0.0016, respectively) compared to 0.20 ± 0.07 in the control larvae. The severity of fin malformations increased significantly at 810 and 21870 µg/L, with GSI scores of 0.44 ± 0.11 and 0.55 ± 0.09 (p = 0.0066 and 0.0032, respectively) compared to 0.06 ± 0.04 in the controls. For spinal deformities, the severity of kyphosis increased significantly at 810 µg/L, with a GSI score of 0.31 ± 0.09 (p = 0.036) compared to 0.03 ± 0.02 in the controls. Scoliosis increased significantly in severity at 270, 810, and 2430 µg/L, with GSI scores of 0.27 ± 0.06, 0.34 ± 0.07 and 0.34 ± 0.11 (p = 0.036, 0.0061, and 0.0073, respectively) compared to 0.03 ± 0.01 in the controls.

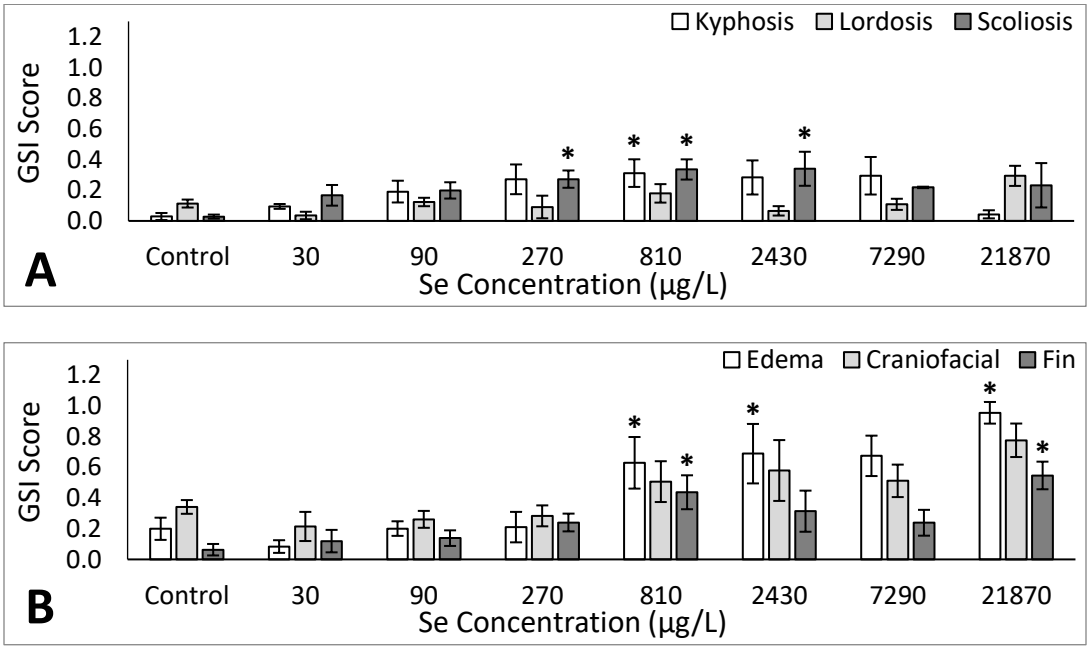


Figure 2.3 Mean (±SE) graduated severity index (GSI) scores of (A) skeletal deformities (kyphosis, lordosis, and scoliosis) and (B) other deformities (craniofacial, finfold, and edema) in larval fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via aqueous exposure. Asterisks represent significant differences compared to the control using either a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test, or a one-way ANOVA followed by a Dunnett's multiple comparisons test ($p < 0.05$); n = 3-6 replicates of 20-30 embryos.

2.4.5 Hatchability and time to hatch

With exception of the greatest test concentrations, SeMet exposure had no significant effect on mean time to hatch or percent hatch of fathead minnow larvae (Figures C2.S5 & C2.S6). A significant reduction in mean time to hatch (approximately 3d compared to 4d in the controls) was observed at 65610 µg/L ($p = 0.0194$; Figure C2.S5). In addition, a significant reduction in embryo hatchability was observed at 21870 µg/L and 65610 µg/L, with percentages of 86.5 ± 3.1% and 21.7 ± 4.4% ($p = 0.0389$ and $p < 0.0001$, respectively) compared to 96.6 ± 1.2% in the controls (Figure C2.S6).

2.5 Discussion

In a selenium-contaminated aquatic system, a developing embryo would be exposed to predominantly inorganic species of selenium such as selenite and selenate (Young et al. 2010). However, these species lack biological relevance as the ecologically relevant Se exposure pathway for embryos is maternal transfer of SeMet accumulated through diet (Janz, 2012). As a result, previous research addressing the embryotoxic effects of Se has largely consisted of yolk microinjections or adult feeding/breeding studies; however, these methods tend to be time consuming, labor intensive, and require specific skills and equipment to conduct. As such, aqueous SeMet exposures were selected as they offer a more rapid and simplified method for determining the effects of SeMet on developing embryos.

2.5.1 Total selenium in stock solutions and tissue

Although the chorion has the potential to act as a barrier to xenobiotics during waterborne exposures (Finn, 2007), previous work has suggested that the chorion is water permeable and potentially exhibits some degree of permeability to smaller organic molecules (Alderdice, 1988;

Finn, 2007) such as SeMet. This was consistent with findings in the present study, as embryos displayed increased Se tissue concentrations following aqueous exposure to SeMet.

2.5.2 Mortality

While Arnold et al. (2016) reported a significant elevation in mortality at a concentration of 100 µg SeMet/L in zebrafish, previous research involving aqueous SeMet embryo exposures with Japanese medaka reported significant elevations in mortality at concentrations ≥ 490 µg/L (Kupsco & Schlenk, 2016a,b). Thus, it appears that fathead minnow embryos are equally and less sensitive to aqueous SeMet exposures than medaka and zebrafish, respectively.

2.5.3 Frequency of deformities

Previous aqueous SeMet embryo exposures with Japanese medaka reported a significant increase in the percentage of total deformities at SeMet concentrations ≥ 490 µg/L (Kupsco & Schlenk, 2016a,b). Despite the elevated proportion of deformed individuals in the controls (34.1 ± 4.3%), previous Se research conducted with the same fathead minnow cultures used in the present study reported background rates of 16.6 ± 5.8%, with the rate of deformities ranging from 0 – 29% in the controls (Lane et al. 2017). The elevated and variable rate of deformities observed in control fish is likely related to variations in egg quality (McDonald & Chapman, 2009), which differs across individual eggs as well as eggs collected from different females. Furthermore, previous Se research conducted in field-based settings have found similar deformity rates at reference sites. For example, Holm et al. (2005) found that background rates of deformities (edema, skeletal, finfold, and craniofacial) in rainbow trout (*Oncorhynchus mykiss*) offspring from one of two reference creeks ranged from 7.9% to 42.2%. Similarly, Rudolph et al. (2008) reported background rates of 37.4 ± 3.6% for skeletal deformities in cutthroat trout (*Oncorhynchus clarkii*) fry from a reference lake.

Previous studies have also reported that spinal deformities tend to be the most common type of deformity observed in Se-exposed embryos, with lordosis representing the most prevalent form of spinal deformity (Muscatello et al. 2006; Kupsco & Schlenk, 2016b). However, edema and fin/craniofacial malformations appeared to be more prevalent than spinal deformities in the present study, and lordosis did not appear to be more common than scoliosis or kyphosis. However, it is important to note that factors such as the developmental stage at which exposure was conducted has the potential to influence the specific types of deformities observed in

developing fish larvae. For example, aqueous SeMet exposures conducted with Japanese medaka during stages 9, 17, 25, 29, 34, and 38 of development, which range from the late morula stage (256-512 cell stage) to the spleen development stage (Iwamatsu, 2004) (i.e. between 5-192 h post fertilization), resulted in differing expression of certain types of deformities depending on the stage at which treatment was initiated (Kupsco & Schlenk, 2016b). It is unsurprising then that different types of deformities were observed in the Japanese medaka, as exposures in the present study were conducted on embryos between stages 5-10 (4 cell - high blastula cell stages; i.e. within 4.5h post fertilization) of embryonic development (US EPA, 1996), and therefore earlier stages of development. In addition to developmental stages, water chemistry parameters (pH, hardness, alkalinity, etc.) also differed across studies. Although not completely understood, previous work has suggested that water chemistry might influence embryo permeability (Alderdice, 1988; Finn, 2007). This indicates the need to exercise caution when making direct comparisons to other aqueous SeMet exposure studies with different water chemistry parameters, as these factors might mediate SeMet toxicity.

2.5.4 Severity of deformities

Similar to the frequency analysis results, edema and fin/craniofacial malformations appeared more responsive to SeMet concentration than spinal deformities. However, further work would be required to determine why these types of deformities were more common in fathead minnows exposed aqueously to SeMet compared to other fish species.

2.5.5 Hatchability and time to hatch

Previously, Japanese medaka embryos exposed aqueously to 0.5, 5, and 50 µM (approximately 100, 1000, and 10000 µg/L) SeMet at six different developmental stages reported a significant reduction in the median days to hatch only in embryos exposed with 5 µM SeMet at stage 34 (Kupsco & Schlenk, 2016b); however, significant reductions in hatchability were reported at concentrations ≥ 5 µM SeMet at all stages of development (Kupsco & Schlenk, 2016b). Similarly, Japanese medaka exposed to 0.05 mM (approximately 10000 µg/L) SeMet in freshwater and three different hypersaline conditions reported a significant reduction in hatchability in all treatment groups (Lavado et al. 2012).

2.6 Conclusions

In summary, our results indicate that aqueous exposures to SeMet increased the incidence and severity of deformities, as well as led to the bioaccumulation of Se within embryonic tissue. The production of deformities and adverse effects similar to those observed with other methods of Se exposure (i.e. dietary and yolk microinjections), suggests that aqueous exposures still represent a simplified and rapid method for studying the embryotoxic effects of Se. However, the lack of clear dose-response relationships for both the frequency and severity of larval deformities suggests that the chorion potentially limits uptake of SeMet, and thus reduces exposure. Furthermore, the apparent tolerance of fathead minnow embryos used in this study relative to other aqueously exposed fish species inspires more questions about the role of factors such as developmental pattern and chorion physiology, and how this potentially influences toxicity across fish species.

CHAPTER 3

3.0 TOXICITY OF AQUEOUS L-SELENOMETHIONINE AND TERT-BUTYL HYDROPEROXIDE EXPOSURE TO ZEBRAFISH (*DANIO RERIO*) EMBRYOS FOLLOWING TERT-BUTYL HYDROQUINONE TREATMENT

Preface

The purpose of this research was to characterize the effects of aqueous L-selenomethionine, tert-butyl hydroperoxide, and tert-butyl hydroquinone exposures to early life-stages of the zebrafish (*Danio rerio*) to facilitate direct comparisons to the fathead minnow SeMet exposures in chapter 2, as well as further investigate the relationship between oxidative stress and the production of larval deformities. Endpoints included mortality, hatchability, and time to hatch were quantified over five days of exposure, with an extensive deformity analysis of 5-day post fertilization larvae carried out afterwards. In addition, changes in the expression of seven selected antioxidant-associated genes was also carried out to identify possible indications of oxidative stress. These experiments allowed for the generation of an LC_{50}, an EC_{50} value for deformities, data related to the incidence and severity of teratogenic abnormalities, as well as fold changes in gene expression induced by aqueous L-Selenomethionine, tert-butyl hydroperoxide, and tert-butyl hydroquinone exposures in *Danio rerio.*

 The proper citation for this article is:

Gerhart AK and Janz DM. 2019. Toxicity of Aqueous L-Selenomethionine and Tert-Butyl Hydroperoxide Exposure to Zebrafish (*Danio rerio*) Embryos Following Tert-Butyl Hydroquinone Treatment. *Toxics*, Special Issue: Contaminant Effects on Zebrafish Embryos 7(3), 44. DOI: 10.3390/toxics7030044

The author contributions to chapter 3 of this thesis were as follows:

Allyson K. Gerhart (University of Saskatchewan) collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript.

David M. Janz (University of Saskatchewan) provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

3.1 Abstract

Aqueous L-selenomethionine (SeMet) embryo exposures represent a rapid and simplified method for investigating the embryotoxic effects of SeMet. Using zebrafish (*Danio rerio*) as a model organism, the objective of the present study was to characterize the effects of waterborne exposure to both SeMet and tert-butyl hydroperoxide (tBOOH) to early life-stages of zebrafish pre-treated with the antioxidant tert-butyl hydroquinone (tBHQ) in an attempt to investigate the mechanism of Se toxicity as it relates to oxidative stress. During the initial concentration range finding experiment, recently fertilized embryos were exposed for 5 days to 5, 25, 125, and 625 µg Se/L (as SeMet). These exposures informed the second experiment in which embryos were exposed to two concentrations of SeMet (25 and 125 µg Se/L) and 75 mg/L tBOOH either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a 4h 100 µg/L tBHQ pre-treatment. Survival, hatchability, time to hatch, the frequency and severity of deformities (total and type), and changes in the expression of seven antioxidant-associated genes were determined. Exposures to SeMet and tBOOH reduced hatchability, increased time to hatch, decreased survival, increased the incidence and severity of deformities, and increased glutathione-disulfide reductase (*gsr*) expression in the pre-treated tBOOH treatment group. Overall, this research provided support for the oxidative stress mechanism of Se toxicity as the pro-oxidant tBOOH was able to produce deformities. Furthermore, exposures suggested that early life-stage zebrafish are more sensitive to aqueous exposure to SeMet relative to Japanese medaka and zebrafish.

3.2 Introduction

Zebrafish (*Danio rerio*) represent one of the most widely used small fish models in toxicological research due to beneficial traits such as a short life cycle, frequent egg production, ease of culture, and a transparent embryo chorion which allows for the determination of fertilization status and morphological analysis throughout development (Ankley & Johnson, 2004). However, the transparent chorion, along with the highly detailed accounts of embryonic development and the substantial volume of toxicity data available in the literature for this species, makes zebrafish embryos an ideal candidate for studying the effects of contaminants with known developmental effects such as selenium (Se).

In most vertebrate organisms, Se is an essential micronutrient due to its role in the synthesis of proteins and enzymes involved in redox homeostasis (Young et al. 2010). However,

the narrow range between essentiality and toxicity of Se is particularly concerning to oviparous vertebrates, which exhibit an increased susceptibility to Se during early life stages (Janz, 2012). Exposure to elevated concentrations of selenomethionine (SeMet), the predominant form of Se in the diet, during the sensitive, early life-stages often results in an increased incidence of edema and mortality, as well as teratogenic effects such as spinal/skeletal deformities, misshapen or missing fins, and craniofacial malformations in developing fish larvae (Lemly, 1997; Janz, 2012). These physical abnormalities are ecotoxicologically relevant as they can directly impair the fishes' ability to swim, feed, and reproduce, which will ultimately lead to diminished population size and diversity over time (Lemly, 2002).

While the relationship between Se exposure and the production of deformities is well established, the mechanism behind the manifestation of these physical abnormalities is still unclear and lacks consensus. Two main theories exist regarding the mechanism of Se toxicity, including Se substitution for sulfur in sulfur-containing amino acids/biomolecules, leading to improper protein folding and function, and the overproduction of reactive molecules as a result of SeMet metabolism, leading to an overwhelmed antioxidant defense system and consequently oxidative stress (Janz et al. 2010). However, oxidative stress seems to have received the most attention in the literature (Spallholz, 1994; Spallholz et al. 2001; Palace et al. 2004).

Previous research investigating the embryotoxic effects of Se have mainly involved dietary exposures (Janz et al. 2010), yolk microinjections (Thomas & Janz, 2016), and more recently, aqueous embryo exposures (Lavado et al. 2012; Arnold et al. 2016; Kupsco & Schlenk, 2016a,b; Gerhart et al. 2019). A previous aqueous SeMet embryo exposure study in zebrafish focused on clarifying the relationship between oxidative stress and Se toxicity employed an antioxidant rescue methodology in which a subset of embryos were pre-treated for 24h with the antioxidant N-acetylcysteine (NAC) prior to SeMet exposure (Arnold et al. 2016). Based on this concept, as well as previous research utilizing electrophilic compounds that elicit an oxidative stress response similar to SeMet (i.e. tert-butyl hydroperoxide [tBOOH] and tert-butyl hydroquinone [tBHQ]) (Kobayashi et al. 2002; Timme-Laragy et al. 2012), we attempted to design and conduct aqueous embryo exposures that would further clarify the relationship between oxidative stress and the manifestation of deformities in larval fish. Therefore, tBOOH, which is an organic peroxide, was selected as a positive control as it should induce oxidative stress (Timme-Laragy et al. 2012). Similarly, tBHQ, which is a synthetic, organic antioxidant,

was selected for use as an antioxidant (Kobayashi et al. 2002). Both chemicals have been used previously in research focused on understanding the antioxidant defense system in mammalian cells, as they have been found to trigger an oxidative stress response through activation of Nrf2, which consequently activates the transcription of phase II and antioxidant enzymes (Kobayashi et al. 2002; Timme-Laragy et al. 2012). Therefore, we predicted that pre-treating with tBHQ would activate/prepare the antioxidant defense system prior to exposure to SeMet or tBOOH, and thus serve a protective function.

As such, the objective of this research was to further investigate the mechanism of Se toxicity, as it relates to oxidative stress, by characterizing the effects of waterborne SeMet, tBOOH, and tBHQ, alone and in combination, on the hatchability, mortality, incidence/severity of deformities, and expression of seven genes relevant to the antioxidant defense system in developing zebrafish embryos.

3.3 Materials and methods

3.3.1 Test compounds

Seleno-L-methionine (SeMet; ≥ 98% purity), tert-Butyl hydroquinone (tBHQ; 97% purity), and tert-Butyl hydroperoxide (tBOOH; 70% solution in water) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.2 Test species

Newly fertilized zebrafish embryos were collected from an in house adult breeding colony that was maintained in an environmental chamber under controlled temperature (27 +/- 1°C) and photoperiod (16:8 light:dark) at the Toxicology Centre, University of Saskatchewan (Saskatoon, Canada). Healthy embryos between 4-cell – high blastula stage (i.e. within 3h of fertilization) of embryonic development (Kimmel et al. 1995) were selected to use in experiments. Animal care and all experimentation were conducted in compliance with the University Committee on Animal Care and Supply (UCACS) and was approved by the Animal Research Ethics Board (AREB) (Animal Use Protocol 20030076).

3.3.3 Aqueous selenomethionine dose-response embryo exposures

Embryos were randomly distributed into glass petri dishes containing 40 mL of test solution. Each individual petri dish contained 20 embryos, with 9-12 replicate petri dishes in

each exposure group. Exposure proceeded for 5 days in facility water alone (control embryos) or facility water spiked with nominal SeMet concentrations of 5, 25, 125, and 625 µg Se/L. Dead embryos were removed, and 75% solution changes were performed daily.

3.3.4 Aqueous selenomethionine, pro- and antioxidant embryo exposures

The 100 µg/L concentration of tBHQ was chosen based on preliminary studies as a concentration that did not induce a rate of mortality or deformities greater than that of the control embryos (unpublished data), while 75 mg/L of tBOOH was chosen based on preliminary studies as a concentration that caused a significant increase in the rate of deformities (unpublished data) relative to control embryos. Based on the aqueous SeMet exposures, 25 and 125 µg Se/L were selected for use in the following exposures as concentrations that caused a significant elevation in both mortality and the frequency of deformities relative to control embryos.

Zebrafish embryos were collected and sorted as described previously. Embryos receiving the antioxidant pre-treatment were randomly distributed into plastic petri dishes containing 40 mL of 100 µg/L tBHQ, while embryos not receiving the pre-treatment were randomly distributed into plastic petri dishes containing 40 mL of facility water. Following 4h, all embryos were re-distributed into glass petri dishes containing 40 mL of facility water alone, 75 mg/L tBOOH, 25 µg Se/L, or 125 µg Se/L. This resulted in 8 treatment groups, including: facility water alone (control embryos), 100 µg/L tBHQ (tBHQ control), 75 mg/L of tBOOH with and without antioxidant pre-treatment (denoted as tBOOH-t and tBOOH, respectively), 25 µg Se/L with and without antioxidant pre-treatment (denoted as 25-t and 25, respectively), and 125 µg Se/L with and without antioxidant pre-treatment (denoted as 125-t and 125, respectively). Each individual petri dish contained 20 embryos, with 32-33 control replicates and 13-21 replicates in each exposure group. Exposure proceeded for 5 days, during which dead embryos were removed, and 75% solution changes were performed daily.

Following the 5-day aqueous exposures, remaining live larvae destined for deformities analysis were euthanized with an overdose of buffered MS-222 (250 mg/L, pH 7.0-7.4), fixed in buffered 10% formalin for 18-24h, and stored in 70% ethanol. The preserved larvae were then examined for malformations in a blind fashion using an Olympus model S261 dissecting microscope (Olympus, Melville, NY, USA). Larvae destined for biochemical analysis were frozen at -80°C until analysis.

3.3.5 Deformities analysis

In the present study, frequency analysis and the Graduated Severity Index (GSI) were used to quantify deformities in fry (Holm et al. 2003; Gerhart et al. 2019). During frequency analysis, larvae in each replicate were individually assessed and categorized as either deformed or not deformed. An overall incidence was calculated by dividing the number of individuals classified as deformed by the total number of individuals in that replicate. This process was then repeated for specific types of deformities (kyphosis, scoliosis, craniofacial, finfold, and edema).

The severity of abnormalities was assessed using severity scores ranging from 0-2, with a score of 0 representing normal; 1 - moderate; and 2 – severe. Total GSI, which describes the overall severity of deformities in each treatment, was calculated by summing the severity score assigned to each category (kyphosis, scoliosis, craniofacial, finfold, and edema) for each individual fry in the replicate. The sums calculated for each fry were then averaged to obtain an overall severity for each replicate. In addition to total GSI, the severity of each distinct type of deformity (kyphosis, scoliosis, craniofacial, finfold, and edema) was calculated by averaging the severity scores assigned to each fry in the category of interest in each replicate.

3.3.6 Total selenium analysis

Stock solution samples were filtered and acidified with 2% high purity nitric acid (Fisher Scientific, Hampton, NH, USA) for total Se analysis. Pooled larvae collected for total Se analysis were euthanized with MS-222, collected in microcentrifuge tubes, and freeze dried. Dried samples were then digested using high purity nitric acid and 30% hydrogen peroxide. Inductively coupled plasma-mass spectrometry (ICP-MS) (8800 ICP-MS Triple Quad, Agilent Technologies, Santa Clara, CA, USA) was used to quantify total Se in both water and tissue samples as described previously (Thomas & Janz, 2016). Instrument performance was verified using the standard reference material 1640a solution (National Institute of Standards and Technology). Analysis of the SeMet stock solutions used in the aqueous embryo exposures produced an average percent recovery of 102% (SD; ± 4.2%). Analysis of tissue samples yielded a 1640a recovery of 102% (SD; ± 1.3%). The tissue digestion process was verified using a certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada) for which a recovery of 103% (SD; ± 8.1%) was obtained. Instrument detection limit differed in each run,

with detection limits ranging from 0.006 – 0.102 µg/L for water samples and 0.061 mg/kg for tissue samples.

3.3.7 Gene expression analysis

Four to five replicates of 11-20 zebrafish larvae exposed to the SeMet, pro- and antioxidant treatment combinations described previously were collected in microcentrifuge tubes and stored at - 80°C until analysis. Total RNA was extracted using the RNeasy Plus Universal Mini Kit (QIAGEN Inc. Toronto, ON, Canada) according to the manufacturer's protocol, quantified using the QIAxpert® microfluidic UV/VIS Spectrophotometer (QIAGEN Inc. Toronto, ON, Canada), and stored at -80°C until analyzed. First-strand cDNA synthesis was performed using the QuantiNova™ Reverse Transcription Kit (QIAGEN) according to the manufacturer's protocol using 2.25µg of total RNA. The cDNA samples were stored at -20°C until analyzed. Quantitative Real-Time PCR was performed in optical, fast, clear 96-well plates using the PowerUp™ SYBR™ Green Master Mix qPCR Kit (Applied Biosystems, Foster City, CA, USA) and the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems).

A 25µL reaction mixture containing PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), an optimized concentration of complementary DNA (cDNA; 100ng) and gene-specific qPCR primers (0.6 µmol), as well as nuclease free water was used for each sample and primer combination. The PCR reaction mixture was incubated/denatured at 50°C for 2 min, then 95°C for 2 min before the first PCR cycle. The thermal cycle profile consisted of denaturing at 95 °C for 1 s and extension at 60°C for 30 s for a total of 40 PCR cycles. This was immediately followed by a dissociation curve. Each reaction was conducted in duplicate. Target gene primer sequences, accession numbers, and efficiencies for nuclear factor erythroid 2-related factor 2a (*nrf2a*), glutathione peroxidase 1a (*gpx 1a*), glutathione s-transferase piscine 1 (*gst p1*), glutathione-disulfide reductase (*gsr*), manganese superoxide dismutase (*sod2*), glutamate-cysteine ligase catalytic subunit (*gclc*), aryl hydrocarbon receptor 2 (*ahr2*), actin beta (*actb*), and elongation factor 1 alpha (*ef1α*) are shown in Table C3.S1. Quantitative real-time PCR data was analyzed and quantified using the QuantStudio™ Real-Time PCR Software v1.3 (Applied Biosystems). Relative changes in target gene expression was determined using the $2^{-\Delta\Delta CT}$ method described by Applied Biosystems (Guide: cms_042380), with *actb* and *ef1α* serving as reference genes. A slight deviation from the suggested calculations involved the use of the control group's

mean ΔC_T to calculate a $\Delta\Delta C_T$ value and fold change value for each replicate as opposed to each treatment group, allowing for the calculation of error terms.

3.3.8 Statistical analysis

All results were reported as mean ± standard error, and all analyses were performed using GraphPad Prism Version 8.1.2 (GraphPad Software: La Jolla, CA, USA). All data were tested for normality and homogeneity of variance using the Shapiro-Wilk normality test and either the Browne-Forsythe test or Bartlett's test as appropriate. When data distribution was normal and exhibited homogeneity of variance, one-way analysis of variance (ANOVA) followed by Holm-Sidak's multiple comparisons test was used to test for significant differences between treatment groups. When data distribution was non-normal, or data could not be satisfactorily transformed, a non-parametric Kruskal–Wallis one-way ANOVA by ranks was used followed by Dunn's multiple comparisons test. Both multiple comparisons tests provided an option to pre-select specific treatment group comparisons. In the aqueous selenomethionine concentration-response embryo exposures, each treatment group was compared to the control. In the aqueous selenomethionine, pro- and antioxidant embryo exposures, each treatment group was compared to the control and each pre-treated and non-pretreated combination was compared to determine if tBHQ pre-treatment had an effect (ex. tBOOH-t vs. tBOOH, 25-t vs. 25, 125-t vs. 125). Alpha values were two-tailed and set at 0.05. A nonlinear regression, dose-response analysis without constraints was performed on the percent cumulative mortality and deformities data to determine an LC_{50} and EC_{50} following SeMet exposure.

3.4 Results

3.4.1 Aqueous selenomethionine dose-response embryo exposures

3.4.1.1 Total selenium in stock solutions and tissue

Total Se quantified in pooled larvae following 5 days of aqueous exposure at concentrations of 5, 25, 125, and 625 µg Se/L had mean total Se tissue concentrations of 34.2, 144, 264, and 291 µg Se/g d.m., respectively, compared to 2.98 µg/g d.m. in control embryos, indicating bioaccumulation in larvae in a clear concentration-dependent manner. To verify exposure concentrations, test solutions were analyzed for total Se using ICP-MS (Table 3.1). Measured concentrations were all in agreement with nominal concentrations.

Table 3.1. Total Se concentration in test solutions (mean ± SE, n = 9-10) and pooled (35-180 larvae; n = 3) *Danio rerio* larvae (5 days post fertilization).

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Tissue Concentration (µg/g dry mass)
Control	0.351 ± 0.026	2.98 ± 0.073
5	5.13 ± 0.30	34.2 ± 3.5
25	24.8 ± 0.41	144 ± 26
125	124 ± 0.81	264 ± 38
625	615 ± 6.4	291 ± 152*

* = Due to the high rate of mortality, tissue mass only allowed for an n = 2

3.4.1.2 Mortality

SeMet exposures ≥ 25 µg/L significantly decreased embryo survival, with percentages of 39.6% ± 7.6%, 52.9% ± 10.1%, and 95.6% ± 3.4% ($p \leq 0.024$), compared to 2.50% ± 1.2% in the controls. An LC_{50} value of 67.9 µg/L (95% CI = 43.4 – 108µg/L) was calculated for SeMet-induced mortality (Figure 3.1). From a time-course perspective, embryo mortality started to increase on the third day, reached a maximum on the 4th day, and was usually followed by a slight decrease on the 5th day of exposure.

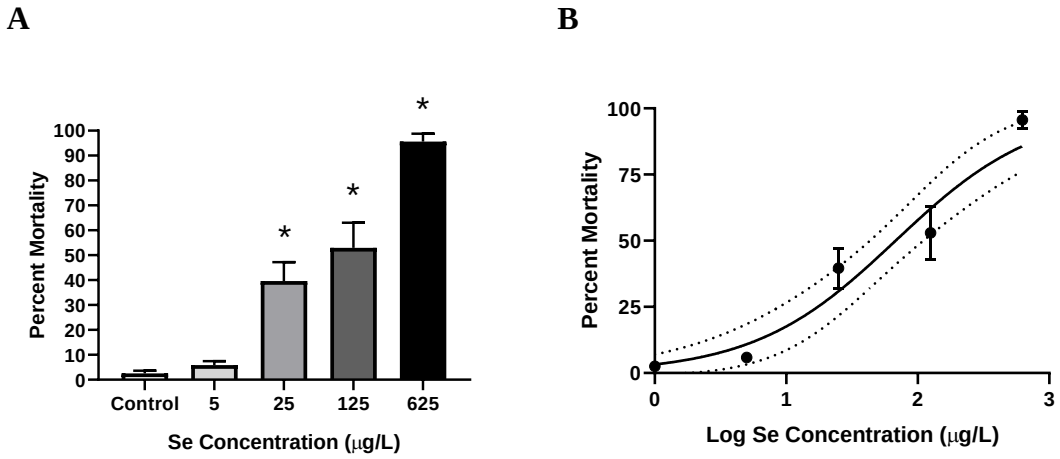


Figure 3.1. Mean (± SE) percent cumulative mortality (1–5 dpf) of zebrafish exposed to increasing concentrations of SeMet via embryo aqueous exposure. (A) Bar graph, asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 8-12 replicates of 20 embryos. (B) Dose response curve used to calculate the LC_{50} value of 67.9 µg/L (95% CI = 43.4 – 108µg/L). Dotted lines represent the 95% confidence interval.

3.4.1.3 Frequency of deformities

Representative images of the deformities observed in this study can be found in Figure C3.S1. SeMet exposures ≥ 25 µg/L significantly increased the percentage of total deformities, with percentages of 44.7% ± 7.8% (p = 0.019) and 62.3% ± 14% ($p \leq 0.0185$) compared to 15.2% ± 2.9% in the controls. An EC_{50} value of 43.8 µg/L (95% CI = 21.4 - 138µg/L) was also calculated for SeMet-induced deformities (Figure 3.2).

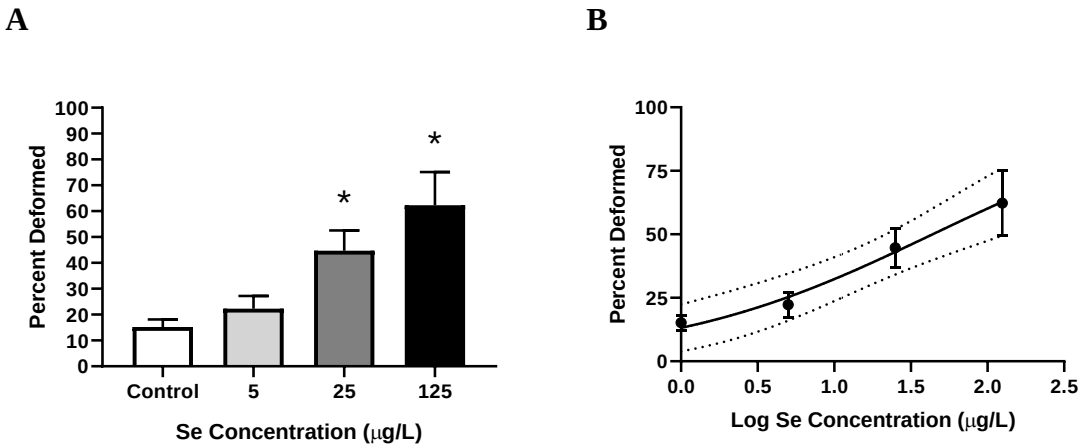


Figure 3.2. Mean (±SE) percentage of total deformities (sum of skeletal, craniofacial, finfold, and edema) in larval zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. (A) Bar graph, asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 9-12 replicates of 20 embryos. (B) Dose response curve used to calculate the EC_{50} value of 43.8 µg/L (95% CI = 21.4 - 138µg/L). Dotted lines represent the 95% confidence interval.

In addition to total deformities, the incidence of edema, craniofacial and fin malformations, as well as spinal curvatures (kyphosis and scoliosis) were also calculated. While exposure to SeMet had no significant effect on the incidence of skeletal deformities in zebrafish larvae, the incidence of edema, fin, and craniofacial malformations increased in a concentration-dependent manner, with significant elevations observed at concentrations ≥ 25 µg/L ($p \leq$ 0.0098). The incidence of edema, craniofacial and fin malformations in the 25 and 125 µg/L treatments were 25.9% ± 7.2% and 39.4% ± 9.4% compared to 0.417% ± 0.42% in the controls;

28.3% ± 8.2% and 30.7% ± 7.2% compared to 3.38% ± 1.4% in the controls; and 31.9% ± 6.3% and 55.1% ± 16% compared to 7.15% ± 2.2% in the controls, respectively (Figure C3.S2).

3.4.1.4 Severity of deformities

In addition to frequency, the severity of deformities was also evaluated. At concentrations ≥ 25 µg/L a significant increase in the severity of deformities was observed, with total GSI scores of 1.80 ± 0.42 and 2.36 ± 0.72 in 25 and 125 µg/L, respectively ($p \leq 0.0036$), compared to 0.237 ± 0.056 in the control larvae (Figure 3.3).

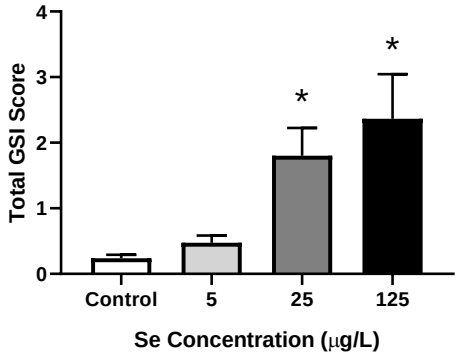


Figure 3.3. Mean (±SE) graduated severity index (GSI) scores for total deformities (sum of skeletal, craniofacial, finfold, and edema scores) in larval zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences compared to the control using a one-way ANOVA followed by a Dunnett's multiple comparisons test ($p < 0.05$); n = 9-12 replicates of 20 embryos.

The severity of individual deformity categories was also assessed. While the severity of skeletal deformities did not significantly differ following exposure, the severity of edema, fin, and craniofacial malformations increased in a concentration-dependent manner, with significant elevations observed at concentrations ≥ 25 µg/L ($p \leq 0.0095$). The severity/GSI scores for edema, craniofacial and fin malformations in the 25 and 125 µg/L treatments were: 0.383 ± 0.11 and 0.460 ± 0.11 compared to 0.004 ± 0.004 in the controls; 0.332 ± 0.082 and 0.307 ± 0.072 compared to 0.0338 ± 0.014 in the controls; and 0.472 ± 0.10 and 0.743 ± 0.23 compared to 0.0882 ± 0.030 in the controls, respectively (Figure C3.S3).

3.4.1.5 Hatchability and time to hatch

SeMet exposure had no significant effect on mean time to hatch (Figure C3.S4); however, a significant reduction in embryo hatchability was observed at concentrations ≥ 25 µg/L, with percentages of 61.3% ± 8.7%, 60.0% ± 8.1%, and 24.4% ± 8.0% ($p \leq 0.0073$) compared to 97.5% ± 1.2% in the controls (Figure C3.S5).

3.4.2 Aqueous selenomethionine, pro- and antioxidant embryo exposures

3.4.2.1 Mortality

Embryos exposed to all SeMet treatments exhibited a significant increase in mortality compared to control embryos ($p < 0.0001$), with percentages of 46.5% ± 7.2%, 50.5% ± 6.6%, 54.5% ± 5.9%, and 73.1% ± 5.0% in the 25-t, 25, 125-t, and 125 treatments, respectively, compared to 4.85% ± 0.74% in the controls. However, no differences were observed between embryos pre-treated with tBHQ and embryos exposed to SeMet alone (Figure 3.4).

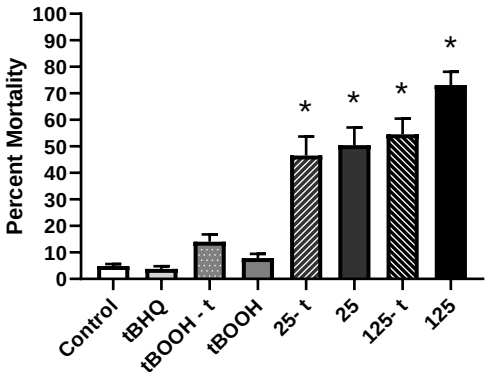


Figure 3.4. Mean (± SE) percent cumulative mortality (1–5 dpf) of zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 20-33 replicates of 20 embryos.

3.4.2.2 Frequency of deformities

A significant increase in the proportion of deformities was observed in all treatment groups ($p < 0.0012$), with percentages of 6.86% ± 2.2%, 32.9% ± 4.9%, 31.9% ± 4.3%, 39.1% ±

5.1%, 37.1% ± 5.6%, 36.2% ± 8.0%, and 48.1% ± 9.4% in the tBOOH-t, tBOOH, 25-t, 25, 125-t, and 125 treatments respectively, compared to 5.30% ± 1.5% in the controls. No significant differences were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone (Figure 3.5).

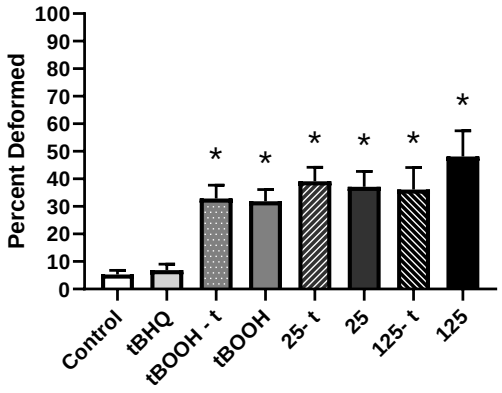


Figure 3.5. Mean (±SE) percentage of total deformities (sum of skeletal, craniofacial, finfold, and edema) in larval zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 13-33 replicates of 20 embryos.

The incidence of kyphosis increased significantly within all SeMet treatment groups ($p \leq 0.045$), with percentages of 7.36% ± 2.9%, 8.62% ± 3.3%, 12.8% ± 5.2%, and 25.6% ± 9.3% in the 25-t, 25, 125-t, and 125 treatments, respectively, compared to 0.479% ± 0.27% in the controls. Scoliosis only showed a significant increase in frequency in the pre-treated 25 µg/L group ($p = 0.0072$), with a percentage of 15.1% ± 3.5% compared to 3.71% ± 1.4% in the controls. The incidence of edema increased significantly in all treatment groups ($p \leq 0.025$), with percentages of 15.5% ± 4.6%, 21.3% ± 4.4%, 26.3% ± 5.2%, 34.7% ± 5.6%, 24.0% ± 6.2%, and 30.6% ± 8.4% in the tBOOH-t, tBOOH, 25-t, 25, 125-t, and 125 treatments, respectively, compared to 0.470% ± 0.26% in the controls. The incidence of fin malformations increased significantly in the pre-treated tBOOH group and within all SeMet treatment groups compared to the controls ($p \leq 0.017$), with percentages of 14.8% ± 3.1%, 17.9% ± 5.7%, 17.0% ± 3.6%, 14.4% ± 4.1%, and 27.0% ± 8.0% in the tBOOH-t, 25-t, 25, 125-t, and 125 treatments, respectively, compared to 1.58% ± 0.62% in the

controls. Finally, craniofacial malformations increased significantly in frequency in both 25 µg/L SeMet treatment groups ($p \leq 0.022$), with percentages of 11.2% ± 3.9% and 11.0% ± 3.2% in the 25-t and 25 treatments, respectively, compared to 0.614% ± 0.36% in the controls. No significant differences were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone (Figure C3.S6).

3.4.2.3 Severity of deformities

Exposure to all treatment groups resulted in a significant increase in the severity of deformities as compared to control larvae ($p \leq 0.0010$), with severity/GSI scores of 0.470 ± 0.086, 0.417 ± 0.062, 0.709 ± 0.17, 0.875 ± 0.16, 0.710 ± 0.19, and 1.32 ± 0.41 in the tBOOH-t, tBOOH, 25-t, 25, 125-t, and 125 treatments, respectively, compared to 0.0766 ± 0.024 in the controls. However, no significant differences were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone (Figure 3.6).

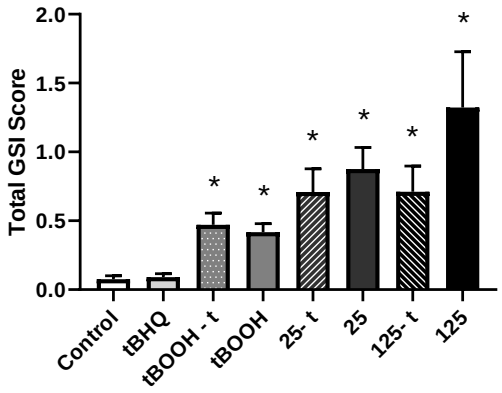


Figure 3.6. Mean (±SE) graduated severity index (GSI) scores for total deformities (sum of skeletal, craniofacial, finfold, and edema scores) in larval zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 13-33 replicates of 20 embryos.

The severity of kyphosis increased significantly in the 25 µg/L group as well as both 125 µg/L treatment groups ($p \leq 0.037$), with severity/GSI scores of 0.0991 ± 0.035, 0.142 ± 0.053,

and 0.340 ± 0.13 in the 25, 125-t, and 125 treatments, respectively, compared to 0.0048 ± 0.003 in the controls. The severity of scoliosis only increased significantly in the pre-treated 25 µg/L group ($p \leq 0.020$), with a severity/GSI score of 0.162 ± 0.038 compared to 0.0422 ± 0.015 in the controls. Exposure to all treatment groups resulted in a significant increase in the severity of edema ($p \leq 0.030$), with severity/GSI scores of 0.155 ± 0.046, 0.213 ± 0.044, 0.229 ± 0.044, 0.375 ± 0.061, 0.240 ± 0.062, and 0.337 ± 0.092 in the tBOOH-t, tBOOH, 25-t, 25, 125-t, and 125 treatments, respectively, compared to 0.0062 ± 0.004 in the controls. In contrast, the severity of craniofacial malformations increased significantly only in the 25 µg/L group ($p \leq 0.0080$), with severity/GSI score of 0.110 ± 0.032 compared to 0.0077 ± 0.005 in the controls. Finally, the severity of fin malformations increased significantly within the pre-treated tBOOH group, 25 µg/L group, and both 125 µg/L treatment groups ($p \leq 0.014$), with severity/GSI scores of 0.151 ± 0.031, 0.205 ± 0.052, 0.178 ± 0.063, and 0.351 ± 0.12 in the tBOOH-t, 25, 125-t, and 125 treatments, respectively, compared to 0.0158 ± 0.0062 in the controls. No significant differences were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone (Figure C3.S7).

3.4.2.4 Hatchability and time to hatch

Significant increases in the mean time to hatch were observed between control embryos and those in both tBOOH treatment groups, as well as the pre-treated 25 µg/L group and 125 µg/L group ($p \leq 0.045$), with mean time to hatch values of 3.90 ± 0.13 days, 3.79 ± 0.11 days, 3.66 ± 0.11 days, and 3.58 ± 0.083 days in the tBOOH-t, tBOOH, 25-t, and 125 treatments, respectively, compared to 3.29 ± 0.045 days in the controls (Figure C3.S8). Furthermore, exposure to all treatment groups resulted in a significant reduction in hatchability relative to control embryos ($p \leq 0.078$), with percentages of 40.7% ± 5.8%, 70.7% ± 5.5%, 51.0% ± 7.3%, 68.1% ± 5.2%, 47.0% ± 6.5%, and 38.6% ± 4.4% in the tBOOH-t, tBOOH, 25-t, 25, 125-t, and 125 treatments, respectively, compared to 95.2% ± 0.74% in the controls. No significant differences in time to hatch or hatchability were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone (Figure C3.S9).

3.4.2.5 Gene expression

Aside from a significant 6.96-fold increase in *gsr* expression relative to control embryos in the pre-treated tBOOH treatment group, exposure did not significantly affect the expression of

the other genes selected. Although not statistically significant, *gsr* also appeared to show a trend towards increased expression following exposure to the 125-t and 125 µg/L treatment groups, with 3.99-fold and 4.23-fold increases in expression relative to control embryos, respectively (= 0.104 for both). Consistent with all previous endpoints, no significant differences were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone (Figure C3.S10).

3.5 Discussion

3.5.1 Aqueous selenomethionine dose-response embryo exposures

3.5.1.1 Total selenium in stock solutions and tissue

Previous studies addressing the embryotoxic effects of Se have chiefly consisted of maternal dietary exposures (Janz et al. 2010) and yolk microinjections (Thomas & Janz, 2016). Despite temporal and species-specific variation in chorion permeability to waterborne contaminants, aqueous embryo exposures (Lavado et al. 2012; Arnold et al. 2016; Kupsco & Schlenk, 2016a,b; Gerhart et al. 2019) have begun to gain traction as they offer a more rapid and simplified method for determining the effects of SeMet on developing fish embryos. Previous aqueous embryo exposure work has suggested that the chorion does exhibit some degree of permeability to SeMet, as embryos displayed increased Se tissue concentrations following aqueous exposure (Kupsco & Schlenk, 2016b; Gerhart et al. 2019). This was consistent with findings in the present study.

3.5.1.2 Mortality

Previous aqueous SeMet embryo exposure work in zebrafish (Arnold et al. 2016), Japanese medaka (*Oryzias latipes*) (Kupsco & Schlenk, 2016b), and fathead minnow (*Pimephales promelas*) (Gerhart et al. 2019) reported significant elevations in mortality at 100, 490, and 810 µg SeMet/L, respectively. Thus, based on the present study and a previous report (Arnold et al. 2016), it appears zebrafish embryos are more sensitive to aqueous SeMet exposures than medaka and fathead minnow.

3.5.1.3 Frequency and severity of deformities

The heightened sensitivity of zebrafish relative to other fish species used in aqueous embryo SeMet exposure experiments remained consistent during evaluations of larval

deformities, as previous aqueous SeMet embryo exposure work in Japanese medaka (Kupsco & Schlenk, 2016a) and fathead minnow (Gerhart et al. 2019) only reported significant elevations in the frequency of deformities at concentrations of 490 and 810 µg SeMet/L, respectively. Further analysis of the incidence of specific categories of deformities suggested that exposure to SeMet had no significant effect on the incidence of skeletal deformities, despite a concentration-dependent increase in the incidence of edema, fin, and craniofacial malformations. While this was consistent with previous work in fathead minnows (Gerhart et al. 2019), previous studies have suggested that spinal deformities are the most representative and common type of deformity observed in Se-exposed embryos (Muscatello et al. 2006; Kupsco & Schlenk, 2016b).

Consistent with the mortality and deformities frequency data, comparisons between the present study and previous aqueous embryo exposure work suggest that zebrafish are more sensitive to aqueous Se exposures, as our previous work in fathead minnows (Gerhart et al. 2019) showed a significant increase in the severity of deformities starting at 810 µg/L compared to 25 µg/L in the present study. Furthermore, the increased responsiveness of edema and fin/craniofacial malformations (to SeMet exposure) relative to spinal deformities, as illustrated by severity, was also observed in fathead minnows (Gerhart et al. 2019). Further investigation into the effect of differing methods of aqueously exposing embryos (i.e. timing, concentrations, etc.) would be required to determine why these types of deformities were not only more common, as observed during frequency analysis, but also more severe in both our fathead minnow and zebrafish embryos compared to previous aqueous embryo exposure studies.

3.5.1.4 Hatchability and time to hatch

The lack of effect on time to hatch is consistent with previous work in fathead minnow (Gerhart et al. 2019) and Japanese medaka (Kupsco & Schlenk, 2016b) embryos, where significant reductions in mean/median time to hatch was only observed at 65610 µg/L (the highest exposure concentration) and 5 µM SeMet (1000 µg/L) at stage 34, respectively. Effects on hatchability were also consistent with the aqueous Se embryo exposure literature. In fathead minnows exposed to Se (as SeMet) concentrations between 30 and 65610 µg/L, a significant reduction in embryo hatchability was observed at 21870 µg/L and 65610 µg/L (Gerhart et al. 2019). Furthermore, Japanese medaka embryos exposed aqueously to 0.5, 5, and 50 µM (approximately 100, 1000, and 10000 µg/L) SeMet at six different developmental stages reported

significant reductions in hatchability at concentrations ≥ 5 µM SeMet at all stages of development (Kupsco & Schlenk, 2016b). Another study involving Japanese medaka exposed to 0.05 mM (approximately 10000 µg/L) SeMet in freshwater and three different hypersaline conditions reported a significant reduction in hatchability in all treatment groups (Lavado et al. 2012).

3.5.2 Aqueous selenomethionine, pro- and antioxidant embryo exposures

Based on the results of the first experiment, 25 and 125 µg Se/L were selected for use in the second experiment, along with two additional pharmacological agents, tert-butyl hydroperoxide (tBOOH), and tert-butyl hydroquinone (tBHQ). These chemicals were chosen with the intention of further investigating the role of oxidative stress in developmental Se toxicity. For example, tBOOH, which is an organic peroxide, was selected as a positive control as it should produce oxidative stress (Timme-Laragy et al. 2012). Similarly, tBHQ, which is a synthetic, organic antioxidant, was selected as an antioxidant (Kobayashi et al. 2002). Both chemicals have been used previously in research focused on understanding the antioxidant defense system in mammalian cells, as they have been found to trigger an oxidative stress response through activation of nrf2, which consequently activates the transcription of phase II and antioxidant enzymes (Kobayashi et al. 2002; Timme-Laragy et al. 2012). Therefore, we predicted that pre-treating with tBHQ would activate/prepare the antioxidant defense system prior to exposure to SeMet or tBOOH, and thus serve a protective function.

3.5.2.1 Mortality

Consistent with the first experiment, exposure to the 25 and 125 µg/L SeMet treatment concentrations caused a significant increase in mortality compared to control embryos; however, no differences were observed between embryos pre-treated with tBHQ and embryos exposed to SeMet alone. This was consistent with previous aqueous SeMet embryo exposure work in zebrafish, where embryos pre-treated with the antioxidant NAC for 24h prior to SeMet exposure showed no significant differences in mortality compared to embryos exposed to SeMet alone (Arnold et al. 2016). As expected, exposure to the tBHQ did not result in significant mortality; however, the same was observed in both pro-oxidant tBOOH treatments. While this initially suggested the exposure concentration was too low, further evaluation of the deformities data suggested otherwise.

3.5.2.2 Frequency and severity of deformities

Consistent with the first experiment, exposure to the 25 and 125 µg/L SeMet treatment concentrations also resulted in a significant increase in both the incidence and severity of deformities compared to control embryos. However, no differences were observed between embryos pre-treated with tBHQ and embryos exposed to SeMet alone. This contrasted with the antioxidant rescue work conducted by Arnold et al. (2016), where embryos exposed to SeMet alone showed a significant increase in the frequency of deformities compared to controls and the embryos pre-treated with NAC for 24h prior to SeMet exposure. Furthermore, embryos pre-treated with NAC showed a qualitative decrease in the severity of deformities (Arnold et al. 2016). As expected, exposure to tBHQ alone did not result in any significant increases in the incidence or severity of deformities. In terms of the tBOOH treatments, it is important to note that despite a lack of mortality following exposure, significant increases in both the incidence and severity of deformities were observed when compared to control embryos, as was in the SeMet treated embryos.

While there were no clear patterns in terms of the occurrence or severity of certain types of deformities observed, we did note that significant elevations in the incidence and severity of skeletal deformities was only observed in the SeMet treatments, and not the tBHQ controls or tBOOH treatments. Furthermore, there seemed to be a general trend towards a higher proportion and severity of deformities observed in the SeMet treatments compared with the tBOOH treatments.

In the present study, lordosis (a form of skeletal curvature) was excluded from the deformities calculations due to an abnormally high incidence within both the control and tBHQ treatment groups (Figures C3.S11-12), as it was not believed to be reflective of selenium exposure. Interestingly, the frequency of lordosis was lower in the SeMet and tBOOH exposure groups compared to control and tBHQ groups. It is uncertain why the rate of lordosis was highest in both control treatments but seemed to decrease in frequency in the other treatment groups; however, it may have been related to either poor egg quality as a result of using an older zebrafish breeding stock, or some sort of nutritional deficiency.

3.5.2.3 Hatchability and time to hatch

In the present study, no consistent patterns were evident during assessment of time to hatch. Furthermore, the biological relevance of these results was considered minimal, as the time to hatch in these treatments only differed from the controls by about a day at most. In terms of effects on hatchability, the reductions observed in the SeMet exposed embryos were consistent with the first experiment, as well as previous aqueous Se embryo work (Lavado et al. 2012; Kupsco and Schlenk, 2016b; Gerhart et al. 2019). While exposure to tBHQ alone did not result in any significant changes in hatchability, reduced hatchability was observed in both tBOOH treatment groups. Of particular interest was the hatch rate in the pre-treated tBOOH group, as it was almost as severely reduced as embryos in the 125 µg/L Se treatment group (40.7% ± 5.8% compared to 38.6% ± 4.4%). Furthermore, it was noted during exposures that larvae in the tBOOH-t treatments generally appeared to be healthy and developmentally normal but appeared to struggle with hatching, inspiring more questions regarding the effect of waterborne exposures on physiochemical properties of the chorion.

3.5.2.4 Gene expression

In an attempt to further reinforce the relationship between oxidative stress and Se induced toxicity, changes in the expression of seven genes relevant to the antioxidant defense system were evaluated. These included: nuclear factor erythroid 2-related factor 2a (*nrf2a*), which is an oxidant-responsive transcription factor that regulates the transcription of antioxidant genes such as *gst p1* (Timme-Laragy et al. 2012); glutathione peroxidase 1a (*gpx 1a*), which is an enzyme that serves a protective role in cells by catalyzing the GSH-dependent reduction/degradation of various hydroperoxides (Tapiero et al. 2003); glutathione s-transferase piscine 1 (*gst p1*), which is one of the phase II detoxification enzymes that catalyze the conjugation of GSH to harmful/electrophilic compounds to aid with excretion (Kobayashi et al. 2002; Oakley, 2011); glutathione-disulfide reductase (*gsr*), which is an enzyme that directly catalyzes the reduction of oxidized glutathione (GSSG) back to reduced glutathione (GSH) (Michiels et al. 1994); manganese superoxide dismutase (*sod2*), which is an enzyme that catalyzes the reduction of the superoxide radical ($O_2^{\bullet -}$) into hydrogen peroxide and oxygen (Fridovich, 1989); glutamate-cysteine ligase catalytic subunit (*gclc*), which is a rate-limiting enzyme that catalyzes the first step in GSH synthesis from l-cysteine and glutamate; and finally aryl hydrocarbon receptor 2

(*ahr2*), which is a ligand-dependent transcription factor involved with the biotransformation of a range of contaminants (Timme-Laragy et. al 2012; Kawajiri & Fujii-Kuriyama, 2017).

As was mentioned previously, only *gsr* expression increased significantly out of the seven genes evaluated, and only in the pre-treated tBOOH treatment group. Since neithertBOOH or tBHQ alone induced a response in *gsr*, it is difficult to conclude on which chemical was responsible for the upregulation of this particular gene. Limited previous data exists for changes in *gsr* expression in fish embryos exposed to tBOOH or tBHQ; however, one study involving 4-dpf zebrafish eleutheroembryos (i.e., hatched but not yet free feeding embryos) exposed to tBHQ for 6 hr reported a significant increase in *gsr* gene expression (Hahn et al. 2014).

3.6 Conclusions

Exposure to the tBOOH treatment groups did not cause significant mortality but did result in significant increases in both the incidence and severity of deformities such as those observed in the SeMet treated embryos, potentially suggesting a similar pathway (i.e. oxidative stress) is playing a role in the generation of deformities. In addition, no differences were observed between embryos pre-treated with tBHQ and embryos exposed to either SeMet or tBOOH alone for all endpoints evaluated in the present study. Here we offer a few potential reasons for this observation:

1. tBHQ is not ideally suited for use as an antioxidant. As noted by Kobayashi et al. (2002), tBHQ is metabolized to an electrophilic quinone in cells, suggesting it could cause oxidative stress itself. Furthermore, it has been referenced to as a weak pro-oxidant in previous work studying oxidative stress (Sant et al. 2017).
2. The concentration of tBHQ (100 µg/L) selected was low enough that no significant differences in mortality or deformities were observed relative to controls, but perhaps not high enough to induce the activation of the antioxidant defense system.
3. The observed results were influenced by the duration and developmental stage of exposure. In the present study, embryos were pre-treated for 4h with 100 µg/L tBHQ immediately following collection. Previous work by Timme-Laragy et al. (2012) treated embryos (at 48 or 72 hpf) with tBHQ for 4h. Another study conducted a 6h tBHQ treatment in 7-day-old zebrafish larvae (Kobayashi et al. 2002), while Arnold et al.

(2016) pretreated zebrafish embryos (within 2 hpf) for 24h with the antioxidant N-acetylcysteine (NAC). Different exposure regimes will likely result in different responses.

To the best of our knowledge, there is no previous research that has looked at the production of deformities in fish embryos following exposure to these chemicals (tBHQ and tBOOH). Therefore, more research is required to better understand whether these pharmacological agents are helpful/suitable for investigating the mechanism of Se toxicity.

CHAPTER 4

4.0 GENERAL DISCUSSION

4.1 Project rationale

The consequences associated with industry-related increases in Se concentrations are well characterized in the literature, with case studies such as Belews Lake, USA (Lemly, 2002) and Lake Macquarie, Australia (Peters et al. 1999) highlighting the effects of coal mine effluent, while Kesterson National Wildlife Refuge, USA (Lemly, 1993) illustrates the effects of runoff generated by the irrigation of arid agricultural soils.

Previous studies addressing the embryotoxic effects of Se have largely consisted of dietary exposures (Thomas & Janz, 2014, 2015; Zee et al. 2016; Mohammad et al. 2017; Pettem et al. 2017, 2018; Lane, 2019) and yolk microinjections (Thomas & Janz, 2016; Lane, 2019). More recently, research employing aqueous Se embryo exposures were also able to generate malformations (Lavado et al. 2012; Arnold et al. 2016; Kupsco & Schlenk, 2016a,b), potentially offering an alternative to the standard methods of Se exposure. However, these aqueous SeMet embryo exposures, especially those employing antioxidant rescue methodologies, are a relatively new concept, and thus warrant further investigation. In addition, previous research involving aqueous embryo exposures to Se have utilized Japanese medaka and zebrafish as model test organisms. As such, fish species native to North America, such as the fathead minnow, have received little attention. Therefore, the overall objective of this thesis was to further investigate the mechanism of Se toxicity, as it relates to oxidative stress, by characterizing the effects of waterborne SeMet exposures in fathead minnow embryos and waterborne exposures to SeMet, tert-butyl hydroperoxide (tBOOH), and tert-butyl hydroquinone (tBHQ) in zebrafish embryos. In addition, this research aimed to critically evaluate the utility of aqueous embryo exposures as a new method of exposure for determining the effects of SeMet on developing embryos of native fishes.

4.2 Se aqueous embryo exposures

More recently, research has suggested that SeMet might not be the most relevant chemical form for use in Se research, as the mechanism of toxicity varies by seleno-compound, chemical form, and speciation (Rigby et al. 2014; Lazard et al. 2017). However, some previous

studies have shown that in fishes collected from contaminated sites, 60-80% of the total Se measured in tissue was in the form of SeMet (Phibbs et al. 2011). Thus, in the present study, SeMet was chosen for exposures as it is still considered to be the predominant form that aquatic organisms are exposed to via diet.

In chapters 2 and 3, range-finding exposures with SeMet were conducted with the objective of characterizing the effects of waterborne SeMet on standard endpoints such as hatchability, time to hatch, mortality, and the incidence/severity of larval deformities in both fathead minnow and zebrafish embryos. Despite concerns regarding chorion permeability to waterborne contaminants, the findings suggested that the chorion does exhibit some degree of permeability to SeMet, as both fathead minnows and zebrafish embryos displayed increased Se tissue concentrations ranging from 53.40 - 90.68 µg Se/g d.m., and 34.2 - 291 µg Se/g d.m. in fathead minnows and zebrafish, respectively. Even with similar background Se tissue concentrations (2.26 vs. 2.98 µg Se/g d.m. in fathead minnow and zebrafish, respectively), zebrafish embryos appeared to take up 3.2 times more Se than fathead minnow embryos, which translated into observable differences in hatchability, the incidence/severity of deformities, and mortality, as illustrated by the 261-fold difference in LC_{50} values for both species. Although significant increases in mortality and the frequency/severity of physical abnormalities at exposure concentrations of 810 and 25 µg/L (in fathead minnows and zebrafish, respectively) seem to suggest that fathead minnows are more tolerant to exposure than zebrafish, tissue concentrations actually suggest that fathead minnows are more sensitive to aqueous SeMet exposures, as significant increases in the same parameters were observed at Se tissue concentrations of 90.68 µg/g d.m. in fathead minnows compared to 144 µg/g d.m. in zebrafish. However, the decision to switch from using E3 Embryo Medium during the fathead minnow exposures to facility water during the zebrafish exposures may have interfered with SeMet assimilation due to differences in water chemistry, thus explaining the requirement for a substantially higher exposure concentration (810 µg/L) to obtain comparable tissue concentrations. Furthermore, the lack of Se tissue concentration measurements for the lower exposure concentrations, as well as the lack of replicate measurements (n =1) for each fathead minnow treatment group calls these species-tissue concentration comparisons into question.

It is also important to note that observing deformities was an extremely important finding in and of itself, as it illustrated that aqueous exposures with SeMet could produce physical

malformations like those observed in Se studies employing traditional methods of exposure (i.e. spiked diets). The frequency/severity of specific types of deformities observed (predominantly edema, fin, and craniofacial malformations) were also very interesting, as the majority of previous Se research has suggested that skeletal deformities are the trademark of *in ovo* Se exposure. Therefore, this finding suggests that this method of exposure might exert its toxicity through a different pathway, thus producing different types of physical malformations than what are typically observed. Overall, the results obtained from the aqueous Se range finding experiments in both data chapters suggest that zebrafish were able to bioaccumulate larger quantities of Se, and thus exhibited an increased susceptibility to aqueous SeMet exposures as compared to fathead minnows.

While similar trends in survival, hatchability, and the rate of deformities were all evident during comparisons to other aqueous SeMet embryo exposure work, the types of deformities observed differed greatly. In chapter 3 of the present study, lordosis exhibited an abnormally high incidence within control treatment groups and seemed to decrease in frequency as the Se exposure concentration increased. This observation was interesting, as the reversal of its appearance during treatment almost suggests Se deficiency. However, it could also be attributed to poor egg quality as a result of using an older zebrafish breeding stock, or some sort of nutritional deficiency. Nonetheless, since it was not believed to be reflective of Se exposure, lordosis was excluded from the deformity calculations in chapter 3. Aside from lordosis, the incidence/severity of the other forms of skeletal deformities were either inconsistent or showed little effect. In contrast, edema, fin, and craniofacial malformations were more consistent and each exhibited a concentration-dependent effect. Previous studies have consistently reported spinal deformities, namely lordosis, more frequently than the other characteristic Se deformities (Muscatello et al. 2006; Kupsco & Schlenk, 2016b). Aside from the numerous differences in study design, including test species, exposure concentrations, exposure conditions, duration of exposure, and developmental stage, it's difficult to say why these differences were observed. It's also important to note that a species sensitivity distribution became apparent (in the context of aqueous exposures), in which zebrafish embryos appeared to be the most sensitive, followed by Japanese medaka and fathead minnows, respectively.

During comparisons to other methods of exposure such as maternal transfer and yolk microinjections, endpoints such as survival and hatchability were largely unaffected (Masse,

2016; Lane, 2019). Aside from skeletal deformities, there were some similarities in terms of the types of deformities increasing in frequency/severity with exposure (edema, craniofacial, finfold) (Thomas & Janz, 2016; Lane, 2019). However, what quickly became evident during comparisons to maternal transfer and yolk microinjection studies was the tissue concentrations linked with effects on survival, hatchability, and deformities were much lower than those observed to cause the same types of effects in the present study. For example, a maternal transfer study involving *Xenopus laevis* females fed a diet spiked with SeMet for 68 days showed significant increases in the frequency/severity of physical abnormalities at tissue concentrations of 81.7 µg/g d.m. (Masse, 2016), compared to 90.7 and 144 µg/g d.m. in fathead minnows and zebrafish, respectively, in the present study. Another maternal transfer study in female fathead minnows fed a diet spiked with SeMet for 28 days reported a significantly increased proportion of deformities at embryo concentrations of 28.4 µg/g d.m. (Lane, 2019). In this same study, SeMet yolk microinjections in recently fertilized fathead minnow embryos reported decreased survival at 18.9 µg/g d.m. and a significant increase in the proportion of deformities at 13.5 and 18.9 µg/g d.m. (Lane et al. 2019). Moreover, in another SeMet yolk microinjection study involving recently fertilized zebrafish embryos, significant increases in mortality were observed at tissue concentrations ≥ 11 µg/g d.m., while reduced hatchability and an increased proportion of deformities were observed at tissue concentrations ≥ 18.7 µg/g d.m. (Thomas & Janz, 2016). It also appeared as though yolk microinjections have an even more pronounced effect than maternal transfer studies. This was also suggested by Lane et al. (2019), who reported a greater proportion of deformities at embryo Se concentrations of 18.9 µg/g d.m. when exposed via microinjection compared to a similar concentration via maternal transfer. Thus, there appears to be observable differences both within aqueous exposures as well as between other methods of exposure.

As was mentioned previously, dietary SeMet that is not metabolized to a usable or excretable form can be readily stored in body proteins in place of Met, leading to increasingly higher Se tissue concentrations (Schrauzer, 2000; Suzuki & Ogra, 2002). Although the route of exposure was different in the present study, Se bioaccumulation was observed in the larvae that were either already hatched or intentionally dechorionated (to prevent chorion-adsorption bias). Thus, it is reasonable to say that SeMet was taken up by the embryos. However, it's difficult to say whether the observed effects (i.e. mortality, deformities, edema, hatchability) in both the

present study and yolk microinjection studies (which both aim to replicate a natural dietary exposure) are the result of the metabolization of SeMet that has become protein-bound, or from exposure to free SeMet during early development. Furthermore, factors such as species-specific differences in the rate/pattern of development as well as the pattern of chorion water hardening/softening further complicate the story but likely explain the differing sensitivities observed in the present study.

4.3 Pharmacological aqueous embryo exposures

In chapter 3, the objective was to characterize the effects of waterborne exposure to both SeMet and tert-butyl hydroperoxide (tBOOH) to early life-stages of zebrafish pre-treated with the antioxidant tert-butyl hydroquinone (tBHQ) in an attempt to investigate the mechanism of Se toxicity as it relates to oxidative stress.

In comparison to the initial range-finding exposures in this chapter (discussed in the prior section), the responses of the SeMet treatment groups in the pharmacological aqueous exposures were consistent, with increased mortality, reduced hatchability, and an increase in the frequency/severity of deformities, which were predominantly edema, finfold, and craniofacial malformations. However, no significant differences were observed between embryos pre-treated with tBHQ and embryos exposed to either tBOOH or SeMet alone for any of the endpoints evaluated. The probable conclusion is that tBHQ is not ideally suited for use as an antioxidant, as it is metabolized to an electrophilic quinone in cells (Kobayashi et al. 2002), and probably induces oxidative stress itself. However, since tBHQ alone had no effect on any of the endpoints evaluated, it can be concluded that the concentration selected (100 µg/L) was too low to induce any significant adverse effects or activation of the antioxidant defense system. In addition, it is possible that the duration of exposure (4h) influenced the results as well, as previous studies with tBHQ (or other antioxidants) exposed embryos for longer lengths of time (Kobayashi et al. 2002; Timme-Laragy et al. 2012; Arnold et al. 2016). Unfortunately, that was one of the difficulties/trade offs that had to be made during the design of the study, as we had to ensure that the embryos were exposed to the antioxidant for an acceptable amount of time but also ensure that the embryos were still exposed during the early developmental stages and avoid chorion water hardening.

Another important point worth noting was the lack of effect exposures had on the expression of genes related to the antioxidant system. Aside from a significant increase in glutathione-disulfide reductase (*gsr*) expression in the pre-treated tBOOH treatment group, SeMet, tBHQ, and tBOOH exposures had no effect on any of the seven genes selected. One proposed reason for this observation was related to the study design, more specifically, the decision to collect larvae for gene expression at the end of the 5-day exposure. Changes in gene expression can occur quite rapidly (within a few hours) and therefore could have occurred earlier on in the exposure period, after which, any perturbations in gene expression would begin to return to baseline levels. As such, we may have missed the major changes in gene expression by sampling at the end of the 5-day exposure, and the elevated levels of *gsr* in the pre-treated tBOOH treatment group might be a lingering effect.

Aside from tBHQ, zebrafish embryo exposures to both tBOOH treatments had no effect on survival but did decrease hatchability and significantly increased the incidence and severity of deformities. The generation of deformities in these treatments was an extremely important finding because it confirmed that a completely different chemical with pro-oxidant properties produced deformities similar to that of the SeMet-treated embryos. This provided some evidence for the proposed role of oxidative stress in the toxicity pathway of Se.

Another interesting finding related to the tBOOH treatments was the lack of effect on survival despite a clear developmental effect (i.e. deformities), as mortality is typically a more sensitive endpoint in Se exposures than deformities. Although statistics were not run across all treatment groups, comparison of the tBOOH treatments to the SeMet treatments revealed a similar frequency of deformities, but there was a trend towards more severe deformities in the SeMet treatments. This makes sense, as more severely deformed fish in the SeMet treatments would translate to increased mortality. Therefore, this suggests that the tBOOH treatments may operate in a similar manner mechanistically, but do not have as pronounced of an effect as SeMet at the exposure concentrations used.

Overall, this data chapter provided some insight into the mechanistic aspect of Se toxicity, as a chemical (other than Se) with pro-oxidant properties was able to produce deformities similar to that of the SeMet exposed embryos. Furthermore, this chapter illustrated the importance of considering timing in relation to altered gene expression, exposure

concentrations, and the developmental stage at which exposure is conducted, and provided lessons/recommendations for better study designs in future aqueous exposure work.

4.4 Evaluation of the utility of aqueous embryo exposures

Previous research investigating the embryotoxic effects of Se has largely consisted of yolk microinjections or maternal transfer studies. More recent research employing aqueous Se embryo exposures generated similar effects as those observed with the classic exposure methods (Lavado et al. 2012; Arnold et al. 2016; Kupsco & Schlenk, 2016a,b), potentially offering an alternative to the standard methods of Se exposure. However, these aqueous embryo exposures, especially those employing antioxidant rescue methodologies, are a relatively new concept and thus warrant further investigation and critique. As such, one of the unofficial objectives of this research was to evaluate the utility of aqueous exposures as a method for determining the effects of SeMet on developing embryos.

Other methods of exposure, (i.e. yolk microinjections or adult feeding/breeding studies) tend to be time consuming, labour intensive, and require specific skills and equipment to conduct. Aqueous exposures represent a simplified method that requires little skill to conduct and rapidly produces results (hatchability, mortality, etc.). They require little resources, as you don't need expensive/specific equipment, and fewer test organisms to conduct. In addition, aqueous exposures have a short time requirement. Feeding studies can last up to 68 days (Masse, 2016) to conduct due to the time required for Se to reach steady state in the tissue, and even longer if a depuration stage is needed. Finally, aqueous exposures require little maintenance/labour (just daily water changes) compared to exposure methods like yolk microinjections, which is an extremely tedious and labor-intensive process, or feeding studies where you care for and feed the fish for an extended period of time.

The main disadvantage of aqueous exposures is the number of factors that can influence the results. This type of method, because it is aqueously based, requires consideration of water quality parameters such as temperature, pH, alkalinity, hardness, and dissolved oxygen. There is also the added complexity of working with a live organism that needs to be exposed within certain windows of time to target the correct developmental stages. As a result, there is a requirement for strict control over the breeding schedule of these live organisms. Timing is extremely important in this type of research because it directly relates to the pattern of chorion

hardening and softening, which interferes with chorion permeability to waterborne contaminants and ultimately the exposure process. Finally, there is also the need to consider any factors that can affect the quality of eggs/embryos you will eventually be exposing, as it would be difficult to distinguish if the observed effects are the result of Se exposure or poor egg quality.

4.5 Factors mediating toxicity in aqueous exposures

As was mentioned previously, the ecologically relevant Se exposure pathway for embryos is maternal transfer of SeMet accumulated through diet (Janz, 2012). As such, concerns regarding chorion adsorption and permeability to xenobiotics during waterborne embryo exposures remains a subject of contention, especially when selenium is concerned. Previous work has reported that the chorion is water permeable and exhibits some degree of permeability to smaller organic molecules (Suga, 1963; Alderdice, 1988; Finn, 2007). However, based on the present study, as well as previous aqueous SeMet exposure studies (Lavado et al. 2012; Kupsco & Schlenk, 2016b), Se bioaccumulation/uptake does take place. However, there are many factors that influence uptake, such as composition of the external medium, developmental stage, and the chorion.

4.5.1 Composition of the external medium

As with any aqueous exposure assay, water chemistry parameters such as temperature, salinity, pH, hardness/alkalinity, and dissolved oxygen are important factors to consider as they can influence availability and speciation, and thus toxicity. For example, previous work has shown that increasing water temperature modified toxicity in juvenile fish exposed aqueously to different contaminants (Patra et al. 2015; Song et al. 2015), while previous aqueous embryo exposure work in Japanese medaka has suggested that hypersaline conditions exacerbated SeMet toxicity (Lavado et al. 2012; Kupsco & Schlenk, 2014, 2016b).

In addition, factors such as pH and hardness/alkalinity have also been shown to alter chemical toxicity and bioaccumulation in aqueous exposure scenarios due to the influence on speciation/ionization state, mobility, and bioavailability of contaminants (Nakamura et al. 2008; Wang et al. 2016). For example, previous research involving aqueous fluoxetine exposures at pH 7-9 reported lower LC_{50} values and increased bioaccumulation as pH increased towards the pK_a in Japanese medaka larvae and juveniles (Nakamura et al. 2008). However, pH is particularly relevant to metal/metalloid exposures as toxicity tends to decrease with increasing

pH (Wang et al. 2016). Previous work involving Coho salmon (*Oncorhynchus kisutch*) eggs exposed to zinc noted that alterations to water pH affected zinc accumulation by the chorion, suggesting that pH may also affect charged binding sites on the chorion and thus chemical binding affinity (Wedemeyer, 1968). Aside from pH, two previous studies involving larval fathead minnow (Sciera et al. 2004) and zebrafish (Bui et al. 2016) exposures to copper noted that increased water hardness significantly reduced copper toxicity. Furthermore, previous work focusing on cadmium uptake in flounder (*Pleuronectes flesus*) and herring (*Clupea harengus* L.) eggs noted that larger amounts of cadmium was detected in fish eggs when exposed in soft water rather than hard water (Westernhagen et al. 1974; Westernhagen & Dethlefsen, 1975).

Dissolved oxygen is particularly relevant in aqueous embryo exposures due to the static renewal aspect of the study design, and its reported influence on embryo development. In the context of an aqueous embryo exposure, factors such as high embryo density relative to test medium volume and low maximal oxygen concentration due to high assay temperatures can lead to oxygen depletion (Küster & Altenburger, 2008). Previous work investigating the effects of hypoxic conditions on developing fish embryos have reported decreases in egg/sperm quality (Wu et al. 2003), suspension of development (Küster & Altenburger, 2008), as well as physical malformations such as missing fins, stunting or deformation of the tail, spinal deformities, and impaired vascular system function/development (Ingalls & Philbrook, 1958; Shang & Wu, 2004; Strecker et al. 2011). As such, considering the influence of water chemistry on toxicity, especially in the context of aqueous embryo exposures, is imperative.

4.5.2 Developmental stage and the chorion

Development is an extremely complex process that follows a well-defined sequence of events. Logically, exposures with chemicals that are known teratogens, such as Se, during this process can result in its disruption, resulting in an incorrectly formed individual or mortality if the malformation is severe enough. Therefore, it is no surprise that the developmental stage at which aqueous exposures are conducted has the potential to influence the observed sensitivity and the specific types of deformities observed in developing fish larvae.

In the present study, aqueous SeMet exposures in both fathead minnow and zebrafish embryos had little effect on the incidence/severity of skeletal deformities, while edema, fin, and craniofacial malformations were more consistent and exhibited a concentration-dependent effect.

This observation contrasts the majority of previous Se research which suggests that skeletal deformities are a trademark of *in ovo* Se exposure (Lemly, 2002; Muscatello et al. 2006; Janz, 2012; Kupsco & Schlenk, 2016b). In addition, it was noted that zebrafish exhibited an increased susceptibility to aqueous SeMet exposures as compared to fathead minnows, which was largely attributed to the observation that zebrafish bioaccumulated larger quantities of Se than fathead minnows.

Based on previous research, we know that the types of deformities observed are related to the developmental stage at which aqueous exposures are conducted. For example, an aqueous exposure study evaluating this exact concept was carried out by Kupsco and Schlenk (2016b). In this study, Japanese medaka embryos were exposed aqueously to SeMet during stages 9, 17, 25, 29, 34, and 38 of development, which range from the late morula stage (256-512 cell stage) to the spleen development stage (Iwamatsu, 2004) (i.e. between 5-192 h post fertilization). As expected, exposures initiated at different time points produced certain types of deformities in a stage-specific manner. Exposures in the present study were conducted on fathead minnow embryos between stages 5-10 (4 cell - high blastula cell stages; i.e. within 4.5h post fertilization) of embryonic development (US EPA, 1996), and zebrafish embryos between 4-cell – high blastula stage (i.e. within 3h of fertilization) of embryonic development (Kimmel et al. 1995), and therefore earlier stages of development than the Japanese medaka embryos. Overall this suggests that timing is incredibly key during these early life-stage exposures, as the stage of development at which the teratogen is introduced will dictate what structure/organ/feature is going to be affected/altered.

In addition, conducting these exposures during certain stages of development may also influence the extent of bioaccumulation, and thus species sensitivity. For example, Kupsco and Schlenk (2016b) noted that embryo Se content was not equal in embryos exposed to the same treatment (5 µM SeMet) but at different stages. Furthermore, certain stages of development (9 and 17) exhibited significantly less Se accumulation than others (Kupsco & Schlenk, 2016b). These fluctuations in Se uptake are likely attributed to structural/physical changes in the chorion over the course of development.

Prior to fertilization, the oocyte undergoes hydration, a process in which the oocyte breaks down yolk proteins to produce free amino acids and peptides (which serve as osmotic

effectors) in order to cause water influx (Le Menn et al. 2007). This water uptake across the membrane (which is thought to serve osmotic balance and buoyancy purposes) is facilitated by aquaporins, which are specialized channels that allow the diffusion of water across cell membranes (Cerdà, 2009).

Following fertilization, a process referred to as water hardening occurs in which a series of chemical reactions within the egg cause the vitelline envelope to differentiate and form the tough protective outer coating referred to as the chorion (Tyler & Sumpter, 1996; Wootton & Smith, 2015). Following completion of this process, which occurs in only 60 minutes in Japanese medaka (Suga, 1963), egg permeability to waterborne contaminants is low. Again, this decreased permeability may be due to structural/physical changes in the chorion. For example, a previous a study involving winter flounder eggs (*Pseudopleuronectes arnericanus*) imaged with a scanning electron microscope before and after fertilization noted physical changes in the chorion surface structure following fertilization (Perry, 1984). Prior to fertilization, the chorion surface exhibited randomly oriented furrows covered with pores (diameter of about 0.26 µm) that sat flush with the membrane. Following fertilization, it was noted that the furrows became more regular, with the depressions oriented parallel to one another. Furthermore, it was noted that the pore diameter appeared to decrease after fertilization (diameter of about 0.18 – 0.19 µm), and the rim of the pores appeared to become thickened and elevated (Perry, 1984). Despite the decreased pore size, the continued presence of aquaporins on the chorion surface following fertilization suggests that the chorion must still exhibit permeability to water as well as other small molecules, but to a smaller extent.

Previous research in Japanese medaka has also suggested that a period of chorion "softening" occurs when the embryo is nearing the end of development (Suga, 1963). It has been proposed that this is due to the release of hatching enzymes (proteases) from secretory cells on the embryo, which slowly breakdown the inner layer of the chorion leading up to hatch, leaving the undissolved, thin outer layer to be eventually torn by the tail of the fry (Suga, 1963; Blaxter, 1988). Evidently, the degree of permeability, and thus susceptibility to waterborne SeMet exposures, changes temporally throughout development. Furthermore, different fish species will exhibit different patterns of hardening and softening, and thus different periods of susceptibility to chemical exposure.

Another important consideration related to developmental stage is the fact that fish larvae tend to be physiologically immature until the end of yolk resorption, and lack the ability to produce their own enzymes, hormones, and growth factors (Lam, 1994). This suggests that fish species exposed during early life-stages might also differ in their capacity to metabolize contaminants and thus respond to exposures differently.

4.5.3 Factors affecting egg quality

In addition to these methodology-related factors that influence aqueous exposures, quality of the eggs/embryos is an entire area of research in and of itself. Generally, good egg quality has been defined by low levels of mortality at fertilization, eying, hatch, and first-feeding, as well as the production of fast-growing and healthy fry (Bromage et al. 1992). In some cases (such as the present study), assessments of quality cannot wait until hatch or first-feeding, but have to be carried out shortly after fertilization in order to achieve early life-stage exposures. In this case, assessments of quality can involve the evaluation of cell symmetry at early stages of cleavage (Brooks et al. 1997). Nonetheless, despite significant research in this area, a lack of consensus exists regarding what levels of mortality or malformations are acceptable, or what factors in the egg itself or the broodfish are responsible for differing levels of quality (Bromage et al. 1992; Brooks et al. 1997). It is evident, however, that egg quality differs between females in the same stocks/tanks (which are maintained under presumably identical conditions), across clutches of eggs from the same females over a single spawning season, as well as across individual eggs from the same clutch. As such, research aimed towards improving egg quality/viability through the modification of both intrinsic and environmental factors has become an area of intense research motivated largely by the need for improvement in the aquaculture industry.

Intrinsic factors thought to influence egg/larval quality can include parental genetics, as well as factors associated with husbandry such as maternal diet, stress, and overripening of eggs. Environmental factors suggested to affect egg quality include photoperiod, water temperature, salinity, pH, dissolved oxygen (DO), as well as the physical environment (substrate, tank volume, stocking density) (Bromage et al. 1992; Brooks et al. 1997; Bromage et al. 2001; Migaud et al. 2013; Bobe, 2015). While most of these factors undoubtedly play a role in the quality of offspring produced, factors of potential relevance to the background rate of

malformations (mostly lordosis) observed in this study include deficiencies in the maternal diet, stress, and overripening of the eggs.

Maternal nutrition/diet is intimately connected to egg/larval quality, as the female is responsible for the production and deposition of energy resources, vitamins, and hormones required for development into the oocyte (Le Menn et al. 2007; Falahatkar et al. 2011). Previous research investigating the effects of diet manipulation on egg/larval quality has focused on the relative proportions and types of components such as essential fatty acids (i.e. lipids) (Harel et al. 1994; Fernández-Palacios et al. 1997; Callan et al. 2012; Mejri et al. 2017), essential amino acids (i.e. proteins) (Cerda et al. 1994; Fernández-Palacios et al. 1997; Mejri et al. 2017), carbohydrates (Cerda et al. 1994), and vitamins (A, E, C) (Palace & Werner, 2006; Falahatkar et al. 2011; Mejri et al. 2017) in the diet of broodstock. Vitamins are of particular interest, as previous work looking at broodstock diets deficient in or supplemented with vitamins reportedly observed an increased or decreased incidence of abnormal/deformed larval offspring (Halver, 2002).

Aside from diet, poor fish husbandry practices can invoke stress in captive broodfish, as well as cause egg overripening (Bromage et al. 1992; Brooks et al. 1997). Some previous work has suggested that stress in captive broodfish can potentially disrupt the endocrine system involved with reproduction, resulting in lower fecundity and fertilization rates, irregular spawning intervals, lower survival rates, and an increased occurrence of abnormal embryos (Schreck, 2010; Bogevik et al. 2012). However, as was discussed by Bobe and Labbé (2010), information on the effect of stress on gamete quality has little consensus, as responses have been quite diverse depending on the type (capture, transport, confinement, overcrowding, net chasing, low water level) and intensity of stressor, the species used, and the length of exposure to the stressor. In addition to stress, egg overripening, which is the process of ageing in unfertilized eggs retained in the body cavity (following ovulation), is potentially one of the most common reasons for poor egg quality in captive broodfish (Brooks et al. 1997; Samarin et al. 2015). Typically, this is due to irregular (and perhaps infrequent) breeding schedules as a result of separating males from females and can result in biochemical and morphological changes that lead to a loss of viability, lower fertilization/hatching rates, as well as an increased incidence of embryo malformations (Springate et al. 1984; Bobe & Labbé, 2010; Bogevik et al. 2012; Samarin et al. 2015).

4.7 Future Research

Overall, the research presented in this thesis demonstrated that aqueous exposures with SeMet produced physical malformations and other adverse effects similar to those observed in Se studies employing traditional methods of exposure (i.e. maternal transfer studies). Furthermore, this research provided support for the oxidative stress aspect of Se toxicity as tBOOH, a chemical with pro-oxidant properties, was able to produce deformities akin to those in SeMet exposed embryos. However, the lack of clear dose-response relationships for the frequency/severity of larval deformities, as well as the apparent differences in tolerance between zebrafish and fathead minnow embryos in the present study (and other aqueously exposed fish species), inspires more questions about the influence species-specific differences in developmental pattern and chorion physiology has on waterborne SeMet exposures.

This research also provided lessons/recommendations for better study designs in future aqueous exposure work. A few areas that could be improved upon or require further investigation are listed below:

- Aqueous SeMet exposures with more fish species, broader concentration ranges, and tissue concentration measurements (relatively sparse in the literature), would allow the development of a species sensitivity distribution for this type of exposure method.
- Additional laboratory-based studies comparing different Se exposure methods (aqueous, maternal transfer, yolk microinjection) to further clarify the reason for the observable differences in sensitivity.
- Additional laboratory-based studies focused on the implications of conducting exposures at different developmental stages would clarify the relationship between developmental stage and the type of deformities observed.
- Research investigating the temporal pattern of chorion water hardening/softening across fish species. This would be useful and applicable to aqueous exposures with any chemical/contaminant, not just Se.
- In chapter 3, the lack of effect on the expression of genes related to the antioxidant system may have been the result of acclimation due to sampling at the end of the 5-day exposure. For future work, it is recommended that sampling for this endpoint occurs either a day after

exposure or involves a different exposure regime in which larvae are reared up to 5dpf and then exposed/sampled to ensure that their molecular machinery is developed enough to respond accurately.

LIST OF REFERENCES

Alderdice DF. 1988. Osmotic and Ionic Regulation in Teleost Eggs and Larvae. In: Hoar WS and Randall DJ (eds). *Fish Physiology: The Physiology of Developing Fish Eggs and Larvae*. Academic Press, San Diego, CA. Vol 11, Part A, 163–251.

Ankley GT and Johnson RD. 2004. Small Fish Models for Identifying and Assessing the Effects of Endocrine-disrupting Chemicals. *ILAR.* 45(4), 469-483.

Ankley GT and V illeneuve DL. 2006. The Fathead Minnow in Aquatic Toxicology: Past, Present and Future. *Aquatic Toxicology*. 78(1), 91-102.

Ankley GT, Jensen KM, Kahl MD, Korte JJ, and Makynen EA. 2001. Description and Evaluation of a Short-term Reproduction Test with the Fathead Minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry.* 20(6), 1276-1290.

Arnér E SJ and Holmgren A. 2000. Physiological Functions of Thioredoxin and Thioredoxin Reductase. *European Journal of Biochemistry*. 267(20), 6102–6109.

Arnold MC, Forte JE, Osterberg JS, and Di Giulio RT. 2016. Antioxidant Rescue of Selenomethionine-Induced Teratogenesis in Zebrafish Embryos. *Archives of Environmental Contamination and Toxicology*. 70(2), 311-320.

Arukwe A and Goksøyr A. 2003. Eggshell and Egg Yolk Proteins in Fish: Hepatic Proteins for the Next Generation: Oogenetic, Population, and Evolutionary Implications of Endocrine Disruption. *Comparative Hepatology*. 2(1), 4.

Baines SB and Fisher NS. 2001. Interspecific Differences in the Bioconcentration of Selenite by Phytoplankton and Their Ecological Implications. *Marine Ecology Progress Series*. 213, 1-12.

BC MoE (British Columbia Ministry of Environment), 2014. Ambient Water Quality Guidelines for Selenium Technical Report Update. Water Protection and Sustainability Branch, Environmental Sustainability and Strategic Policy Division, Victoria, BC, pp.1-270.

Björnstedt M, Xue J, Huang W, Akesson B, and Holmgren A. 1994. The Thioredoxin and Glutaredoxin Systems Are Efficient Electron Donors to Human Plasma Glutathione Peroxidase*. *The Journal of Biological Chemistry*. 269(47), 29382-29384.

Blaxter JHS. 1988. Pattern and Variety in Development. In: Hoar WS, Randall DJ (eds) *Fish Physiology: The Physiology of Developing Fish Eggs and Larvae*. Academic Press, San Diego, CA, USA, 11A, 1-58.

Bobe J and Labbé C. 2010. Egg and Sperm Quality in Fish. *General and Comparative Endocrinology*. 165(3), 535-548.

Bobe J. 2015. Egg Quality in Fish: Present and Future Challenges. *Animal Frontiers*. 5(1), 66-72.

Böck A, Forchhammer K, Heider J, Leinfelder W, Sawers G, Veprek B, and Zinoni F. 1991. Selenocysteine: The 21st Amino Acid. *Molecular Microbiology*. 5(3), 515-520.

Boehler CJ, Raines AM, and Sunde RA. 2014. Toxic-Selenium and Low-Selenium Transcriptomes in *Caenorhabditis Elegans*: Toxic Selenium Up-Regulates Oxidoreductase and Down-Regulates Cuticle-Associated Genes. *PLoS ONE*. 9(6): e101408.

Bogevik AS, Natário S, Karlsen Ø, Thorsen A, Hamre K, Rosenlund G, and Norberg B. 2012. The Effect of Dietary Lipid Content and Stress on Egg Quality in Farmed Atlantic Cod *Gadus morhua*. *Journal of Fish Biology*. 81(4), 1391-1405.

Bromage N, Jones J, Randall C, Thrush M, Davies B, Springate J, Duston J, and Barker G. 1992. Broodstock Management, Fecundity, Egg Quality and the Timing of Egg Production in the Rainbow Trout (*Oncorhynchus mykiss*). *Aquaculture*. 100(1), 141-166.

Bromage N, Porter M, and Randall C. 2001. The Environmental Regulation of Maturation in Farmed Finfish with Special Reference to the Role of Photoperiod and Melatonin. *Aquaculture*. 197(1), 63-98.

Brooks S, Tyler CR, and Sumpter JP. 1997. Egg Quality in Fish: What Makes a Good Egg?" *Reviews in Fish Biology and Fisheries*. 7(4), 387-416.

Bui T-KL, Do-Hong LC, Dao T-S, and Hoang TC. 2016. Copper Toxicity and the Influence of Water Quality of Dongnai River and Mekong River Waters on Copper Bioavailability and Toxicity to Three Tropical Species. *Chemosphere*. 144, 872-878.

Bunnell TM, Burbach BJ, Shimizu Y, and Ervasti JM. 2011. β-Actin Specifically Controls Cell Growth, Migration, and the G-actin Pool. *Molecular Biology of the Cell*. 22(21), 4047-4058.

Callan CK, Laidley CW, Forster IP, Liu KM, Kling LJ, and Place AR. 2012. Examination of Broodstock Diet Effects on Egg Production and Egg Quality in Flame Angelfish (*Centropyge loriculus*). *Aquaculture Research*. 43(5), 696-705.

CCME (Canadian Council of Ministers of Environment), 2007. Canadian Water Quality Guidelines for the Protection of Aquatic Life: Summary table. In: Canadian Environmental Quality Guidelines, 1999, Canadian Council of Ministers of Environment, Task Force on Water Quality Guidelines, Winnipeg, MB, pp.1-9.

Cerdà J. 2009. Molecular Pathways During Marine Fish Egg Hydration: The Role of Aquaporins. *Journal of Fish Biology*. 75(9), 2175-2196.

Cerdá J, Carrillo M, Zanuy S, Ramos J, and De La Higuera M. 1994. Influence of Nutritional Composition of Diet on Sea Bass, *Dicentrarchus labrax* L., Reproductive Performance and Egg and Larval Quality. *Aquaculture*. 128(3-4), 345-361.

cms_042380 - Guide to Performing Relative Quantitation of Gene Expression Using RT qPCR. ThermoFisher Scientific. Available online: https://www.thermofisher.com/document-connect/documentconnect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFSAssets%2FLSG%2Fmanuals%2Fcms_042380.pdf&title=R3VpZGUgdG8gUGVyZm9ybWluZyBSZWxhdGl2ZSBRdWFudGl0YXRpb24gb2YgR2VuZSBFeHByZXNzaW9uIFVzaW5nIFJlYWwtVGltZSBRdWFudGl0YXRpdmUgUENS. (Accessed on 05/06/2019).

Cold Spring Harbor Protocols. 2012. E3 Embryo Medium (60x). doi:10.1101/pdb.rec070540

Conley JM, Funk DH, and Buchwalter DB. 2009. Selenium Bioaccumulation and Maternal Transfer in the Mayfly *Centroptilum triangulifer* in a Life-Cycle, Periphyton-Biofilm Trophic Assay. *Environmental Science & Technology.* 43(20), 7952-7957.

Conley JM, Funk DH, Hesterberg DH, Hsu L-C, Kan J, Liu Y-T, and Buchwalter DB. 2013. Bioconcentration and Biotransformation of Selenite versus Selenate Exposed Periphyton and Subsequent Toxicity to the Mayfly *Centroptilum triangulifer*. *Environmental Science & Technology*. 47(14), 7965-7973.

Diplock AT and Hoekstra WG. 1976. Metabolic Aspects of Selenium Action and Toxicity. *CRC Critical Reviews in Toxicology*. 4(3), 271-329.

Falahatkar B, Dabrowski K, and Arslan M. 2011. Ascorbic Acid Turnover in Rainbow Trout, *Oncorhynchus mykiss*: Is There a Vitamin Enrichment Effect during Embryonic Period on the Juvenile Fish "Sensitivity" to Deficiency? *Aquaculture.* 320(1-2), 99-105.

Fan T W-M, Teh SJ, Hinton DE, Higashi RM. 2002. Selenium Biotransformations Into Proteinaceous Forms by Foodweb Organisms of Selenium-Laden Drainage Waters in California. *Aquatic Toxicology.* 57(1), 65−84.

Fernández-Martínez A and Charlet L. 2009. Selenium Environmental Cycling and Bioavailability: A Structural Chemist Point of View. *Reviews in Environmental Science and Bio/Technology*. 8(1), 81-110.

Fernández-Palacios H, Izquierdo M, Robaina L, Valencia A, Salhi M, and Montero D. 1997. The Effect of Dietary Protein and Lipid from Squid and Fish Meals on Egg Quality of Broodstock for Gilthead Seabream (*Sparus aurata*). *Aquaculture*. 148(2), 233-246.

Ferrie GM, Alford VC, Atkinson J, Baitchman E, Barber D, Blaner WS, Crawshaw G, Daneault A, Dierenfeld E, Finke M, Fleming G, Gagliardo R, Hoffman EA, Karasov W, Klasing K, Koutsos E, Lamkton J, Lavin SR, Lentini A, Livingston S, Lock B, Mason T, McComb A, Morris C, Pessier AP, Olea-Popelka F, Probst T, Rodriguez C, Schad K, Semmen K, Sincage J, Stamper MA, Steinmetz J, Sullivan K, Terrell S, Wertan N, Wheaton CJ, Wilson B, and Valdes EV. 2014. Nutrition and Health in Amphibian Husbandry. *Zoo Biology*. 33(6), 485-501.

Finn RN. 2007. The Physiology and Toxicology of Salmonid Eggs and Larvae in Relation to Water Quality Criteria. *Aquatic Toxicology*. 81(4), 337–354.

Franklin CC, Backos DS, Mohar I, White CC, Forman HJ, and Kavanagh TJ. 2009. Structure, Function, and Post-translational Regulation of the Catalytic and Modifier Subunits of Glutamate Cysteine Ligase. *Molecular Aspects of Medicine*. 30(1), 86-98.

Fridovich I. 1989. Superoxide Dismutases - An Adaptation to a Paramagnetic Gas. *Journal of Biological Chemistry*. 264(14), 7761-7764.

Friguet B. 2006. Oxidized Protein Degradation and Repair in Ageing and Oxidative Stress. *Federation of European Biochemical Societies Letters.* 580(12), 2910-2916.

Fukui M and Zhu BT. 2010. Mitochondrial Superoxide Dismutase SOD2, but Not Cytosolic SOD1, Plays a Critical Role in Protection Against Glutamate-induced Oxidative Stress and Cell Death in HT22 Neuronal Cells. *Free Radical Biology and Medicine.* 48(6), 821-830.

Ganther HE. 1986. Pathways of Selenium Metabolism Including Respiratory Excretory Products. *Journal of the American College of Toxicology*. 5(1), 1-5.

Gerhart AK, Hecker M, and Janz DM. 2019. Toxicity of Aqueous L -Selenomethionine Exposure to Early Life-Stages of the Fathead Minnow (*Pimephales promelas*). *Bulletin of Environmental Contamination and Toxicology*. 102(3), 323-328.

Greenwood NN and Earnshaw A. 1997. *Chemistry of the Elements* (2nd ed.). Butterworth-Heinemann. p. 780.

Hahn ME, McArthur AG, Karchner SI, Franks DG, Jenny MJ, Timme-Laragy AR, Stegeman JJ, Woodin BR, Cipriano MJ, Linney E. 2014. The Transcriptional Response to Oxidative Stress during Vertebrate Development: Effects of *tert*- Butylhydroquinone and 2,3,7,8-Tetrachlorodibenzo-p-Dioxin. *PLoS ONE*. 9(11), e113158.

Halver JE. 2002. The Vitamins. In: Halver JE and Hardy RW (eds). *Fish Nutrition*. Academic Press, San Diego, California, USA. 62-143.

Hamilton SJ. 2004. Review of Selenium Toxicity in the Aquatic Food Chain. *Science of the Total Environment*. 326, 1-31.

Handy DE, Lubos E, Yang Y, Galbraith JD, Kelly N, Zhang Y-Y, Leopold JA, and Loscalzo J. 2009. Glutathione Peroxidase-1 Regulates Mitochondrial Function to Modulate Redox-dependent Cellular Responses. *The Journal of Biological Chemistry*. 284(18), 11913-11921.

Harel M, Tandler A, Kissil GW, and Applebaum SW. 1994. The Kinetics of Nutrient Incorporation into Body Tissues of Gilthead Seabream (*Sparus aurata*) Females and the Subsequent Effects on Egg Composition and Egg Quality. *British Journal of Nutrition*. 72(1), 45-58.

Hoffman DJ and Heinz GH. 1988. Embryotoxic and Teratogenic Effects of Selenium in the Diet of Mallards. [*Anas Platyrhynchos*]. *Journal of Toxicology and Environmental Health*. 24(4), 477-490.

Holm J, Palace VP, Siwik P, Sterling G, Evans R, Baron C, Werner J, Wautier K. 2005. Developmental Effects of Bioaccumulated Selenium in Eggs and Larvae of Two Salmonid Species. *Environmental Toxicology and Chemistry.* 24(9), 2373-2381.

Holm J, Palace VP, Wautier K, Evans RE, Baron CL, Podemski C, Siwik P, Sterling G. 2002. An Assessment of the Development and Survival of Wild Rainbow Trout (*Oncorhynchus mykiss*) and Brook Trout (*Salvelinus fontinalis*) Exposed to Elevated Selenium in an Area of Active Coal Mining. In: *The Big Fish Bang.* Proceedings of the 26th Annual Larval Fish Conference, Os, Norway, July 22-26. Browman HI, Skiftesvik AB (eds). Institute of Marine Research: Bergen, Norway, 2003, 257-273.

Holmgren A, Johansson C, Berndt C, Lonn ME, Hudemann C, and Lillig CH. 2005. Thiol Redox Control Via Thioredoxin and Glutaredoxin Systems. *Biochemical Society Transactions.* 33, 1375-1377.

Ingalls TH and Philbrook FR. 1958. Monstrosities Induced by Hypoxia. *The New England Journal of Medicine*. 259(12), 558-564.

Iwamatsu T. 2004. Stages of Normal Development in the Medaka *Oryzias latipes. Mechanisms of Development*. 121(7), 605-618.

Jain SK and Micinski D. 2013. Vitamin D Upregulates Glutamate Cysteine Ligase and Glutathione Reductase, and GSH Formation, and Decreases ROS and MCP-1 and IL-8 Secretion in High-glucose Exposed U937 Monocytes. *Biochemical and Biophysical Research Communications*. 437(1), 7-11.

Janz DM, DeForest DK, Brooks ML, Chapman PM, Gilron G, Hoff D, Hopkins WD, McIntyre DO, Mebane CA, Palace VP, Skorupa JP, Wayland M. 2010. Selenium Toxicity to Aquatic Organisms. In: Chapman PM, Adams WJ, Brooks ML, Delos CG, Luoma SN, Maher WA, Ohlendorf HM, Presser TS, Shaw DP (eds). *Ecological Assessment of Selenium in the Aquatic Environment.* CRC Press Pensacola, FL, pp 141-231.

Janz, DM. 2012. Selenium. In: Wood CM, Farrell AP, and Brauner CJ (eds). *Fish Physiology: Homeostasis and Toxicology of Essential Metals.* Elsevier, San Diego, CA. 31A, pp. 327-373.

Kajander E, Harvima R, Eloranta T, Martikainen H, Kantola M, Kärenlampi S, and Åkerman, K. 1991. Metabolism, Cellular Actions, and Cytotoxicity of Selenomethionine in Cultured Cells. *Biological Trace Element Research*. 28(1), 57-68.

Kawajiri K and Fujii-Kuriyama Y. 2017. The Aryl Hydrocarbon Receptor: A Multifunctional Chemical Sensor for Host Defense and Homeostatic Maintenance. *Experimental Animals*. 66(2), 75-89.

Kimmel CB, Ballard WW, Kimmel SR, Bonnie U, and Schilling TF. 1995. Stages of Embryonic Development of the Zebrafish. *Developmental Dynamics*. 203(3), 253-310.

Kobayashi M, Itoh K, Suzuki T, Osanai H, Nishikawa K, Katoh Y, Takagi Y, and Yamamoto M. 2002. Identification of the Interactive Interface and Phylogenic Conservation of the Nrf2-Keap1 System. *Genes to Cells*. 7(8), 807-820.

Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigó R, and Gladyshev VN. 2003. Characterization of Mammalian Selenoproteomes. *Science*. 300(5624), 1439-1443.

Kupsco A and Schlenk D. 2016a. Molecular Mechanisms of Selenium-Induced Spinal Deformities in Fish. *Aquatic Toxicology*. 179, 143-150.

Kupsco A and Schlenk D. 2016b. Stage Susceptibility of Japanese Medaka (*Oryzias Latipes*) to Selenomethionine and Hypersaline Developmental Toxicity. *Environmental Toxicology and Chemistry*. 35(5), 1247-1256.

Küster E and Altenburger R. 2008. Oxygen Decline in Biotesting of Environmental Samples—Is There a Need for Consideration in the Acute Zebrafish Embryo Assay? *Environmental Toxicology*. 23(6), 745-750.

Lam TJ. 1994. Hormones and Egg/Larval Quality in Fish. *Journal of the World Aquaculture Society*. 25(1), 2-12.

Lane T, Janz DM, Liber K, Green D, Bluhm K, Raes K, Doig LE, and Hecker M. 2017. Developmental Effects of Maternally Transferred Selenium to Early Life Stage Fathead Minnow (*Pimephales promelas*). Poster WP031 at SETAC North America 38th Annual Meeting.

Lane T. 2019. Reproductive and Developmental Effects of Selenomethionine in the Fathead Minnow *Pimephales promelas* (Master's thesis, College of Graduate Studies Research, University of Saskatchewan, Saskatchewan, Canada).

Lavado R, Shi D, and Schlenk D. Effects of Salinity on the Toxicity and Biotransformation of L-Selenomethionine in Japanese Medaka (*Oryzias latipes*) Embryos: Mechanisms of Oxidative Stress. *Aquatic Toxicology*. 108, 18-22.

Lazard M, Dauplais M, Blanquet S, and Plateau P. 2017. Recent Advances in the Mechanism of Selenoamino Acids Toxicity in Eukaryotic Cells. *Biomolecular Concepts.* 8(2), 93-104.

Le Menn F, Cerdà J, and Babin PJ. 2007. Ultrastructural Aspects of the Ontogeny and Differentiation of Ray-Finned Fish Ovarian Follicles. In: Babin PJ, Cerdà J, Lubzens E. (eds). *The Fish Oocyte: From Basic Studies to Biotechnological Applications.* Springer, Dordrecht, The Netherlands. 1-38.

LeBlanc KL, and Wallschläger D. 2016. Production and Release of Selenomethionine and Related Organic Selenium Species by Microorganisms in Natural and Industrial Waters. *Environmental Science & Technology*. 50(12), 6164-6171.

Lemly DA. 1993. Subsurface Agricultural Irrigation Drainage: The Need for Regulation. *Regulatory Toxicology and Pharmacology.* 17(2), 157-180.

Lemly DA. 1997. A Teratogenic Deformity Index for Evaluating Impacts of Selenium on Fish Populations. *Ecotoxicology and Environmental Safety*. 37(3), 259-266.

Lemly DA. 2002. Symptoms and Implications of Selenium Toxicity in Fish: The Belews Lake case example. *Aquatic Toxicology*. 57(1-2), 39–49.

Lemly DA. 2004. Aquatic Selenium Pollution is a Global Environmental Safety Issue. *Ecotoxicology and Environmental Safety*. 59(1), 44-56.

Liu C, Xu H, Lam SH, Gong Z. 2013. Selection of Reliable Biomarkers from PCR Array Analyses Using Relative Distance Computational Model: Methodology and Proof-of-Concept Study. *PLoS ONE*. 8(12): e83954.

Lobanov AV, Hatfield DL, and Gladyshev VN. 2009. Eukaryotic Selenoproteins and Selenoproteomes. *Biochimica et Biophysica Acta - General Subjects*. 1790(11), 1424–1428.

Luoma SN and Presser TS. 2009. Emerging Opportunities in Management of Selenium Contamination. *Environmental Science & Technology*. 43(22), 8483-8487.

Maher WA, Roach A, Doblin M, Fan T, Foster S, Garrett R, Möller G, Oram L, and Wallschlager D. 2010. Environmental Sources, Speciation, and Partitioning of Selenium. In: Chapman PM, Adams WJ, Brooks ML, Delos CG, Luoma SN, Maher WA, Ohlendorf HM, Presser TS, and Shaw DP (eds). *Ecological Assessment of Selenium in the Aquatic Environment*. CRC Press, Boca Raton, FL. pp. 47–92.

Maier KJ. 1990. The Toxicity and Bioaccumulation of Selenium and Boron to *Daphnia magna* and *Chironomus decorus.* (Doctoral dissertation). Retrieved from ProQuest Dissertations & Theses Global. (Order No. 9102095).

Malandrakis EE, Exadactylos A, Dadali O, Golomazou E, Klaoudatos S, and Panagiotaki P. 2014. Molecular Cloning of Four Glutathione Peroxidase (GPx) Homologs and Expression Analysis during Stress Exposure of the Marine Teleost *Sparus aurata*. *Comparative Biochemistry and Physiology*, Part B. 168(1), 53-61.

Mariotti M, Ridge PG, Zhang Y, Lobanov AV, Pringle TH, Guigo R, Hatfield DL, and Gladyshev VN. 2012. Composition and Evolution of the Vertebrate and Mammalian Selenoproteomes. *PLoS ONE*. 7(3), E33066.

Marsili A, Zavacki A, Harney J, and Larsen P. 2011. Physiological Role and Regulation of Iodothyronine Deiodinases: A 2011 update. *Journal of Endocrinological Investigation.* 34(5), 395-407.

Masse A. 2016. Reproductive and Developmental Effects of Elevated Maternal Dietary Selenium in the Model Amphibian *Xenopus laevi*s (Master's thesis, College of Graduate Studies Research, University of Saskatchewan, Saskatchewan, Canada).

Massé AJ, Muscatello JR, and Janz DM. 2015. Dose-Dependent Early Life Stage Toxicities in *Xenopus laevis* Exposed In Ovo to Selenium. *Environmental Science & Technology*. 49(22), 13658-3666.

McDonald B and Chapman P. 2009. The Need for Adequate Quality Assurance/Quality Control Measures for Selenium Larval Deformity Assessments: Implications for Tissue Residue Guidelines. *Integrated Environmental Assessment and Management.* 5(3), 470-475.

Mejri S, Tremblay R, Vandenberg G, Moren M, Khemis IB, and Audet C. 2017. Differences in Nutrient Content of Eggs and Larvae as Indicators for Improvement of Broodstock Nutrition in Walleye (*Sander vitreus*) Production. *Canadian Journal of Zoology*. 95(5), 299-310.

Meseck SL and Cutter GA. 2006. Evaluating the Biogeochemical Cycle of Selenium in San Francisco Bay Through Modeling. *Limnology and Oceanography*. 51(5), 2018-2032.

Michibata H. 1981. Uptake and Distribution of Cadmium in the Egg of the Teleost, *Oryzias latipes*. *Journal of Fish Biology*. 19(6), 691-696.

Michiels C, Raes M, Toussaint O, and Remacle J. 1994. Importance of SE-Glutathione Peroxidase, Catalase, and CU/ZN-SOD for Cell Survival Against Oxidative Stress. *Free Radical Biology and Medicine*. 17(3), 235-248.

Migaud H, Bell G, Cabrita E, McAndrew B, Davie A, Bobe J, Herraez MP, and Carrillo M. 2013. Gamete Quality and Broodstock Management in Temperate Fish. *Reviews in Aquaculture*. 5(S1), S194-S223.

Misra S, Hamilton C, and Niyogi S. 2012. Induction of Oxidative Stress by Selenomethionine in Isolated Hepatocytes of Rainbow Trout (*Oncorhynchus mykiss*). *Toxicology in Vitro*. 26(4), 621-629.

MoEE (Ministry of Environment and Energy), 1994. Water Management Policies, Guidelines, Provincial Water Quality Objectives of the Ministry of Environment and Energy. Ontario Ministry of Environment and Energy, Toronto, ON, pp.1-67.

Mohammad N, Salahinejad A, Jamwal A, Chivers DP, and Niyogi S. 2017. Chronic Dietary Selenomethionine Exposure Induces Oxidative Stress, Dopaminergic Dysfunction, and

Cognitive Impairment in Adult Zebrafish (*Danio rerio*). *Environmental Science & Technology*. 51(21), 12879-2888.

Moroder L. 2005. Isosteric Replacement of Sulfur with Other Chalcogens in Peptides and Proteins. *Journal of Peptide Science*. 11(4), 187-214.

Muscatello JR, Bennett PM, Himbeault KT, Belknap AM, and Janz DM. 2006. Larval Deformities Associated with Selenium Accumulation in Northern Pike (*Esox lucius*) Exposed to Metal Mining Effluent. *Environmental Science & Technology*. 40(2), 6506-6512.

Nakamura Y, Yamamoto H, Sekizawa J, Kondo T, Hirai N, and Tatarazako N. The Effects of pH on Fluoxetine in Japanese Medaka (*Oryzias latipes*): Acute Toxicity in Fish Larvae and Bioaccumulation in Juvenile Fish. *Chemosphere*. 70(5), 865-873.

Nasiadka A and Clark MD. 2012. Zebrafish Breeding in the Laboratory Environment. *ILAR*. 53(2), 161-168.

Neumann PM, De Souza MP, Pickering IJ, and Terry N. 2003. Rapid Microalgal Metabolism of Selenate to Volatile Dimethylselenide. *Plant, Cell & Environment*. 26(6), 897−905.

Nguyen T, Nioi P, and Pickett CB. 2009. The Nrf2-Antioxidant Response Element Signaling Pathway and Its Activation by Oxidative Stress. *Journal of Biological Chemistry*. 284(20), 13291-13295.

Niimi AJ and Laham QN. 1976. Relative Toxicity of Organic and Inorganic Compounds of Selenium to Newly Hatched Zebrafish (*Brachydanio rerio*). *Canadian Journal of Zoology*. 54(4), 501-509.

Oakley A. 2011. Glutathione Transferases: A Structural Perspective. *Drug Metabolism Reviews*. 43(2), 138-151.

Okuno T, Kubota T, Kuroda T, Ueno H, and Nakamuro K. (2001). Contribution of Enzymic α,γ-Elimination Reaction in Detoxification Pathway of Selenomethionine in Mouse Liver. *Toxicology and Applied Pharmacology*. 176(1), 18-23.

Ookawara T, Imazeki N, Matsubara O, Kizaki T, Oh-Ishi S, Nakao C, Sato Y, and Ohno H. 1998. Tissue Distribution of Immunoreactive Mouse Extracellular Superoxide Dismutase. *American Journal of Physiology-Cell Physiology*. 275(3), C840-847.

Orr PL, Guiguer KR, and Russel CK. 2006. Food Chain Transfer of Selenium in Lentic and Lotic Habitats of a Western Canadian Watershed. *Ecotoxicology and Environmental Safety*. 63(2), 175-188.

Palace VP and Werner J. 2006. Vitamins A and E in the Maternal Diet Influence Egg Quality and Early Life Stage Development in Fish: A Review. *Scientia Marina*. 70(2), 41-57.

Palace VP, Spallholz JE, Holm J, Wautier K, Evans RE, and Baron CL. 2004. Metabolism of Selenomethionine by Rainbow Trout (*Oncorhynchus mykiss*) Embryos Can Generate Oxidative Stress. *Ecotoxicology and Environmental Safety*. 58(1), 17-21.

Papp LV, Lu J, Holmgren A, and Khanna KK. 2007. From Selenium to Selenoproteins: Synthesis, Identity, and Their Role in Human Health. Antioxidants & Redox Signaling. 9(7), 775-806.

Patra RW, Chapman JC, Lim RP, Gehrke PC, and Sunderam RM. 2015. Interactions Between Water Temperature and Contaminant Toxicity to Freshwater Fish. *Environmental Toxicology and Chemistry*. 34(8), 1809-1817.

Pedrero Z and Madrid Y. 2009. Novel Approaches for Selenium Speciation in Foodstuffs and Biological Specimens: A Review. *Analytica Chimica Acta*. 634(2), 135-152.

Perry DM. 1984. Post-fertilization Changes in the Chorion of Winter Flounder, Pseudopleuronectes Americanus Walbaum, Eggs Observed with Scanning Electron Microscopy. *Journal of Fish Biology*. 25(1), 83-94.

Peters GM, Maher WA, Jolley D, Carroll BI, Gomes VG, Jenkinson AV, and McOrist GD. 1999. Selenium Contamination, Redistribution and Remobilisation in Sediments of Lake Macquarie, NSW. *Organic Geochemistry*. 30(10), 1287-1300.

Pettem CM, Briens JM, Janz DM, and Weber LP. 2018. Cardiometabolic Response of Juvenile Rainbow Trout Exposed to Dietary Selenomethionine. *Aquatic Toxicology*. 198, 175-189.

Pettem CM, Weber LP, and Janz DM. 2017. Cardiac and Metabolic Effects of Dietary Selenomethionine Exposure in Adult Zebrafish. *Toxicological Sciences.* 159(2), 449-460.

Phibbs J, Franz E, Hauck D, Gallego M, Tse JJ, Pickering IJ, Liber K, and Janz DM. 2011. Evaluating the Trophic Transfer of Selenium in Aquatic Ecosystems Using Caged Fish, X-ray Absorption Spectroscopy and Stable Isotope Analysis. *Ecotoxicology and Environmental Safety*. 74(7), 1855-1863.

Plateau P, Saveanu C, Lestini R, Dauplais M, Decourty L, Jacquier A, Blanquet S, and Lazard M. 2017. Exposure to Selenomethionine Causes Selenocysteine Misincorporation and Protein Aggregation in *Saccharomyces cerevisiae*. *Scientific Reports.* 7(1), 44761.

Presser TS, Piper DZ, Bird KJ, Skorupa JP, Hamilton SJ, Detwiler SJ, and Huebner MA. 2004. The Phosphoria Formation: A Model for Forecasting Global Selenium Sources to the Environment. In: Hein, J (ed). Life Cycle of the Phosphoria Formation from Deposition to the Post-Mining Environment. *Handbook of Exploration and Environmental Geochemistry.* Elsevier, San Diego, CA. 8, pp. 299-319.

Riedel GF, Ferrier DP and Sanders JG. 1991. Uptake of Selenium by Freshwater Phytoplankton. *Water, Air, & Soil Pollution.* 57(1), 23-30.

Rigby MC, A. Lemly DA, and Gerads R. Fish Toxicity Testing with Selenomethionine Spiked Feed - What's the Real Question Being Asked? *Journal of Environmental Monitoring.* 16(3), 511-517.

Ruan W and Lai M. 2007. Actin, a Reliable Marker of Internal Control?" *Clinica Chimica Acta*. 385(1-2), 1-5.

Rudolph B-L, Andreller I, Kennedy CJ. 2008. Reproductive Success, Early Life Stage Development, and Survival of Westslope Cutthroat Trout (*Oncorhynchus clarki* L.) exposed to elevated selenium in an area of active coal mining. *Environmental Science & Technology.* 42(8), 3109–3114.

Rushmore TH, Morton MR, and Pickett CB. 1991. The Antioxidant Responsive Element. Activation by Oxidative Stress and Identification of the DNA Consensus Sequence Required for Functional Activity. *The Journal of Biological Chemistry*. 266(18), 11632-11639.

Samarin AM, Policar T, and Lahnsteiner F. 2015. Fish Oocyte Ageing and Its Effect on Egg Quality. *Reviews in Fisheries Science & Aquaculture*. 23(3), 302-314.

Sant KE, Hansen JM, Williams LM, Tran NL, Goldstone JV, Stegeman JJ, Hahn ME, Timme-Laragy A. 2017. The Role of Nrf1 and Nrf2 in the Regulation of Glutathione and Redox Dynamics in the Developing Zebrafish Embryo. *Redox Biol.* 13(C), 207-218.

Schrauzer GN. 2000. Selenomethionine: A Review of Its Nutritional Significance, Metabolism and Toxicity. *The Journal of Nutrition.* 130(7), 1653-1656.

Schreck CB. 2010. Stress and Fish Reproduction: The Roles of Allostasis and Hormesis. *General and Comparative Endocrinology*. 165(3), 549-556.

Schwarz K and Foltz CM. 1957. Selenium as an Integral Part of Factor 3 Against Dietary Necrotic Liver Degeneration. *Journal of the American Chemical Society*. 79(12), 3292-3293.

Sciera KL, Isely JJ, Tomasso JR, and Klaine SJ. 2004. Influence of Multiple Water-quality Characteristics on Copper Toxicity to Fathead Minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry*. 23(12), 2900-2905.

Shang EHH and Wu RSS. 2004. Aquatic Hypoxia Is a Teratogen and Affects Fish Embryonic Development. *Environmental Science & Technology*. 38(18), 4763-4767.

Singleman C and Holtzman NG. 2014. Growth and Maturation in the Zebrafish, *Danio rerio*: A Staging Tool for Teaching and Research. *Zebrafish*. 11(4), 396-406.

Song L, Vijver MG, Peijnenburg WJGM, Galloway TS, and Tyler CR. 2015. A Comparative Analysis on the In Vivo Toxicity of Copper Nanoparticles in Three Species of Freshwater Fish. *Chemosphere*. 139, 181-189.

Spallholz JE, Shriver BJ, and Reid TW. 2001. Dimethyldiselenide and Methylseleninic Acid Generate Superoxide in an In Vitro Chemiluminescence Assay in the Presence of Glutathione: Implications for the Anticarcinogenic Activity of L-Selenomethionine and L-Se-Methylselenocysteine. *Nutrition and Cancer.* 40(1), 34-41.

Spallholz JE. 1994. On the Nature of Selenium Toxicity and Carcinostatic Activity. *Free Radical Biology and Medicine*. 17(1), 45-64.

Springate JRC, Bromage NR, Elliott JAK, and Hudson DL. 1984. The Timing of Ovulation and Stripping and Their Effects on the Rates of Fertilization and Survival to Eying, Hatch and Swim-up in the Rainbow Trout (*Salmo gairdneri* R.). *Aquaculture*. 43(1-3), 313-322.

Stadtman TC. 1996. Selenocysteine. *Annual Review of Biochemistry*. 65, 83-100.

Stewart R, Grosell M, Buchwalter D, Fisher N, Luoma S, Mathews T, Orr P, and Wang W. 2010. Bioaccumulation and Trophic Transfer of Selenium. In: Chapman PM, Adams WJ, Brooks ML, Delos CG, Luoma SN, Maher WA, Ohlendorf HM, Presser TS, and Shaw DP (eds). *Ecological Assessment of Selenium in the Aquatic Environment*. CRC Press, Boca Raton, FL. pp. 93-139.

Strecker R, Seiler T-B, Hollert H, and Braunbeck T. 2011. Oxygen Requirements of Zebrafish (*Danio rerio*) Embryos in Embryo Toxicity Tests with Environmental Samples. *Comparative Biochemistry and Physiology*, Part C. 153(3), 318-327.

Stürzenbaum SR and Kille P. 2001. Control Genes in Quantitative Molecular Biological Techniques: The Variability of Invariance. *Comparative Biochemistry and Physiology*, Part B. 130(3), 281-289.

Suga N. 1963. Change of the Toughness of the Chorion of Fish Eggs. *Embryologia*. 8(1), 63-74.

Sunde RA. 1984. The Biochemistry of Selenoproteins. *Journal of the American Oil Chemists Society*. 12, 1891-900.

Suzuki KT and Ogra Y. 2002. Metabolic Pathway for Selenium in the Body: Speciation by HPLC-ICP MS with Enriched Se. *Food Additives and Contaminants.* 19(10), 974-983.

Suzuki T, Takagi Y, Osanai H, Li L, Takeuchi M, Katoh Y, Kobayashi M, and Yamamoto M. 2005. Pi Class Glutathione S-transferase Genes are Regulated by Nrf 2 Through an Evolutionarily Conserved Regulatory Element in Zebrafish. *The Biochemical Journal*. 388(1), 65-73.

Tapiero H, Townsend DM, and Tew KD. 2003. The Antioxidant Role of Selenium and Seleno-Compounds. *Biomedicine & Pharmacotherapy*. 57(3), 134–144

Thomas JK and Janz DM. 2014. In Ovo Exposure to Selenomethionine via Maternal Transfer Increases Developmental Toxicities and Impairs Swim Performance in F1 Generation Zebrafish (*Danio rerio*). *Aquatic Toxicology*. 152, 20-29.

Thomas JK and Janz DM. 2015. Developmental and Persistent Toxicities of Maternally Deposited Selenomethionine in Zebrafish (*Danio rerio*). *Environmental Science & Technology*. 49(16), 10182-10189.

Thomas JK, and Janz DM. 2016. Embryo Microinjection of Selenomethionine Reduces Hatchability and Modifies Oxidant Responsive Gene Expression in Zebrafish. *Scientific Reports*. 6, 26520.

Tiedke J, Cubuk C, Burmester T. 2013. Environmental Acidification Triggers Oxidative Stress and Enhances Globin Expression in Zebrafish Gills. *Biochemical and Biophysical Research Communications.*441(3), 624–629.

Timme-Laragy AR, Karchner SI, Franks DG, Jenny MJ, Harbeitner RC, Goldstone JV, Mcarthur AG, and Hahn ME. 2012. Nrf2b, Novel Zebrafish Paralog of Oxidant-responsive Transcription Factor NF-E2-related Factor 2 (NRF2). *Journal of Biological Chemistry.* 287(7), 4609-4627.

Tyler CR and Lancaster P. 1993. Isolation and Characterization of the Receptor for Vitellogenin from Follicles of the Rainbow Trout, *Oncorhynchus mykiss*. *Journal of Comparative Physiology B.* 163(3), 225-233.

Tyler CR and Sumpter JP. 1996. Oocyte Growth and Development in Teleosts. *Reviews in Fish Biology and Fisheries*. 6(3), 287-318.

Unrine JM, Jackson BP, Hopkins WA, and Romanek C. 2006. Isolation and Partial Characterization of Proteins Involved in Maternal Transfer of Selenium in the Western Fence Lizard (*Sceloporus occidentalis*). *Environmental Toxicology and Chemistry.* 25(7), 1864-867. Web.

US EPA (United States Environmental Protection Agency). 2016. Aquatic Life Ambient Water Quality Criterion for Selenium (Freshwater). EPA 822-R-16-006 Review. Office of Water, Washington, DC, pp. 15.

US EPA. 1987. Guidelines for the Culture of Fathead Minnows, *Pimephales promelas* for Use in Toxicity Tests.

US EPA. 1996. Prehatching Development of the Fathead Minnow *Pimephales promelas* Rafinesque. US EPA, Duluth, MN, EPA/600/R-96/079.

Veres Z, Kim IY, Scholz TD, and Stadtman TC. 1994. Selenophosphate Synthetase. *The Journal of Biological Chemistry.* 269(14), 10597-10603.

Wang Z, Meador JP, and Leung KMY. 2016. Metal Toxicity to Freshwater Organisms as a Function of pH: A Meta-analysis. *Chemosphere*. 144, 1544-1552.

Wedemeyer G. 1968. Uptake and Distribution of ^{65}Zn in the Coho Salmon Egg (*Oncorhynchus kisutch*). *Comparative Biochemistry and Physiology*. 26, 271-279.

Westernhagen H and Dethlefsen VW. 1975. Combined Effects of Cadmium and Salinity on Development and Survival of Flounder Eggs. *Journal of the Marine Biological Association of the United Kingdom*. 55, 945-957.

Westernhagen H, Rosenthal H, and Sperling K-R. 1974. Combined Effects of Cadmium and Salinity on Development and Survival of Herring Eggs. *Helgolander wiss. Meeresunters*. 26, 416 433.

Wootton RJ and Smith C. 2015. Reproductive Biology of Teleost Fishes. John Wiley & Sons, Ltd., Chichester, West Sussex, UK.

Wu RSS, Zhou BS, Randall DJ, Woo NYS, and Lam PKS. 2003. Aquatic Hypoxia Is an Endocrine Disruptor and Impairs Fish Reproduction. *Environmental Science & Technology.* 37(6), 1137-1141.

Young TF, Finley K, Adams WJ, Besser J, Hopkins WD, Jolley D, McNaughton E, Presser TS, Shaw DP, and Unrine J. 2010. What You Need to Know About Selenium. In: Chapman PM, Adams WJ, Brooks ML, Delos CG, Luoma SN, Maher WA, Ohlendorf HM, Presser TS, and Shaw DP (eds). *Ecological Assessment of Selenium in the Aquatic Environment*. CRC Press, Boca Raton, FL. pp. 7-45.

Zee J, Patterson S, Wiseman S, and Hecker M. 2016. Is Hepatic Oxidative Stress a Main Driver of Dietary Selenium Toxicity in White Sturgeon (*Acipenser transmontanus*)? *Ecotoxicology and Environmental Safety.* 133, 334-340.

Zhang DD and Hannink M. 2003. Distinct Cysteine Residues in Keap1 Are Required for Keap1-Dependent Ubiquitination of Nrf2 and for Stabilization of Nrf2 by Chemopreventive Agents and Oxidative Stress. *Molecular and Cellular Biology*. 23(22), 8137-8151.

Zhang Y and Moore JN. 1996. Selenium Fractionation and Speciation in a Wetland System. *Environmental Science & Technology*. 30(8), 2613-2619.

Zhong L, Arnér E SJ, and Holmgren A. 2000. Structure and Mechanism of Mammalian Thioredoxin Reductase: The Active Site is a Redox-Active Selenolthiolyselenenylsulfide Formed from the Conserved Cysteine-Selenocysteine Sequence. *Proceedings of the National Academy of Sciences of the United States of America*. 97(11), 5854-5859.

APPENDIX[a]

[a]The supplementary data included in this appendix have been published in both the *Bulletin of Environmental Contamination and Toxicology* (2019), 102(3), 323-328, under joint authorship with Markus Hecker (University of Saskatchewan) and David M. Janz (University of Saskatchewan), as well as in *Toxics*, Special Issue: Contaminant Effects on Zebrafish Embryos (2019), 7(3), 44, under joint authorship with David M. Janz (University of Saskatchewan). References cited in the supporting information are included in the reference section. The figure or table number is presented as Cx.Sy format, where 'Cx' indicates chapter number; 'Sy' indicates figure or table number.

Table C3.S1 Target genes and their primer sequences, accession numbers, and efficiencies used for real time qRT-PCR (Liu et al. 2013; Tiedke et al. 2013; Arnold et al. 2016; Thomas & Janz, 2016)

Target Gene	**Accession #**	**Primer sequences (5'-3')**	**Efficiency (%)**
nrf2a	NM_182889.1	F: ACACACACCTGAAGCAGACG R: GGCATCATGAGATCAGTGGA	135.054
gpx1a	NM_001007281.2	F: GAAATACGTCCGTCCTGGAA R: CATAAGGGACACAGGGTCGT	106.439
gst p1	NM_131734.3	F: TGGTGCTTTGAAGATCATGC R: CTGAAACAGCACCAGGTCAC	94.656
sod2	NM199976	F: TATGCAGCTTCATCACAGCAAGCA R: GGTTGTCACATCACCCTTGGCC	92.043
gsr	NM001020554	F: ACAGTCAGTGAGGATGATGTGCCAG R: TAGACCCAAGAGTGGAAAGAATACCAGC	101.401
gclc	NM_199277	F: AAGTGGATGAGGGAGTTTGTTGCC R: CTTGTGGAGCAGGTCGTAGTTGAT	96.885
ahr2	AF063446.1	F: CCAGAGCCCTACACAAGCAT R: TCCTTAAGTGGACGGTTTGC	109.186
actb	AF057040	F: AAG ATC AAG ATC ATT GCT CCC R: CCA GAC TCA TCG TAC TCC T	101.269
EF1a	NM_131263.1	F: CTTCAACGCTCAGGTCATCA R: CGGTCGATCTTCTCCTTGAG	98.262

Figure C2.S1. Morphological malformations at varying degrees of severity observed in larval fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure during deformities analysis. Letters indicate level of severity; Control (C), Moderate (M), and Severe (S).

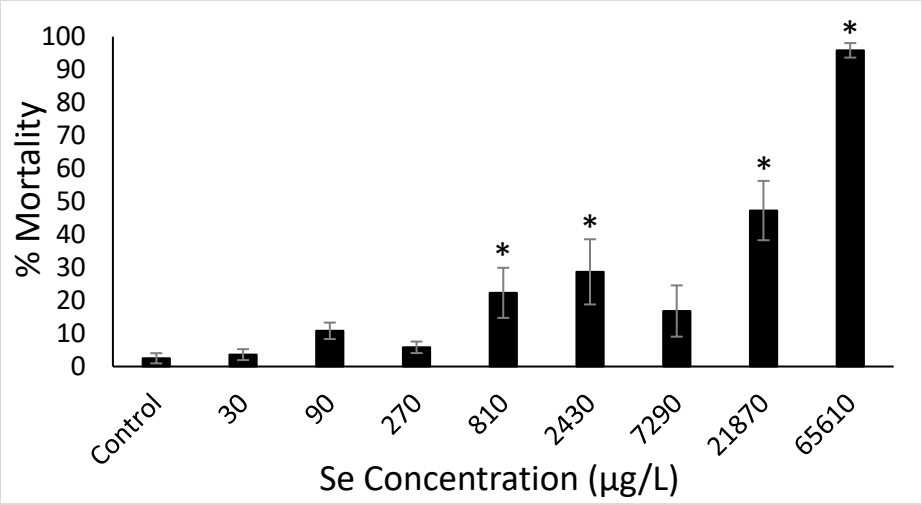


Figure C2.S2. Mean (± SE) percent cumulative mortality (1–6 dpf) of fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 6-16 replicates of 20-30 embryos.

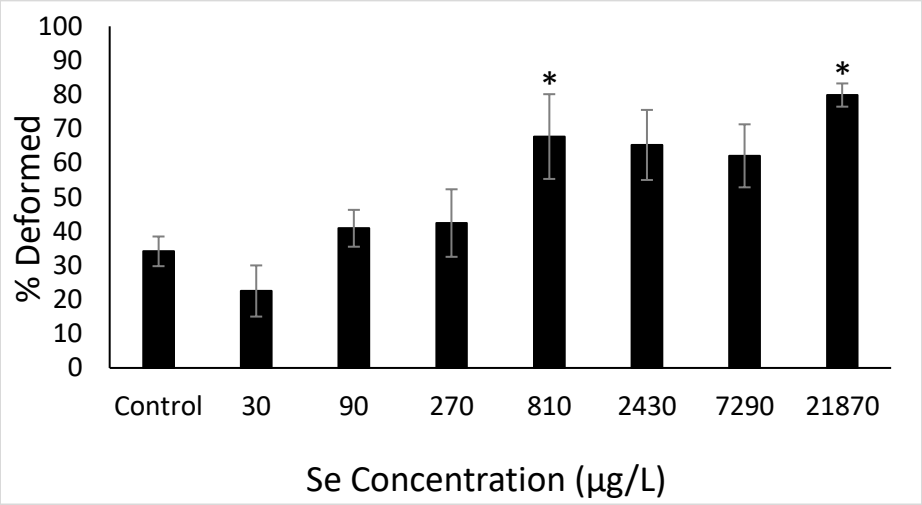


Figure C2.S3. Mean (±SE) percentage of total deformities (sum of skeletal, craniofacial, finfold, and edema) in larval fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences compared to the control using a one-way ANOVA followed by a Dunnett's multiple comparisons test ($p < 0.05$); n = 3-6 replicates of 20-30 embryos.

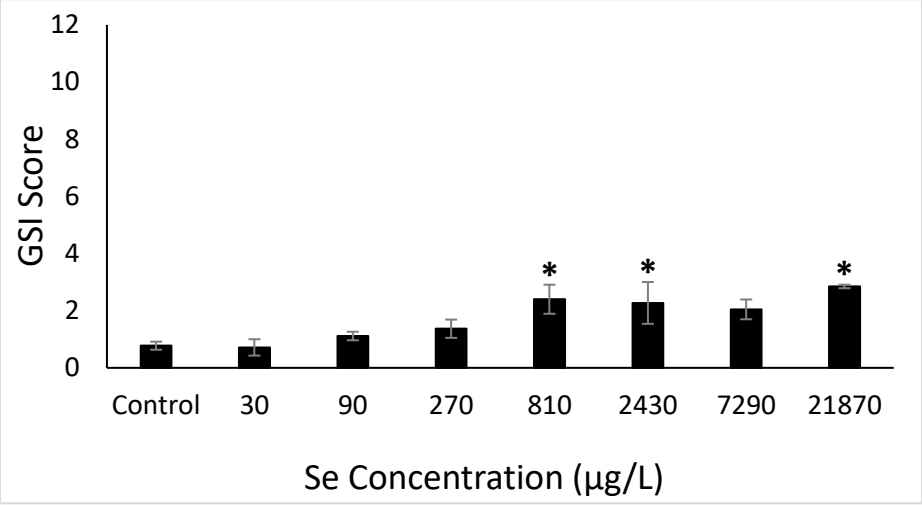


Figure C2.S4. Mean (±SE) graduated severity index (GSI) scores for total deformities (sum of skeletal, craniofacial, finfold, and edema scores) in larval fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences compared to the control using a one-way ANOVA followed by a Dunnett's multiple comparisons test ($p < 0.05$); n = 3-6 replicates of 20-30 embryos.

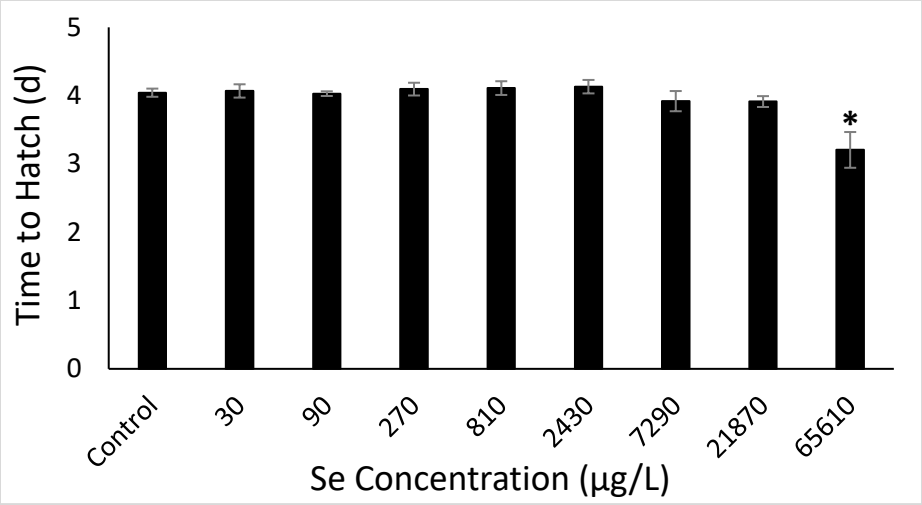


Figure C2.S5. Mean (± SE) time to hatch in fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 6-17 replicates of 20-30 embryos.

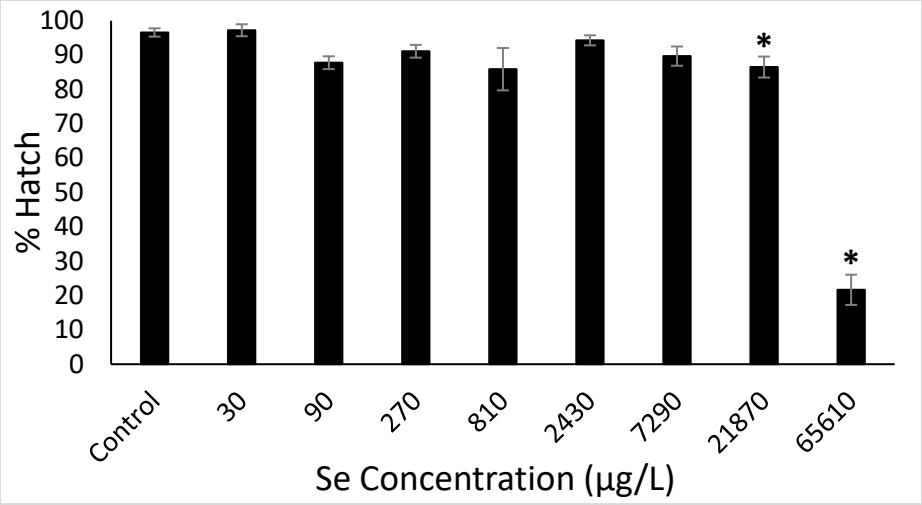


Figure C2.S6. Mean (± SE) percent hatch of fathead minnows exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 6-17 replicates of 20-30 embryos.

Figure C3.S1. Morphological malformations at varying degrees of severity observed in larval zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure during deformities analysis. Letters indicate level of severity; Control (C), Moderate (M), and Severe (S)**.**

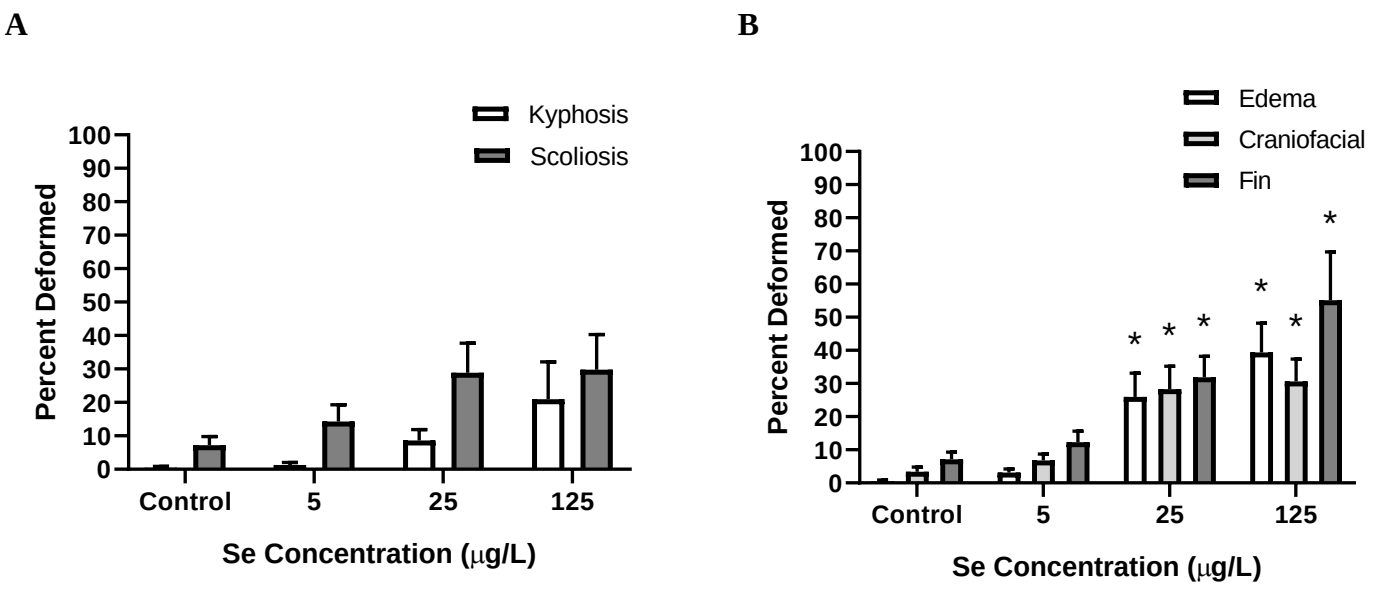


Figure C3.S2. Mean (±SE) percentage of (A) skeletal deformities (kyphosis and scoliosis) and (B) other deformities (craniofacial, finfold, and edema) in larval zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by Dunn's multiple comparisons test ($p < 0.05$); n = 9-12 replicates of 20 embryos.

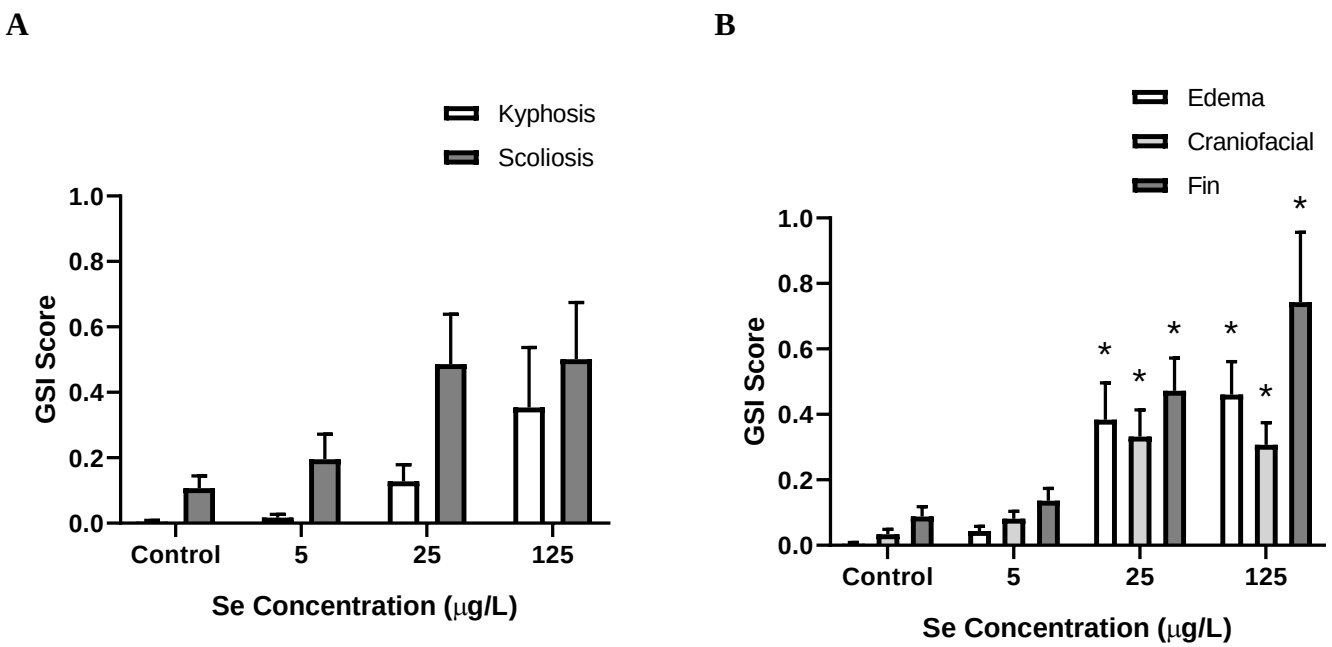


Figure C3.S3. Mean (±SE) graduated severity index (GSI) scores of (A) skeletal deformities (kyphosis and scoliosis) and (B) other deformities (craniofacial, finfold, and edema) in larval zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons ($p < 0.05$); n = 9-12 replicates of 20 embryos.

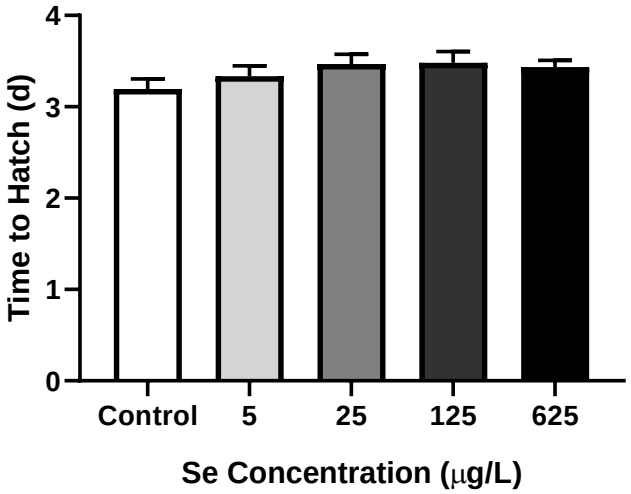


Figure C3.S4. Mean (± SE) time to hatch in zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 8-12 replicates of 20 embryos.

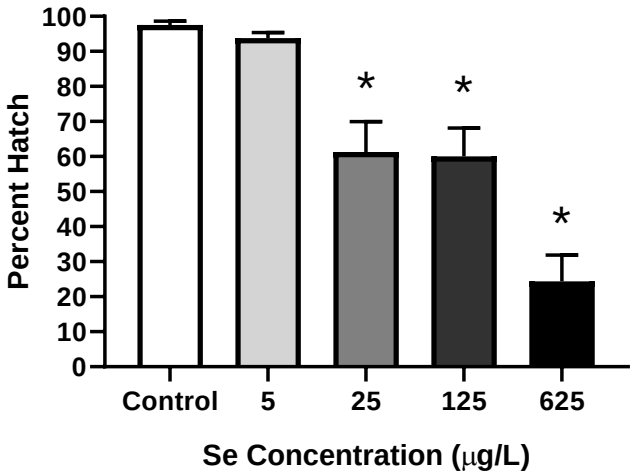


Figure C3.S5. Mean (± SE) percent hatch of zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 8-12 replicates of 20 embryos.

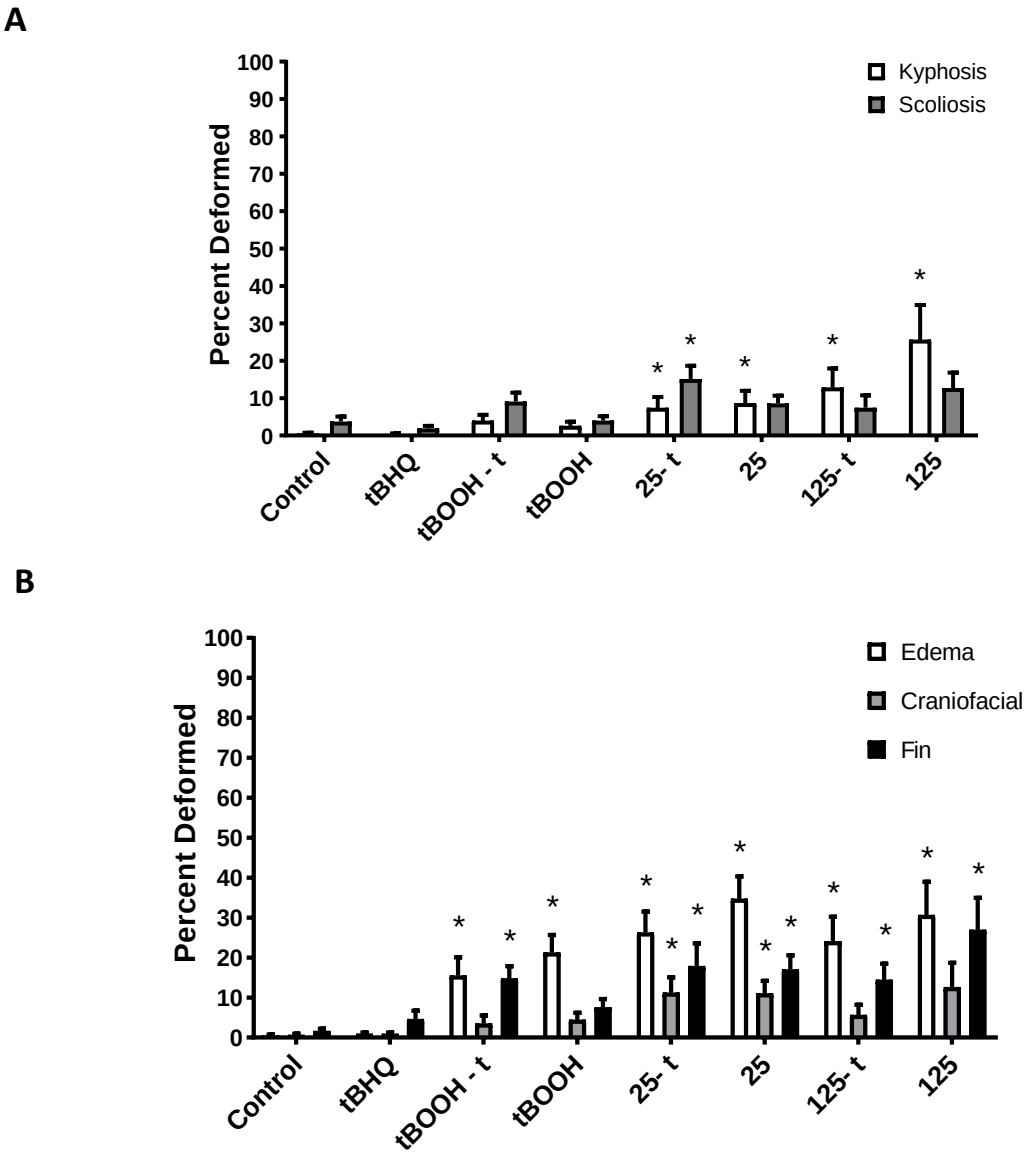


Figure C3.S6. Mean (±SE) percentage of (A) skeletal deformities (kyphosis and scoliosis) and (B) other deformities (craniofacial, finfold, and edema) in larval zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 13-33 replicates of 20 embryos.

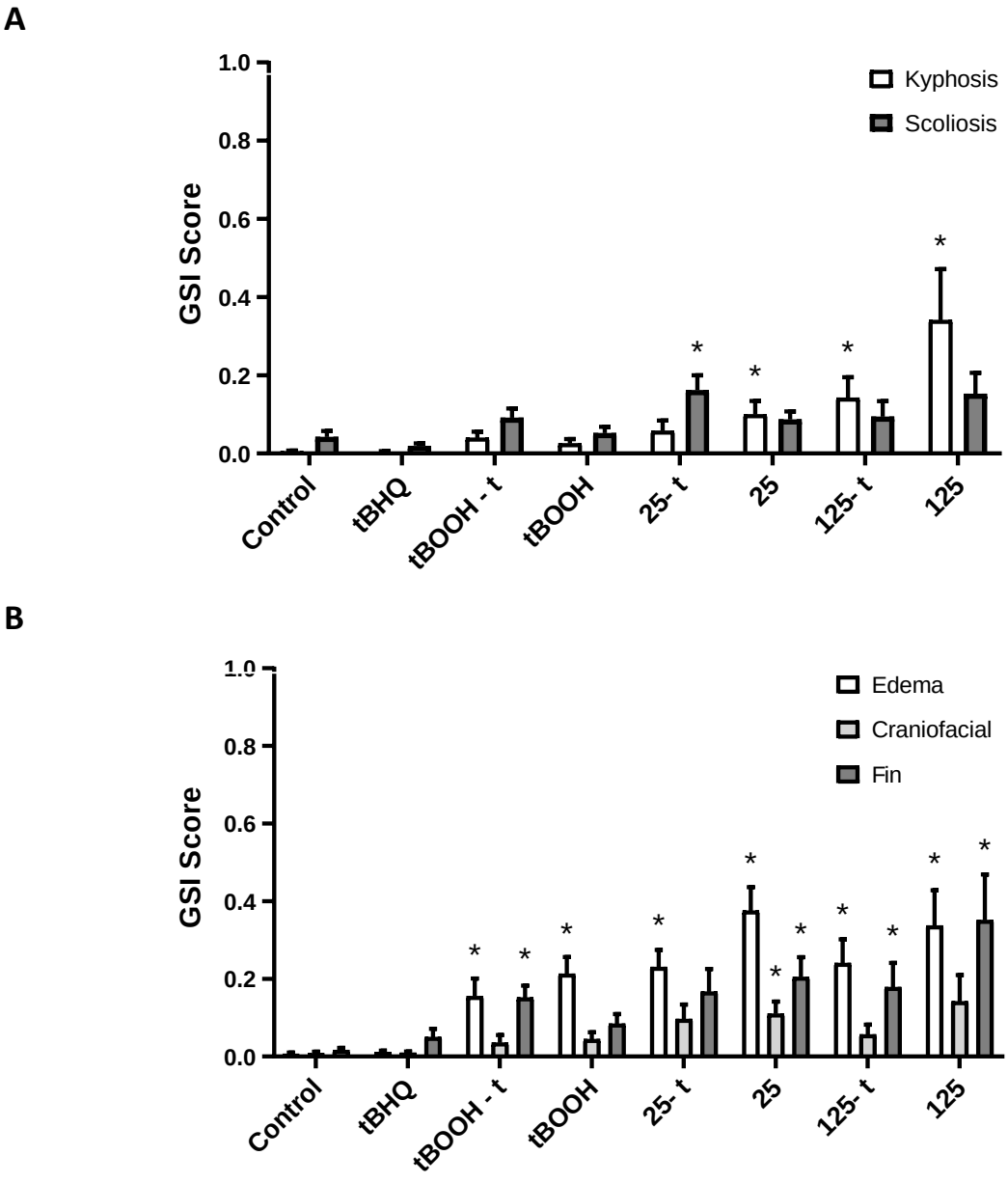


Figure C3.S7. Mean (±SE) graduated severity index (GSI) scores of (A) skeletal deformities (kyphosis and scoliosis) and (B) other deformities (craniofacial, finfold, and edema) in larval zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 13-33 replicates of 20 embryos.

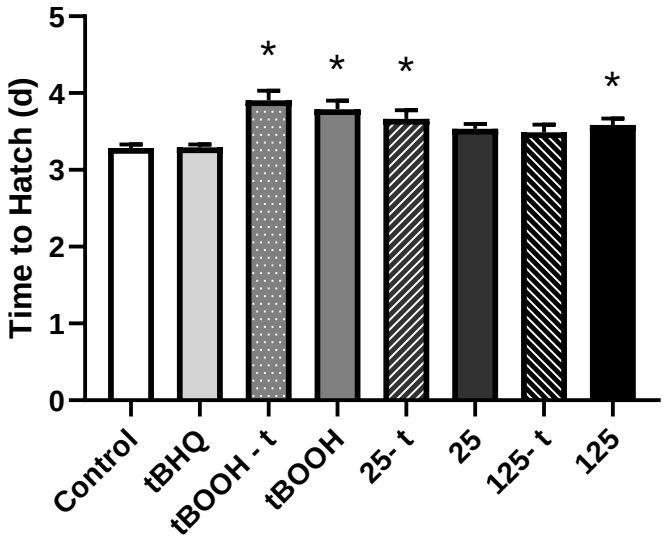


Figure C3.S8. Mean (± SE) time to hatch in zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 20-33 replicates of 20 embryos.

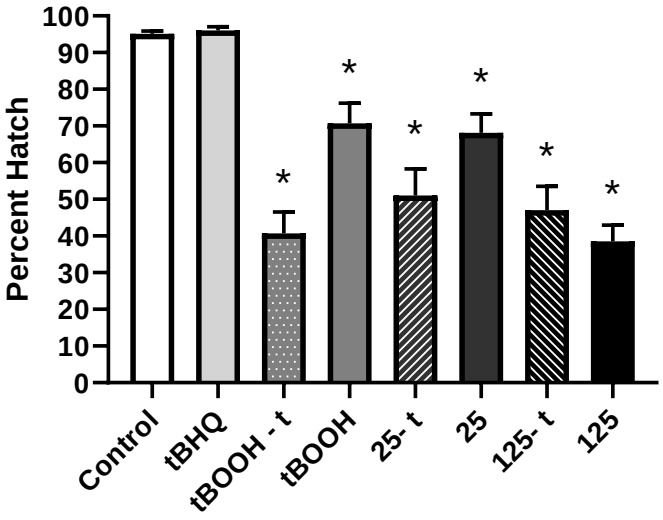
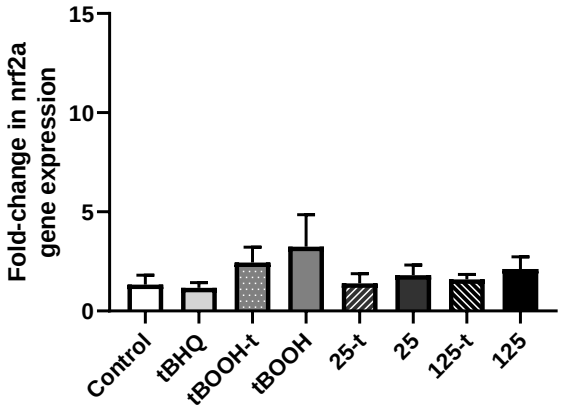
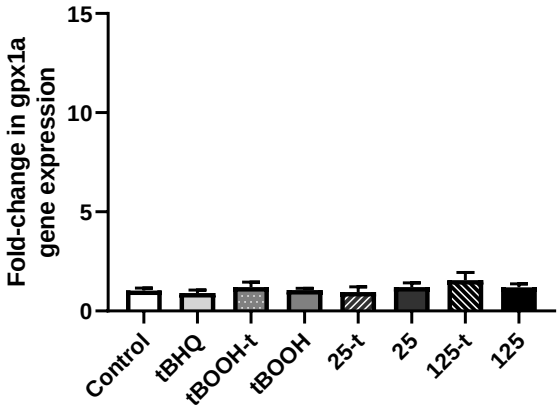
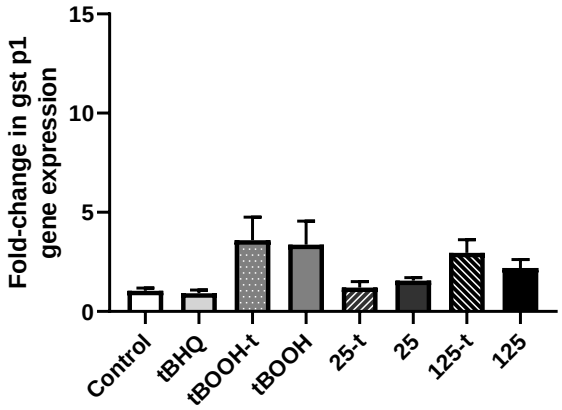
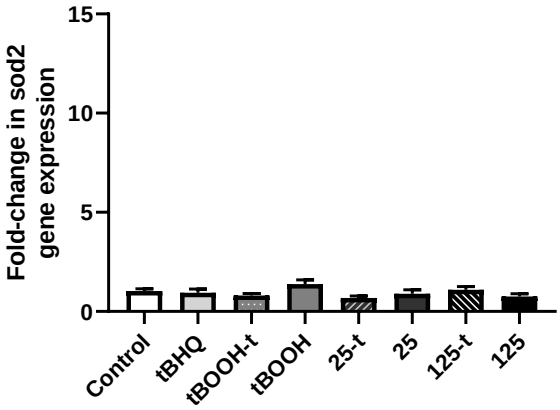


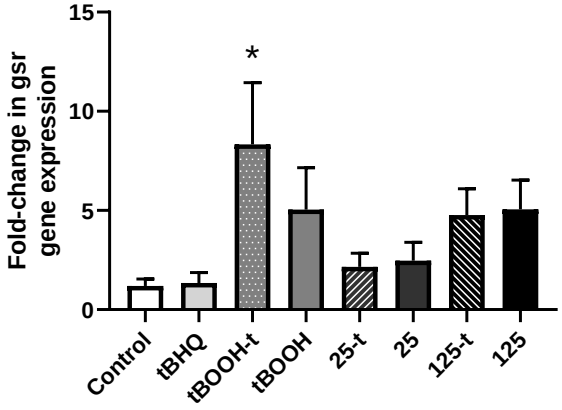
Figure C3.S9. Mean (± SE) percent hatch of zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 20-33 replicates of 20 embryos.

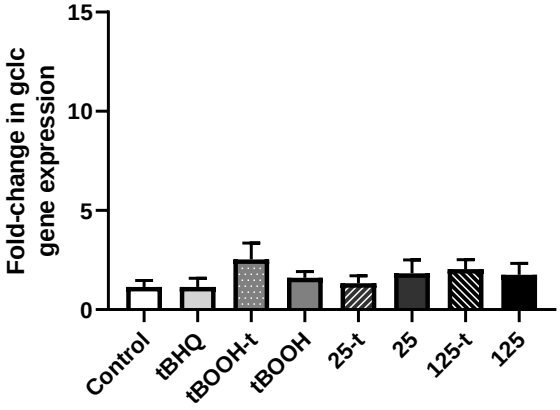

Fold-change in nrf2a gene expression
15
10
5
0
Control
tBHQ
tBOOH-t
tBOOH
25-t
25
125-t
125


Fold-change in gpx1a gene expression
15
10
5
0
Control
tBHQ
tBOOH-t
tBOOH
25-t
25
125-t
125


Fold-change in gst p1 gene expression
15
10
5
0
Control
tBHQ
tBOOH-t
tBOOH
25-t
25
125-t
125


Fold-change in sod2 gene expression
15
10
5
0
Control
tBHQ
tBOOH-t
tBOOH
25-t
25
125-t
125


Fold-change in gsr gene expression
*
15
10
5
0
Control
tBHQ
tBOOH-t
tBOOH
25-t
25
125-t
125


Fold-change in gclc gene expression
15
10
5
0
Control
tBHQ
tBOOH-t
tBOOH
25-t
25
125-t
125

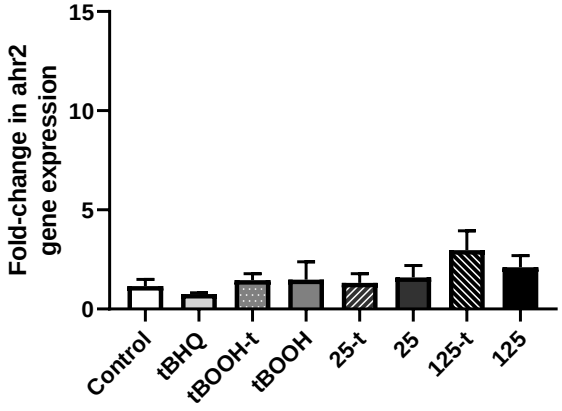

Fold-change in ahr2 gene expression
15
10
5
0
Control
tBHQ
tBOOH-t
tBOOH
25-t
25
125-t
125

Figure C3.S10. Mean (± SE) fold change in gene expression in zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences compared to the control using either a one-way analysis of variance (ANOVA) followed by Holm-Sidak's multiple comparisons test or a Kruskal–Wallis one-way ANOVA followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 4-5 replicates of 11-20 zebrafish larvae.

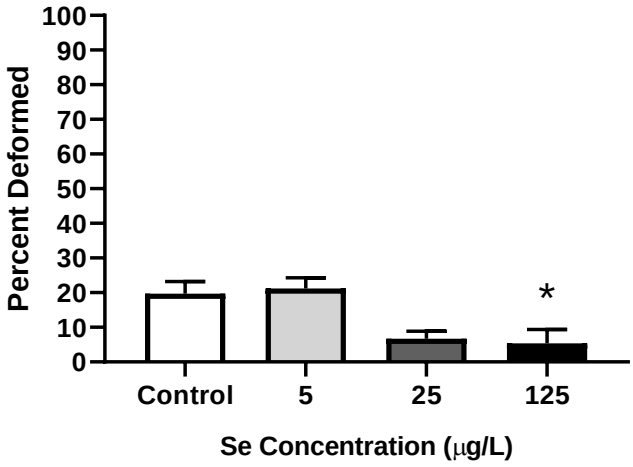


Figure C3.S11. Mean (±SE) percentage of lordosis in larval zebrafish exposed to increasing concentrations of L-selenomethionine (SeMet) via embryo aqueous exposure. Asterisks represent significant differences compared to the control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by Dunn's multiple comparisons test ($p < 0.05$); n = 9-12 replicates of 20 embryos.

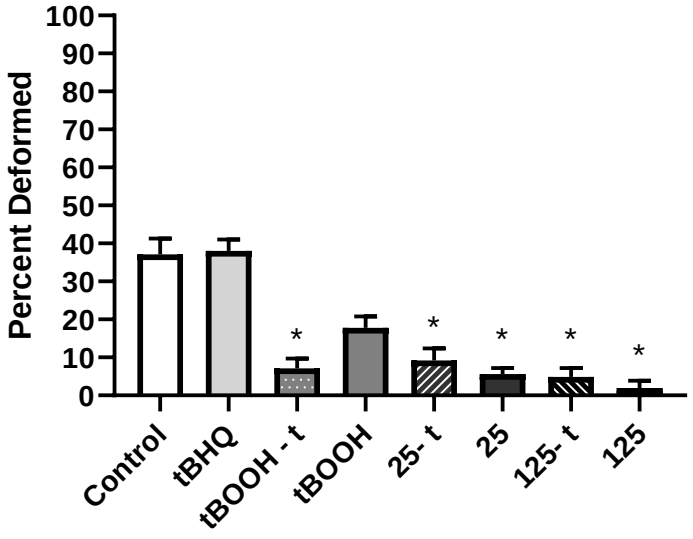


Figure C3.S12. Mean (±SE) percentage of lordosis in larval zebrafish exposed via embryo aqueous exposure to facility water (control), tBHQ, tBOOH, and two concentrations of SeMet (25 and 125 µg Se/L) either with (tBOOH-t, 25-t, 125-t) or without (tBOOH, 25, 125) a tBHQ pre-treatment. Asterisks represent significant differences from control using a Kruskal–Wallis one-way analysis of variance (ANOVA) by ranks followed by a Dunn's multiple comparisons test ($p < 0.05$); n = 13-33 replicates of 20 embryos.