January 2018

Effects Of Early Cadmium And Selenium Exposure On Zebrafish Neural Development And Behavior

Marissa Wold

EFFECTS OF EARLY CADMIUM AND SELENIUM EXPOSURE ON ZEBRAFISH NEURAL DEVELOPMENT AND BEHAVIOR

by

Marissa K. Wold
Bachelor of Science, University of Minnesota, 2015

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of Master of Science

Grand Forks, North Dakota

August
2018

This thesis, submitted by Marissa Wold, in partial fulfillment of the requirements for the degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Tristan Darland

Diane Darland

Seema Somji

This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Grant McGimpsey
Dean of the School of Graduate Studies

July 24, 2018
Date

PERMISSION

Title	Effects of Early Cadmium and Selenium Exposure on Zebrafish Neural Development and Behavior
Department	Biology
Degree	Master of Science

In presenting this thesis in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my thesis work or, in his absence, by the Chairperson of the department or the dean of the School of Graduate Studies. It is understood that any copying or publication or other use of this thesis or part 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 North Dakota in any scholarly use which may be made of any material in my thesis.

Marissa Wold
July 26, 2018

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to express particular gratitude to Dr. Tristan Darland for his mentorship and patience throughout this project. I could not have asked for a better advisor, and I am forever grateful. I also wish to express my sincerest appreciation to the other members of my advisory committee, Dr. Diane Darland and Dr. Seema Somji, for their guidance and support during my time in the master's program at the University of North Dakota. Thanks also to the members of the Darland lab for their contributions to the project; special thanks to Cody Boyle for his assistance. Thank you to Danielle Roush for her advice and support throughout this endeavor. Thanks to Dr. David Pierce and Mike Wang of the UND Chemistry Department. And, finally, thank you to our funding sources: ND EPSCoR and INBRE.

ABSTRACT

Cadmium is a naturally-occurring trace metal frequently found in soil that has been linked with increased prevalence of various cancers via formation of reactive oxygen species. Selenium, a widely-prevalent metalloid, antagonizes the detrimental action of cadmium and has been demonstrated to exert a rescue effect. The present work focuses on the short- and long-term effects of exposure to ecologically-relevant cadmium concentrations on zebrafish development and behavior, and compares this to co-treatment with selenium. This study has demonstrated a decrease in overall brain size, specifically telencephalic area, in response to cadmium exposure, and has documented a sparing effect of selenium treatment. A similar effect is seen in larval body size and eye diameter. This study has also reported an effect on spinal morphology and hatching delay. Longitudinally, cadmium treatment affects survival to six months, and has an impact on adult brain metrics. The results of behavioral assay indicate an effect of larval cadmium exposure on adult learning behavior, as well as a rescue effect of selenium.

INTRODUCTION

Background on Cadmium

Trace metals, also called 'heavy metals' for their high atomic weights, are a suite of elements including arsenic, cadmium, chromium, lead, and mercury. All of these are naturally-occurring substances found in varying concentrations throughout the earth's crust. Due to their utility, these metals are often mined for use by humans. Cadmium, especially, is sought after in industry, with an array of applications ranging from use in electroplating to inclusion in long-life batteries. In trace amounts, it is a useful soil enricher. As a transition metal, cadmium (Cd^{2+}) is most chemically similar to mercury and zinc; it is commonly co-mined with zinc because of their affinity for one another. Mining activity liberates metals not previously made bioavailable, bringing to the surface an assortment of toxins that would otherwise have remained largely inaccessible to groundwater supplies.

Cadmium is a known environmental toxin posing a hazard to both wildlife and humans. It is increasingly prevalent in both urban and rural settings, especially near long-life battery factories and in agricultural areas (Jarup & Akesson, 2009; Lopez et al., 2006). Certain industrial workers are at high risk of direct exposure to trace metals through occupational contact, including miners, construction workers, smelters, and some agricultural workers (OSHA, 2018; Tchounwou et al., 2012). Smokers are also at high risk, inhaling about 1

μg per cigarette (ATSDR, 2012a). This is due in part to the tendency of tobacco to accumulate cadmium from the soil (Satarug & Moore, 2004). In spite of this, the primary source of human contact with metals remains via food. Northeastern North Dakota, along with a number of other regions in the U.S., has a high natural soil content of cadmium (Tolcin, 2017; Jyoti, 2015). This cadmium is largely due to glacial shale deposits, which are rich in metals, particularly cadmium in the form of cadmium sulfide (Page, Chang, & El-Amamy, 1987; USGS, 1980). This high soil content, combined with cadmium's presence in pesticides and industrial waste, produces a high risk of exposure. An abundance of cadmium in soil means much of it is available for absorption by crops, particularly by leafy greens and cereal grains. Recent estimates suggest that the average human ingests 8 to 25 μg per day (ATSDR, 2012b; EFSA, 2009; Jarup & Akesson, 2009). The Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives and Contaminants (JECFA) has declared a Provisional Tolerable Weekly Intake (PTWI) of 5.8 μg/kg body weight, but more recent studies from the U.S. Agency for Toxic Substances and Disease Registry (ATSDR) and the European Food Safety Authority (EFSA) place this estimate much lower, at around 2.5 μg/kg (ATSDR, 2012a; EFSA, 2012; JECFA, 2011).

Cadmium Toxicity & Human Health

Toxicity to humans is well-documented, and many cadmium-induced disease phenotypes have been identified and described. Affected systems include urinary, digestive, musculoskeletal, cardiovascular, neural, and reproductive, all of which may also develop

cancer. Increased dosage and prolonged exposure amplify these effects. Heavy metals are known endocrine disruptors, with cadmium being reported to reduce levels of thyroid hormone, inhibit estrogen receptors, and disrupt growth hormone expression (Jones, Kille, & Sweeney, 2005; Le Guevel et al., 2000; Hontela, Daniel, & Ricard, 1996). Cadmium may also exert a genotoxic effect in the form of genomic instability, acting as a potent mutagen and working to counteract the actions of DNA repair systems as well as inducing formation of aberrant nuclei (Filipic, 2011; Cavas, Garanko, & Arkhipchuk, 2005). It may also have effects on epigenetic regulation in the form of chromatin modification and changes in histone structure and microRNA expression (Koedrith et al., 2013). The affinity of cadmium for thiol groups may result in the inhibition of sulfur-containing proteins, especially cysteine and glutathione (Jezierska, Lugowska, & Witeska, 2009). In addition to these concerns, cadmium is known to reduce the activity of various enzymes related to oxidative metabolism, including citrate synthase, succinate dehydrogenase (SDH), glucose-6-phosphate dehydrogenase (G6PDH), and lactate dehydrogenase (LDH). Cadmium may also interfere with hemoglobin production by inhibiting the activity of ferrochelatase and gamma levulinic acid dehydrogenase (ALA-D). This is consistent with the behavior of other trace metals, particularly lead (Jezierska, Lugowska, & Witeska, 2009a).

The human body is capable of metabolizing some trace metals, like arsenic, but is unable to do so with cadmium. In small doses, cadmium can be processed and disposed of as waste; at higher levels, bioaccumulation occurs in tissues like the brain, liver, lung, testis, kidney, bone, and blood, where it has a half-life of around 30 years (Jarup, 2009;

Bernard, 2008). This may lead to a whole host of problems ranging from developmental defects to cancer (Koedrith et al., 2013; Esteban-Vasallo et al., 2012; Chow et al., 2008; Ali, Murthy, & Chandra, 1986). One of the most rapidly affected organs is the kidney, largely due to breakdown of mitochondrial membrane potential and subsequent degradation of Na^+/K^+ -ATPase (Bernard, 2008; Nordberg, 2007; Jarup et al., 1998). Cadmium exposure can lead to tubule and glomerular damage, exacerbation of diabetic renal pathology, and ultimately renal failure (Gonick, 2008). These effects are amplified by prolonged or repeated exposure (Jarup & Akesson, 2009; Jarup et al., 1998). Kidney pathogenesis can in turn create problems in bone, where cadmium can induce and accelerate development of osteoporosis and osteomalacia. It can also cause a painful condition known as *itai-itai*, in which sufferers experience frequent fractures and long bone distortion (Schutte et al., 2008; Jarup et al., 1998). The cardiovascular system is also affected – vascular damage can result from oxidation within endothelial cells. Affected persons may experience atherosclerosis, impaired vasorelaxation, hypertension, and even heart failure (Steinbrenner & Sies, 2009). Another concern with cadmium is its carcinogenic nature. Cadmium has been linked to renal, lung (at rates comparable to long-term smokers), endometrial, bladder, testicular, prostate, and breast cancers (Jarup & Akesson, 2009; Darbre, 2006; Waalkes, 2000). Effects are more pronounced in women, as cadmium appears to be a metalloestrogen – an endocrine disrupting factor that alters gene expression in estrogen-responsive cells, though it has also been suggested that this effect may be due in part to iron deficiency (Nawrot, 2015; EFSA, 2011; Jarup & Akesson, 2009). Xenoestrogens such as cadmium have demonstrated an affinity for estrogen receptors, thus competing with naturally occurring estrogen for binding sites,

though at low levels this has not been shown to influence reproductive capacity (Pollack et al., 2011; Denier et al., 2009; Darbre, 2006). Cadmium has also been linked to birth defects and developmental disorders, including decreased birth weight and length. These effects may be mitigated by placental accumulation of cadmium (Esteban-Vasallo et al., 2012).

Development of the Vertebrate Nervous System & Dopaminergic Reward Pathways

Cadmium can also have devastating effects on the nervous system due in part to its ability to cross the blood-brain barrier and exert neurotoxic effects (Favorito et al., 2011). Neural pathology includes diminished brain function and significant decreases in critical neurotransmitters (Lopez et al., 2006). While much is known about the chemical interactions of cadmium in the body, comparatively little is known about its effects on embryological development, particularly neural development. Of primary interest to the present work is the formation of the ventral midbrain and its populations of dopaminergic neurons. In mammals, the majority (75%) of all dopaminergic neurons are located in the ventral midbrain (see Figure 1).

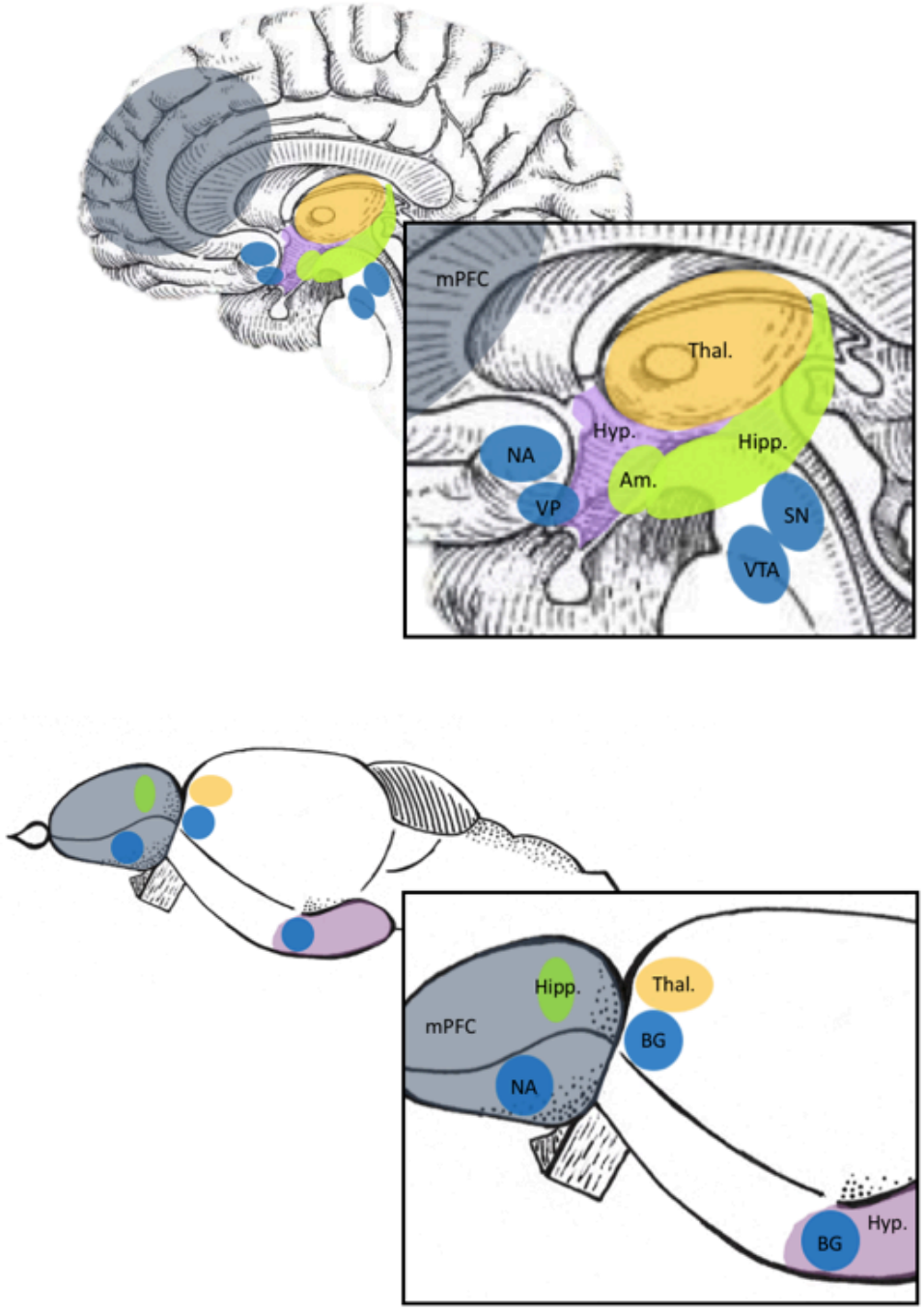

Figure 1. Comparative anatomy of human (top) and zebrafish (bottom) brain structures. Humans have a cortical region in the form of the medial prefrontal cortex (mPFC), which is represented in zebrafish in the form of the telencephalon (shown in grey). The human limbic system is contained in the thalamus (Thal., orange), hypothalamus (Hyp., purple), and hippocampus/amygdala (Hipp./Am., green). The zebrafish has analogous structures located in the forebrain, with a hippocampus-like region contained within the medial aspect of the telencephalon. The equivalent of the thalamus is located just caudal to the

telencephalon-diencephalon boundary, while the structure representing the hypothalamus is both more caudal and more ventral. The human basal ganglia (shown in blue) involved in the dopaminergic reward pathway include the nucleus accumbens (NA), ventral pallidum (VP), substantia nigra (SN), and ventral tegmental area (VTA). The bulk of all dopaminergic neuronal cell bodies are located in the SN and VTA. The zebrafish has basal ganglia-like clusters of dopaminergic neurons (BG) localized to the posterior tubercle of the ventral forebrain and the rostral portion of the hypothalamic region. The analogue to the NA lies in the ventral telencephalon, just rostral to the hippocampal region.

Development sees these neurons generated in the floor plate of the mesencephalon, giving rise to three distinct neuronal clusters. These clusters (A8-10) develop into the substantia nigra, retrorubal field, and ventral tegmental area, respectively. Axonal projections reach the dorsal striatum via the nigrostriatal pathway, and the prefrontal cortex and ventral striatum via the mesocorticolimbic system. These last are involved in regulation of emotion and the mediation of reward pathways, making them particularly critical functioning units that may be vulnerable to early cadmium exposure (Hegarty, Sullivan, & O'Keeffe, 2013).

Vertebrate brain development is a tightly regulated process induced by a number of extrinsic and intrinsic genes and transcription factors, including bone morphogenetic protein (BMP) and wingless type (Wnt) antagonists (Noggin, Chordin, & Follistatin; Dickkopf, Frzb, & Cerberus, respectively), *Fgf*, and *SoxB1* gene families; this is summarized in Figure 2 (Gilbert, 2016; Schmidt, 2013; Chow et al., 2008; Weinstein & Hemmati-Brivanlou, 1999). Typical vertebrate neural development begins during gastrulation with ectodermal formation of the neural plate via suppression of BMP and expression of forkhead box protein 4 (*FoxD4*) transcription factor. Cells of the organizer (in zebrafish, the embryonic shield) are responsible for inducing neighboring cells to

form the neural tube (Gilbert, 2016; Weinstein & Hemmati-Brivanlou, 1999). The neural tube is then acted upon by a Wnt gradient to determine the anterior-posterior organization (e.g. forebrain precedes hindbrain on the basis of lower Wnt presence); blockage of Wnt signaling by antagonists signals head and brain formation. Fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) are responsible for initiation of the receptor tyrosine kinase signaling cascade, which also antagonizes both BMP and Wnt (Gilbert, 2016). It is at this time development of the ventral mesencephalon begins. The floor plate of the neural tube secretes sonic hedgehog (*Shh*), the expression pattern of which dictates the formation of different populations of dopaminergic neurons, with the early medial pool producing neurons of the ventral tegmental area and the later intermediate pool contributing largely to the substantia nigra (Hegarty et al., 2013; Joksimovic et al., 2009). Following this primordial neural formation, proneural genes are expressed, inducing neural progenitor development (Schmidt, 2013). At this point, *FGF8, Shh, FoxA2*, and *Wnt1* induce ventral midbrain dopaminergic (VM DA) precursor formation from radial glia-like cells of the floor plate. *Nurr1, Lmx1a, En1/2, Otx2, FoxA1/2, Ngn2*, and *Pitx3* then play a role in the differentiation of these precursors into neurons (Fu et al., 2016; Luo & Huang, 2016; Hegarty et al., 2013). Later, VM DA projections are formed under the influence of *Nolz1, EphrinB2* and *Ephrin A5*; this is summarized in Figure 2 (Hegarty et al., 2013; Ko et al., 2013; Ikemoto, 2007).

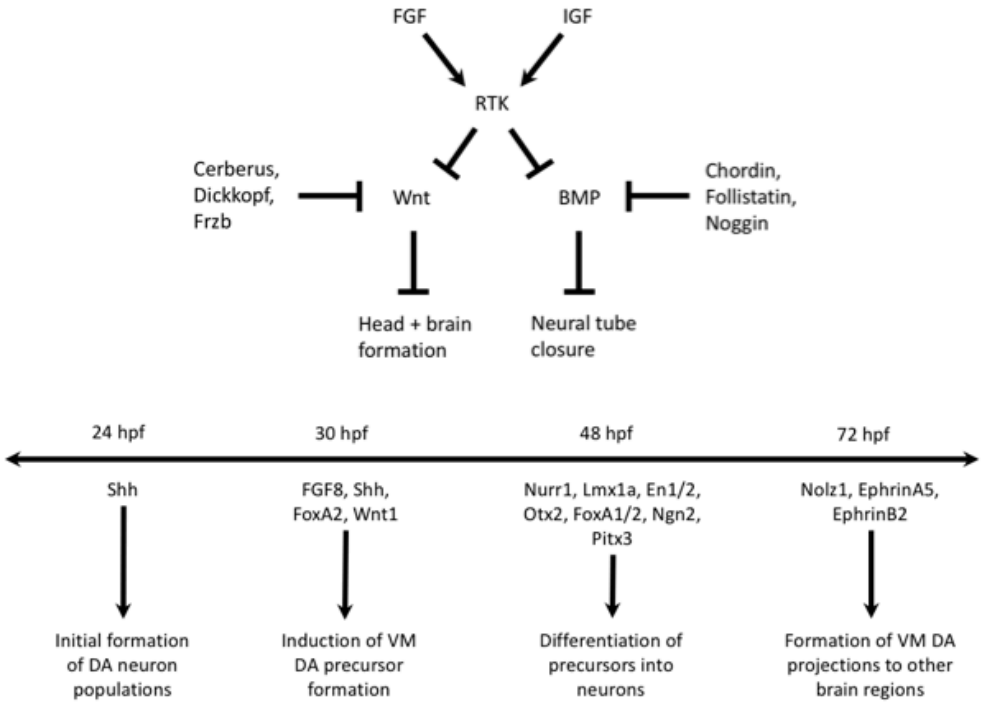

Figure 2. Genes and transcription factors involved at critical points in the formation of the vertebrate dopaminergic reward pathway. A schematic of the transcription factors involved in initial vertebrate head and brain formation is shown above, with a summary of those involved in development of dopaminergic neuron populations (overlaid with zebrafish timeline of formation) below. Fibroblast growth factors (FGF) and insulin-like growth factors (IGF) are responsible for the initiation of receptor tyrosine kinase (RTK) signaling cascade. This inhibits both BMP and Wnt signaling. BMP is also inhibited by Chordin, Follistatin, and Noggin, while Wnt is inhibited by Cerberus, Dickkopf, and Frzb. These inhibitions allow for initial head and brain formation, as well as neural tube closure, which the presence of Wnt and BMP inhibits. Around 24 hpf, secretion of *Shh* initiates early formation of dopaminergic (DA) neuronal populations. Shortly thereafter, at 30 hpf, *FGF8, Shh, FoxA2,* and *Wnt1* induce ventral midbrain DA precursor formation. At 48 hpf, *Nurr1, Lmx1a, En1/2, Otx2, FoxA1/2, Ngn2,* and *Pitx3* effect precursor differentiation. Finally, by 72 hpf, *Nolz1, EphrinA5*, and *EphrinB2* stimulate the formation of ventral midbrain DA axonal projections.

There are some minor differences in mode of neural development in zebrafish compared to humans, but the end result is very similar. For instance, during zebrafish neurulation, the neural plate forms a structure called a neural keel before developing the neural tube (called ‘secondary neurulation’); in other vertebrates, the neural plate forms directly into

the neural tube (Schmidt, 2013; Strahle & Blader, 1994). In spite of these differences, neural development is a conserved process, and zebrafish exhibit standard vertebrate brain morphology and formation (Chow et al., 2008). In zebrafish, neurogenesis is initiated by basic helix-loop-helix (bHLH) factors *neurogenin1* (*ngn1*) and *achaete-scute1* (*acs1*) (Schmidt, 2013). Several homeobox genes are responsible for pattern formation: *emx1* and *dlx2* are important for forebrain organization; *otx* dictates diencephalon and midbrain division; and *pax2.1* informs the midbrain-hindbrain boundary (MHB). Cadmium disrupts the action of most of these, leading to indistinct boundaries within the brain as well as affecting neuronal cell fate decisions (Chow et al., 2008).

In mammals, the dopaminergic system is contained primarily within the substantia nigra and ventral tegmental area, where cell bodies are localized (Boehmler et al., 2004; Rink & Wullimann, 2002a). The zebrafish does not have these exact structures (see Figure 1), but homologues exist in the form of a dopaminergic projection pathway akin to the mesostriatal pathway in humans (Boehmler et al., 2004). No dopaminergic cells are found in the midbrain, but clusters are present in the posterior tubercle. Rink & Wullimann (2002a) found three groups of neurons localized to the rostral portion of the posterior tubercle that labeled as both dopaminergic and projecting to the ventral telencephalon. Zebrafish polypeptides have 52-72% amino acid sequence identity with amniote D2 and D3 receptors, with the most similarity being to the D2a (zebrafish analogue D2c) receptor. This high structural and sequential identity makes the zebrafish a valuable model for examining the functional changes observed in the dopaminergic

reward pathway in humans exposed to substances like cadmium. Development of zebrafish DA neurons proceeds in a similar fashion to that of other vertebrates.

Zebrafish dopaminergic neuron development has been observed in the ventral telencephalic area at 24 hpf through 5 dpf as shown in Figure 2 (Du et al., 2016). The earliest DA neuron formation is typically seen at about 24 hpf in the basal forebrain, with Ngn1 being expressed by neural progenitor cells (Jeong et al., 2006). Earlier detection has been reported, with DA neurons observed in the ventral diencephalon as early as 17-18 hpf (Holtzscuch et al., 2001), but this is not common. Between 24 and 48 hpf, the hypothalamic region of the diencephalon containing DA neurons undergoes expansion (Guo et al., 1999). The diencephalons 1 and 2 (DC1/2) are detectable in the periventricular nucleus of the posterior tubercle at 24 hpf (Du et al., 2016). At 30 hpf, DA neurons in the PT begin to differentiate into distinct neuronal populations as well as diencephalons 2, 3, and 4 (DC2/3/4). Also at this time the dorsal/ventral nuclei of the telencephalic area begin to be detected (Guo et al., 1999, Du et al., 2016). This process of differentiation is regulated at least in part by NR4A2 (Blin et al., 2008). At 48 hpf, all neuronal populations of the ventral DC, locus coeruleus (LC), raphe nuclei (Ra), and telencephalon are detectable. Further differentiation occurs through 54 hpf. By 3 dpf, there is axonal projection from DC2 to the telencephalon, and all neuronal populations are developed (Du et al., 2016).

Chemistry of Reactive Oxygen Species

The hazard associated with cadmium exposure lies in its mechanism of action. As a heavy metal, cadmium can induce formation of reactive oxygen species (ROS); however, because cadmium is not a redox-active molecule, it accomplishes this indirectly (see Figure 3). Cadmium tends to free up iron (II) and copper (II) by replacing them in various compounds, thus increasing the bioavailability of these redox-reactive metals in the organism. These, in turn, undergo the Fenton (Haber-Weiss) reaction, wherein the metal interacts with H_2O_2 to produce a metal cation along with either a hydroxide anion and a hydroxide radical (Cu^{2+}, Fe^{2+}) or a proton and a hydroperoxyl radical (Fe^{3+}) (Nair et al., 2013; Cuypers et al., 2010; Casalino, Sblano, & Landriscina, 1997).

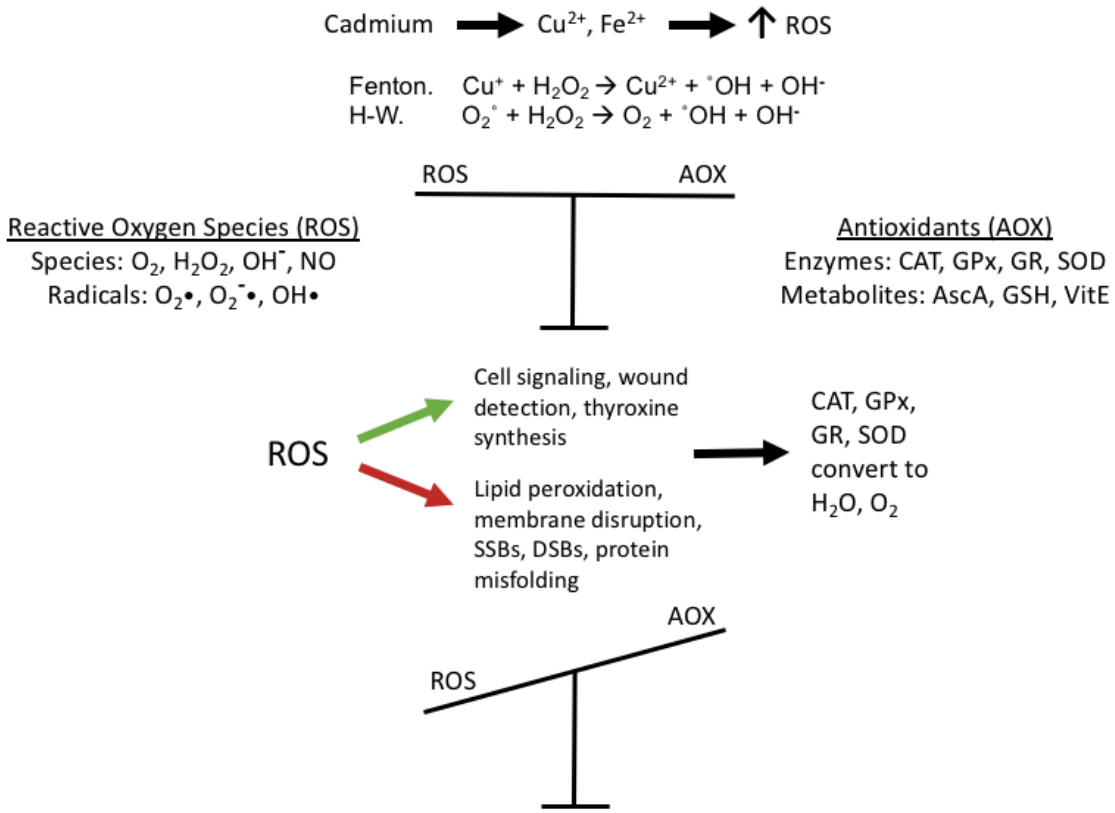

Figure 3. The systemic effect of cadmium introduction on oxidative balance. Cadmium enters the body and liberates Fenton-active metals like copper (Cu^{2+}) and iron (Fe^{2+}). These then undergo the Fenton (Haber-Weiss) reaction to induce formation of reactive oxygen species (ROS). Oxidative metabolism is a fine balance between ROS and the antioxidant defense system (AOX). When the system is functioning normally, ROS are used by the cell. When excess ROS are present, they are free to move around and interact with macromolecules, inducing damage. AOX enzymes then have to work harder to convert excess ROS to water and oxygen, striving to maintain that balance.

Some of these ROS, including oxygen, hydrogen peroxide, hydroxyl ions, and nitric oxide, are harmless in their natural state and will not interfere with cell function. In many cases these are intentionally generated by the cell in order to carry out crucial functions, including use as transient second messengers, modulation of protein activity, and wound detection (Ralston & Raymond, 2016; Niethammer, 2009; Hanson, 2004). Others, like peroxides, superoxide anions, and hydroxyl radicals, contain an unpaired electron – making them 'radical', which increases their propensity toward reactivity and makes their reactionary intermediates less stable (Held, 2015). Most ROS are formed as the natural byproducts of cellular respiration, but certain substances like cadmium indirectly necessitate their production as intermediates of metal-catalyzed oxidation reactions. Increased production of ROS leads to an imbalance in cellular oxidation levels, which produces a state of oxidative stress (Steinbrenner & Sies, 2009). Although ROS are used in cell signaling pathways, as well as in thyroxine synthesis and bacteriophage action, an overabundance can result in cellular withdrawal from the cell cycle (entry into stasis) and inappropriate apoptosis (Held, 2015; Koedrith et al., 2013).

The problem with radicals, or 'free radicals', is their increased propensity for interaction with macromolecules such as DNA, lipids, and proteins. The presence of excess radicals is especially concerning with regard to their tendency to induce lipid peroxidation, an oxidative degradation of the lipid molecule that results in disruption of lipid-based membranes (Koedrith et al., 2013). This process involves radical removal of electrons from lipid molecules and causes structural breakdown (Koedrith et al., 2013). Aside from the issue of cell membrane destruction, there is degeneration of mitochondrial membranes. As a result of radical interference, there is deterioration of membrane potential and cristae deformation leading to a dysfunction in ATP production, thereby causing a decrease in intracellular ATP (Lopez et al., 2006). The mitochondrial effects can result in significant neurological consequences.

Neurons have the highest energy requirements of all somatic cells and are the largest consumers of oxygen – the brain consumes oxygen at a rate tenfold that of any other tissue, making it much more vulnerable to oxidative damage (Ralston & Raymond, 2016; Bolam & Pissadaki 2012; Vander et al., 2012). In addition, cells have difficulty repairing distal ends of dendrites and axons because of their distance from the cell body (Ralston & Raymond, 2016). This is compounded by oxidative damage to mitochondria, which are largely responsible for maintaining axonal health (O'Donnell, 2013). Agnihotri et al. (2015) compared oxidative biomarkers across five different tissues in juvenile mice treated orally with doses ranging from 0.1 to 2.0 mg/L cadmium for 30 days and determined that the brain was most susceptible to oxidative stress. They reported hippocampal disruption and shrinking, as well as a decrease in the prevalence of neurons

in the dentate gyrus. They also observed decreased activity of catalase, superoxide dismutase, and glutathione peroxidase, while lipid peroxidation and tissue breakdown biomarkers were more highly expressed in the brain than other tissues. Concentration of malate dehydrogenase (MDH) was also remarkably high, indicating severe tissue damage. A similar effect was seen in adult rats (Carageorgiou et al., 2005; Carageorgiou, 2004). Cadmium has also been shown to damage astrocytes, inducing morphological changes and causing cell death (Jiang et al., 2015). Tobwala et al. (2014) demonstrated oxidative stress related disruption in human brain microvascular endothelial cells exposed to varying concentrations of cadmium, indicating the possibility of blood brain barrier disruption as a mode of metal toxicity.

The high oxidative requirements of neurons require them to have an abundance of mitochondria to meet this need. Perturbations are a danger to synaptic junctions, where minor alterations to mitochondrial morphology, function, or concentration can be detrimental to neural signaling (Chauhan et al., 2011). It has also been noted that cadmium induces apoptosis and necrosis in cortical neurons mediated by the caspase pathway (Lopez et al., 2003). Cadmium may also cause a decrease in several critical brain enzymes, including acetylcholinesterase, acid phosphates, alkaline phosphatase, ATPase, and catalase (Antonio et al., 2003). Rat pups exposed to low levels of cadmium (10 mg/L) *in utero* displayed a decrease in dopamine content of the cortex, dorsal hippocampus, and medio-basal hypothalamus (Antonio et al., 2010).

Routes of Exposure

In addition to its presence in soil, cadmium leaches readily into the water supply due to its tendency to ionize in polar solvents. Potential sources for this interaction include soil to groundwater movement as well as direct contact with bodies of water through mine tailings. Recent water contamination incidents including the 2015 Gold King Mine incident in Colorado, the 2014 event at Mount Polley Mine in British Columbia, and the 2015 water crisis in Flint, Michigan have raised concerns about the toxic effects of widescale exposure to trace metals on human populations, as well as their broader ecological impact. A prevalence of heavy metal ions increases the acidity of water, producing myriad potential problems for humans and wildlife alike. Adding to these concerns, cadmium may enter organisms directly and create a number of other issues. Environmental concerns have been raised due to the tendency of cadmium to bioaccumulate; species higher in the food chain are at greater risk for bioaccumulation via ingestion of many metal-containing prey species. Recent interest in cadmium is due in part to its high toxicity and tendency to bioaccumulate, though much of this concern is related to the abundance of human activity that releases it (Liao et al., 2011; Kalman et al., 2010).

While most humans are primarily exposed via ingestion, fish accumulate heavy metals primarily through absorption in the gills, though uptake may also occur through the gut and skin. Cadmium enters passively through calcium channels before being actively transported to the blood; from there it is stored in tissue (Favorito et al., 2011). It may also be absorbed physiologically in the form of an inorganic salt, as mercury is (Klinck et

al., 2004). In frogs, cadmium has been found to alter nucleic acid structure, interfere with the action of several critical enzymes, and disrupt uptake of catecholamines and other neurotransmitters (Cooper, 1984). Aquatic species are most vulnerable to water contamination; this, in addition to the growing popularity of aquatic organisms in the lab makes the study of metal effects on fish highly relevant.

Effects on Aquatic Species

Cadmium enters the fish primarily through calcium channels in the gills, where it competes with calcium transport and disrupts ionic balance (Verbost et al., 1987). It is also known to cause damage to enzymes within the gills, dramatically decreasing the activity of carbonic anhydrase and Na/K-ATPase in the European eel (Lionetto, Vilella, & Lin, 2000). The disturbance of calcium uptake subsequently disrupts Ca^{2+} ATPase activity, further impacting ATP production. Cd can also bind to calmodulin and other Ca^{2+} binding proteins, allowing it to deposit in bone. Cd^{2+} is the primarily absorbed species; this is consistent with the belief that the most bioavailable form of metals is the dissolved ionic state, resulting in increased toxicity (Sfakianakis et al., 2015). It is believed to enter cells by mimicking essential metals (namely Ca^{2+}, Cu^{2+}, and Fe^{2+}) and has been proposed to bind to an Fe-binding site in addition to its calcium channel entry (Cooper, Handy, & Bury, 2006). It has been observed to accumulate in the gills, and to induce upregulation of metal binding proteins (Komjarova & Bury, 2014). Once inside the fish, cadmium can cause a number of genetic aberrations, including inhibition of DNA repair, downregulation of mismatch recognition proteins, and both double- and single-strand breaks; all of this may be due to its ability to replace zinc in enzymes

(Bertin & Averbeck, 2006; Giaginis, Gatzidou, & Theocharis, 2006; Mikhailova et al., 1997). These genetic effects are compounded by the ability of cadmium to decrease expression of proteins associated with DNA mismatch repair. Zebrafish embryos treated acutely (4-9 hrs) with 0.5-3.0 μM Cd displayed downregulation of DNA mismatch recognition protein MutS homolog 6 (MSH6) expression at both the mRNA and protein levels (Hsu et al., 2013; Hsu et al., 2010). Oxidative stress biomarkers including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) are significantly increased with exposure to cadmium, while acetylcholinesterase activity and thiol prevalence are decreased in brain and muscle tissue, leading to issues associated with decreased neurotransmitter breakdown (Costa-Silva, 2015).

On a broader scale, cadmium induces reproductive impairments, including inhibition of estrogen signaling in females and decreased sperm viability in males (Acosta et al., 2016; Chouchene et al., 2016). There are also issues of behavioral anomaly, stemming at least in part from damage to the olfactory epithelium as a result of increased cell death (Krone, 2007). Affected fish demonstrate decreased response to environmental chemical cues, which may interfere with feeding, reproductive, and survival behaviors (Kusch & Chivers, 2007). Jin et al. (2015) reported decreased swimming speed and distance, as well as decreased response to light-dark stimulation in fish treated with 10 μM Cd. In addition, embryonically exposed fish may display physical deformities due to genotoxicity and increased apoptosis (Cheng, So, & Wu, 2000). Xie et al. (2009) proposes that cadmium-induced apoptosis may be mediated by calcium release into the cells from intracellular storage. In zebrafish treated with 100 μM Cd from 5-28 hpf, Chan

& Cheng (2003) observed much higher concentrations of apoptotic cells in embryos also displaying developmental deformity. There was overall a much higher abundance of apoptotic cells in cadmium-treated embryos compared with controls; this was especially true in the neural tube and developing gut. When observed histologically, the neural tube of cadmium-treated fish lacked a clear boundary, and there was some disruption of cellular organization. They did not check for volume changes as a result of this tissue disorganization.

Of chief concern is the effect cadmium has on the central nervous system. Cadmium exposure is known to compromise astroglia and oligodendrocytes, as indicated by a decrease in GFAP expression (Monaco, Grimaldi, & Ferrandino, 2016). Developmentally speaking, embryonic cadmium exposure is known to inhibit both neurogenesis and retinogenesis. It has been shown to stunt cranial growth, inhibit formation of clear boundaries between brain regions, and decrease numbers of differentiated neurons and glia (Chow et al., 2008). Larval exposure can result in ocular malformation that may present as functional blindness (Chow et al., 2009). In adults, cadmium accumulation causes neural tissue disorganization, especially in the optic tectum, ventricles, and medulla oblongata (Favorito et al., 2011). There is evidence that acute exposure to lead and mercury decreases levels of acetylcholinesterase activity in the zebrafish brain, though this effect was not seen with cadmium treatment (Richetti et al., 2011).

Much of what is known about the effects of cadmium on the nervous system is based on experiments that used incredibly high concentrations to treat their subjects. Often,

exposure was in excess of 100 μM, which is very much a knockout dose; it generally kills most of the subjects. The present work was more interested in examining the subtler effects seen at lower concentrations, to look at how comparatively small changes in the brain, and subsequent behavior, ultimately affect fitness.

Zebrafish (Danio rerio) Model

In recent years, the zebrafish has become a popular model in biomedical research, outstripping even the mouse as a preferred laboratory organism (Stewart et al., 2014). Zebrafish possess many attractive qualities as a model, not least of which is the relative ease of care and maintenance of a colony. They are a cost-effective option, requiring comparatively little space or resources, without sacrificing the genetic similarity to humans so prized in a model. They reproduce quickly and prolifically; a single healthy female is able to lay hundreds of eggs in a week. In addition to this, they develop rapidly, with formation of all major organ primordia occurring within 24 hours of fertilization. Within 5 days of fertilization they are free swimming, and they reach sexual maturity within 4 months. In addition to these benefits, their external fertilization and development allows for easy manipulation of the microenvironment during development with the added benefit of minimizing confounding variables. This mode of development also affords an unprecedented opportunity to study vertebrate development noninvasively (Rieger et al., 2011). Their relatively small genome allows for easy manipulation and production of a wide range of mutant and reporter lines, and their recent popularity has made this abundance of mutants readily available to researchers. In addition to this, the

zebrafish research community has directed significant resources to a major database of reagents in the form of the Zebrafish Information Network (ZFIN) website.

The zebrafish has previously demonstrated its utility in neurological and behavioral studies, particularly those pertaining to the monoaminergic reward pathway and reward-based learning (Gerlai, 2017; Kalueff, Stewart, & Gerlai, 2014; Darland et al., 2012). Its 87% homology with the human genome as well as its high amino acid sequence fidelity with amniote dopamine transporters renders it an ideal model in examining these interactions (Du et al., 2016; Rink, 2001). All major mammalian brain structures have homologues in the zebrafish brain, some of which are illustrated in Figure 1 (Du et al., 2016; Boehmler et al., 2004; Rink & Wullimann, 2002b). The zebrafish possesses physiological similarity as well, having highly developed neurotransmitter systems consistent with those of mammals (Chatterjee & Gerlai, 2009). In addition to these anatomical and physiological similarities to humans, the zebrafish is transparent during formation of critical structures, allowing development to be observed in real time as well as visualization of any morphological changes induced by treatment. The existence of a variety of mutants and reporters, including a reporter line expressing green fluorescent protein (GFP) in the central nervous system, allows for more detailed observation of morphology. Because of this, the zebrafish provides an effective toxicological model, and is often used as a biomarker for aquatic health (Hill, 2005; Linney, 2004).

In light of these advantages, the zebrafish provides a useful model for studying the effects of acute cadmium exposure on neural development. The zebrafish has previously been

shown to be sensitive to acute metal exposure, with effects observed in multiple organ systems (Alsop & Wood, 2011). A number of recent studies have used zebrafish for toxicological research, including investigation into metal effects on cytotoxicity, development, gene expression, and survival (Green & Planchart, 2018; Hu et al., 2011; Cambier et al., 2010; Hsu et al., 2010; Cheuk, Chan, & Chan, 2008; Chan & Cheng, 2003). The effects of cadmium on neurological development and behavior have been less studied (Wold et al., 2017). Previous work by the Darland lab has demonstrated the efficacy of the zebrafish model for use in behavioral studies, providing evidence for a dose-dependent response to cocaine exposure in monitoring learning behaviors as well as providing a framework for the present study (Mersereau et al., 2016; Darland et al., 2012).

Interactions of Selenium with Cadmium

Given the relative abundance of cadmium and its toxic effects, there is great interest in identifying ways to ameliorate its impact. There is growing evidence that selenium, another naturally occurring substance, may be able to accomplish this. Selenium is an essential metalloid that tends to be present in arid, alkaline soils. In small to moderate amounts, it is harmless, though overdose is possible. It has similar chemistry to sulfur and tellurium, the elements respectively above and below it on the periodic table. It is a versatile substance, readily combining with metals and non-metals alike to form both organic and inorganic compounds. Selenium is used in glass manufacturing, pigmentation, and electroplating; it is a useful anticorrosive, vulcanizing agent, and metallurgy additive. One of its largest uses by percent is in the production of electrolytic

manganese for use in electrolytic cells. It is also used in solar cells (Tolcin, 2017). In areas with selenium-poor soil, selenium is a fertilizer additive in the form of sodium selenite (Schuyler-Anderson, 2016). It may behave as an oxidant (selenate) or reductant (selenite), making it an important component of soil chemistry in regard to pH balance. In turn, selenium may exist in several species depending upon soil composition – for instance, alkaline soils favor selenates (Saha, Fayiga, & Sonon, 2017). Selenites are fine; selenates are toxic (Bauer, 1997).

From a human health perspective, selenium is a primary component of a class of compounds called selenoproteins, which are responsible for a number of physiologic processes, including reproduction, production and regulation of hormones and growth factors, thyroid hormone metabolism, DNA synthesis, and oxidative protection (NIH, 2017). The adult RDA for selenium is 55 μg per day; supplements usually take the form of sodium selenite (Na_2SeO_3) (NIH, 2017). Chronic deficiency can produce juvenile cardiomyopathy (Keshan disease) and osteoarthropathy (Kashin-Beck disease); these pathologies tend to be localized to northeastern China, where estimated daily selenium intake is ≤ 10 μg per day (Zwolack & Zaparowska, 2012). Instances of selenium toxicity in the form of selenosis are very rare. Selenium is incorporated into some proteins as selenocysteine or selenomethionine, though selenoscysteine is more reactive. In fact, dietary selenomethionine is incorporated into proteins in place of methionine, and is then converted to cysteine (Chen & Berry, 2003). Selenium is a critical element in the development of spermatozoa, contributing to the formation of glutathione peroxidase 4, an important structural component of mature spermatozoa (Kurokawa & Berry, 2013).

Selenoprotein P (SelP) is the major transporter of selenium to Sertoli cells, and mice with SelP knockout are infertile. Indeed, selenium can be used as a biomarker of sperm viability as it also serves a protective role against environmental stressors (Ahsan et al., 2014, Michaelis et al., 2014). Selenoenzymes—specifically the deiodinases—are responsible for activation and inactivation of the thyroid hormones and are crucial for thyroid hormone regulation. Not only are deiodinases important regulators of adult thyroid hormone metabolism; they also serve a critical role in local thyroid hormone coordination during vertebrate development (Galton et al., 2014; Dentice et al., 2012; St. Germain, Galton, & Hernandez, 2009). Thyroid hormone is believed to be critical to proper cerebellum development; this is perhaps why selenium is always present in the brain, even during prolonged periods of dietary insufficiency (Bellinger et al., 2010; Chen & Berry, 2003). The highest concentrations of selenium in the brain are found in regions with more gray matter, specifically the cerebellum. One fifth of all selenium present in the rat brain is in the form of GPx (Chen & Berry, 2003). Selenium derivatives are also important to the cell cycle; they induce upregulation of certain cell cycle related genes, including cyclin C and cyclin-dependent kinases, which are responsible for initiation of DNA synthesis (Zeng, 2009). The selenium function of most interest to us is the latter – the role of selenium in antioxidant compounds and resultant cellular protection.

Selenium Uptake

Selenium evidently enters fish through the gills, as evidenced by histopathological changes observed in the gills of fish exposed to high concentrations of selenium. There is

also absorption through the gut, as fish ingest invertebrates with accumulated selenium in waters with high selenium content due to mining activity or agricultural runoff (Arnold et al., 2014; Tashjian, 2006). Tashjian (2006) examined tissue levels of selenium after exposure to concentrations ranging from 250-1000 μg Se/kg body weight, and found increased accumulation with increasing exposure. The highest concentrations were seen in the liver, followed by skeletal muscle, plasma, and RBCs.

Cellular uptake of selenium is dependent upon the extracellular redox state. Thiol levels are strongly correlated with the degree of selenium uptake in lung cancer cells; increases in extracellular thiol correspond to greater presence of selenium in the cells. Olm et al. (2009) found that the anion channel blocker 4,4-diisothiocyanostilbene-2,2-disulphonic acid (DIDS) prevented selenium uptake into keratinocytes, both with and without glutathione. This suggests both selenide and its reduced form were using this pathway. The x_c- cysteine/glutamate antiporter, which exchanges extracellular L-cysteine for intracellular L-glutamate, may also play a role. Given the chemical similarity between cysteine and selenocysteine, it is possible this is a mechanism for selenium entry to the cell. However, Olm et al. (2009) asserts it is not the antiporter itself, but rather its effect on extracellular thiol, that affects selenium uptake. Tobe (2017) proposes a new modality: transport of the metabolite selenodiglutathione (SDG) through cysteine transporters. This compound is formed through conjugation of selenium trisulfide with glutathione, and during production of selenide. Cells incubated with SDG, when compared with cells incubated with H_2SeO_3, displayed a remarkable increase in cellular accumulation of selenium, indicating SDG is the preferred form for uptake into the cell.

Role of Selenium in Protective Chemical Species

The human genome codes for 25 selenoproteins (Pillai, Uyehara-Lock, & Bellinger, 2014). Most selenoproteins catalyze redox reactions and have selenocysteine (Sec) at their active site. Sec, sometimes called the twenty-first amino acid, is an essential compound, as evidenced by knockout experiments that proved embryonic lethal (Bulteau & Chavatte, 2015). Unlike the twenty base amino acids, Sec is synthesized from serine, and contains a selenol group that is highly reactive. This high reactivity makes it a more efficient participant in redox reactions (Kurokawa & Berry, 2013). The structure of Sec is identical to that of Cys, with the exception that selenium is substituted for sulfur; this substitution is functional due to the chemical similarity between the two. In mammals, this form has the biologic advantage because the selenol group is a stronger ion than the thiol group at physiologic pH (Bulteau & Chavette, 2015; Driscoll & Copeland, 2003). Heavy metals tend to have an affinity for thiol-based compounds (Poole, 2015; Su et al., 2008). Because of this, thiol groups do play a significant role in oxidative metabolism: a number of antioxidants, particularly glutathione peroxidase, are thiol-based compounds (Solovyev, 2015). Selenomethionine, the other selenium-containing amino acid, is obtained from plants rather than being synthesized by humans. Due to the covalent bonds holding the selenium molecule in place, this compound is significantly less reactive than its cousin (Kurokawa & Berry, 2013).

The role of selenium in regulating the status of glutathione allows it to play an indirect role in the action of metallothioneins. These cysteine-rich proteins are a key component of trace metal processing, and are critical to both the sequestration and detoxification of

metals (Chen, 2007; Bertin & Averbeck, 2006). There are four known promoters of metallothionein in zebrafish, but their activation and the subsequent expression of metallothionein has not been studied in vivo, nor has it been examined in neural tissue. Yan & Chan (2002) found that zinc exposure resulted in upregulation of a metallothionein promoter in a caudal fin cell line. Cheuk et al. (2008) examined the effects of cadmium on expression in liver and caudal fins *in vitro*. Metallothioneins are known to be upregulated in zebrafish in response to heavy metal exposure as shown by Chen et al. (2004). Cadmium has been demonstrated to have a potent effect on embryonic zebrafish toxicity, second only to mercury in the heavy metals (Chen et al., 2004). The embryonic treatment groups are expected to show a dose-dependent response, which is expected to increase in an approximately linear fashion with each ten-fold increase of cadmium (Favorito et al., 2011). Adult treatment groups are expected to display a decreased upregulatory response with increasing embryonic treatment.

Antioxidant compounds are formed to counteract reactive oxygen species (ROS). Selenium is incorporated into a number of antioxidant enzymes, usually as a selenoprotein. Selenium exerts its main effects through production of selenocysteine, a critical component of important selenoenzymes (Steinbrenner & Sies, 2009). Three major classes of selenoproteins are directly involved in oxidative protection: the glutathione peroxidases, thyroid hormone deiodinases, and thioredoxin reductases. There are eight known glutathione peroxidases (GPx); of these, GPx1-4 and GPx6 contain Sec. These enzymes break down hydrogen peroxides in a glutathione-dependent reductive reaction to eventually produce water and the oxidized form of glutathione, glutathione disulfide. The

iodothyronine deiodinases, of which there are three (DI1-3), are responsible for regulating the activity of thyroid hormones by catalyzing their reductive deiodination. Finally, the three thioredoxin reductases (Thx1-3) use an NADPH cofactor to reduce oxidized thioredoxin (Kurokawa & Berry, 2013). Thioredoxin is a small protein containing a dithiol active site that scavenges hydrogen peroxide and hydroxyl radicals (Arner & Holmgren, 2000). In addition to these, selenoproteins P, S, and W are either known to play an antioxidant role or are expected to do so (Zwolak & Zaparowska, 2012). GPx and Thx are involved in the actual ROS transformation pathway; SelP is a transporter protein responsible for supplying tissue with selenium and it has a possible role in ROS deactivation (Pillai et al., 2014; Steinbrenner & Sies, 2009). Selenium has also been observed to exert a protective effect against cadmium-induced oxidative damage in plants, notably a decrease in toxicity biomarker malondialdehyde prevalence in plants co-treated with selenium (Pedrero, 2008).

In this way, selenium is integral to the antioxidant defense system. It is involved especially in the formation of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The most important of these is GPx, as it is known to be the most widely expressed antioxidant compound with its variants participating in cytosolic, plasma, intercellular space, and membrane antioxidation processes (Rusetskaya & Borodulin, 2013). SOD is responsible for converting superoxide anion to hydrogen peroxide, which catalase, peroxidase, or glutathione peroxidase then change to water (Fukai 2011). Selenium has been shown to exert a significant protective effect on

GPx against cadmium exposure in zebrafish liver and ovarian tissue, and to a lesser extent, on CAT and SOD (Banni et al., 2011).

The mechanism of this protection is twofold: first, selenium compounds actively participate in countering oxidative damage induced by heavy metals; and second, selenium plays a key role in opposing metal-induced antioxidant suppression (Rusetskaya et al., 2013; Banni et al., 2011). This means that selenium functions in cells in three main ways: 1) it binds heavy metal ions and ROS metabolites, rendering them inert; 2) it increases expression of cytoprotective genes; and 3) it composes GPx, which is largely responsible for breakdown of excess H_2O_2 to restore balance to the system.

Neural Interactions of Selenium with Cadmium

Neurons are more susceptible to oxidative damage than other cells (Sadek et al., 2017; Solovyev, 2015; Rayman, 2012; Halliwell, 2001). Oxidative damage in the brain may be due in part to low innate levels of antioxidants as well as an abundance of polyunsaturated fatty acids and a higher oxidative metabolism (Solovyev, 2015). El-Boshy et al. (2015) reported a decrease in malondialdehyde (MDA), a molecular marker of lipid peroxidation, with selenium treatment.

Liu et al. (2014) demonstrated a neural rescue by selenium in chicken brains. They reported a decrease in nitric oxide (NO) and MDA levels in both the cerebrum and cerebellum of treated fowl. In addition, they saw a rebound in antioxidant activity and noted attenuated damage to Purkinje and granular cell layers with selenium treatment.

Further histological analysis revealed nearly normal myelin sheaths and mitochondrial membranes.

Selenium-Cadmium Interactions & Rescue Effect

Regardless of point of entry, cadmium progresses into the bloodstream; this is chiefly the location of its interaction with selenium. Once there, it complexes with selenide, binding SelP and decreasing the reactive availability of cadmium. This occurs in the presence of GSH, which reduces selenite (the reduced form is what complexes with metals). The interaction between cadmium and metalloenzymes may also affect the metabolism of essential metals, specifically copper, iron, and zinc (Lazarus et al., 2008). Sasakura & Suzuki (1998) found that the interactions between cadmium and selenium served to decrease whole blood Cd and plasma Se. A similar effect has been observed in cow serum, with a significant negative correlation observed between Cd and Se (Tomza-Marciniak et al., 2011). Zhao et al. (2014) reported a significant difference between mRNA levels of selenoproteins K, N, T, and S in Se-Cd treatment groups versus Cd treatment in chicken lymphocytes. Oxidative stress in the endoplasmic reticulum has been linked to regulation of apoptosis, though the toxic effects of cadmium are alleviated by the presence of selenium (Liu et al., 2015; Zhao et al., 2014). Chen et al. (2017) confirmed this, determining that cadmium-induced oxidative stress in the endoplasmic reticulum of chicken neutrophils was related to apoptosis; this effect was also alleviated by selenium. Messaoudi et al. (2010) reported a similar prevention of cadmium damage by selenium in rat erythrocytes.

A histologic examination of mouse brain, liver, and kidney revealed a significant reduction of pathology in Se-Cd treated subjects compared with Cd-only subjects (Lazarus et al., 2011). Similarly, El Heni et al. (2008) reported a decrease in histopathological damage of both hepatic and renal tissue in male Wistar rats. Of note is their lack of significant difference in tissue cadmium sequestration between selenium co-administration and cadmium control. This is contrary to other findings in which selenium was shown to exert a significant protective effect on treated tissue (Jamba, Nehru, & Bansal, 2000; Chen, Wanger, & Weswig, 1975).

Lynch et al. (2016) incubated porcine jejunal cells with organic and inorganic selenium prior to cadmium exposure and reported a significant decrease in seven of the eight DNA damage parameters they measured, most markedly in a yeast-derived selenium compound. Liu et al. (2015) found significant reductions in both tissue cadmium concentration and MDA presence in chicken kidneys; this was accompanied by significant increases in the activity of both GPx and SOD. Zhang et al. (2017) reported similar findings in chicken hepatocytes. Thus, the protective effects of selenium against oxidative damage have been reported across tissues.

Several studies have examined tissue levels of heavy metals in fish, and have found the highest amounts of them in the liver (Burger et al., 2012; Can et al., 2011). Burger et al. (2012) reported the lowest levels of mercury in the brain, and the highest selenium also in the liver. Can et al. (2011) compared gill, liver, and muscle, finding the highest selenium in the gill. Su et al. (2008), working with rats, found the highest concentrations of

mercury in the kidney, followed by the liver, and then the blood. Co-dosing with selenium resulted in decreased mercury accumulation in the kidney, but an increased overall body load of mercury, particularly in the liver. They found that the body load remains high despite the decreased toxicity associated with selenium. They believe this to be the result of a Se-Hg-SelP complex in the blood forming a neutral compound. The altered form then results in redistribution of mercury throughout the organism or decreased mercury absorption. El-Boshy et al. (2015) demonstrated that co-administration of selenium significantly reduced cadmium concentrations in liver tissue of male Sprague-Dawley rats. Interestingly, this study also found significantly reduced selenium in the liver tissue with co-dosing (as compared with selenium-only dosing), suggesting that there is a selenium-cadmium interaction occurring external to the tissue. This is supported by the observation that both selenium and cadmium concentrations decrease by one third of their individual concentrations when administered together. They also report an ameliorative effect on antioxidant enzymes levels and blood count in selenium-treated tissues.

In light of the well-documented sparing effect of selenium in other tissues, we sought to examine the protective effect of selenium in nervous tissue. Little has been reported on selenium rescue of trace metal effects on development and how this impacts brain formation. Even less is known about any effect that might persist into adulthood and impact behavior. Therefore, the present work endeavors to address this by examining both the immediate developmental impact of selenium co-treatment as well as any longitudinal effects that may exist.

We hypothesized that cadmium would impact zebrafish brain development. Based on this, we then predicted that this impact would be detrimental, and that the effect would increase with increasing concentration. We also hypothesized that selenium would alter cadmium's effect; we predicted that selenium would work to counter the damage caused by cadmium, and would produce a sparing effect. The main questions we sought to answer were as follows:

1) What are the embryonic and longitudinal effects of cadmium exposure in the range we are examining?
2) Can selenium ameliorate the effects of cadmium treatment?
3) How might it accomplish this, and to what extent?

MATERIALS AND METHODS

Fish Maintenance

All fish were housed in the University of North Dakota aquatic laboratory in accordance with IACUC standards (Animal Welfare Assurance #A3917-01, protocol 1403-7). Adults were kept in 3L tanks on a system of filtered water. System water consisted of reverse osmosis water conditioned to a pH of 7.6-7.8 and conductivity of 800 microsiemens achieved via addition of 0.1g/L sodium bicarbonate, 0.2-0.3g/L Instant Ocean (Instant Ocean Spectrum Brands, Blacksburg, VA, USA), and calcium carbonate from crushed coral (Aquatic Habitats, Apopka, FL) – hereafter called "fish water". Fish were raised in racks on the system with 10% daily water change and triple filtration (including biological, mechanical, and charcoal) plus UV sterilization (Aquatic Habitats, Apopka, FL). Fish were kept on a 14-10 light-dark cycle. Feeding occurred twice daily with artemia given in the morning and pellet food source in the afternoon. Larvae were placed on the system at 5 dpf and raised to adulthood at 8-10 months of age.

The fish used in this study were a green fluorescent protein (GFP) reporter line driven by α1-tubulin in the central nervous system (CNS), received as a gift from Goldman (2001). For breeding, adults were set up in either individual or basket crosses, with egg collection occurring the following day. Eggs were cleaned and nonviable eggs discarded. At one day post fertilization (1 dpf), healthy-looking (e.g. the embryo had progressed to the point

where a tail bud was visible and had not clouded over), viable eggs were placed in a six well plate at a density of 50 eggs per well. Each well represented a different treatment group.

Experimental Paradigm

Two experiments were conducted: one with cadmium-only treatment, and another with cadmium plus selenium. Treatment paradigm was consistent for both, with initial exposure occurring 24 hours after fertilization to allow for axis formation and patterning to occur. This also allows for organ primordia to form. During the treatment window, organs continue to develop; in particular brain, heart, and eyes. At 48 and 72 hours (2-3 dpf), water was changed out and a fresh solution administered to ensure consistent exposure across the treatment window. At 96 hours (4 dpf), treatment was stopped and plain fish water was applied. This was done both to allow larvae a small recovery period before additional experimentation and to account for inflation of the swim bladder, to simulate the point at which the fish moves higher in the water column and is no longer in contact with the sediment. The full treatment paradigm is outlined in Figure 4; treatment groups are summarized in Figure 5.

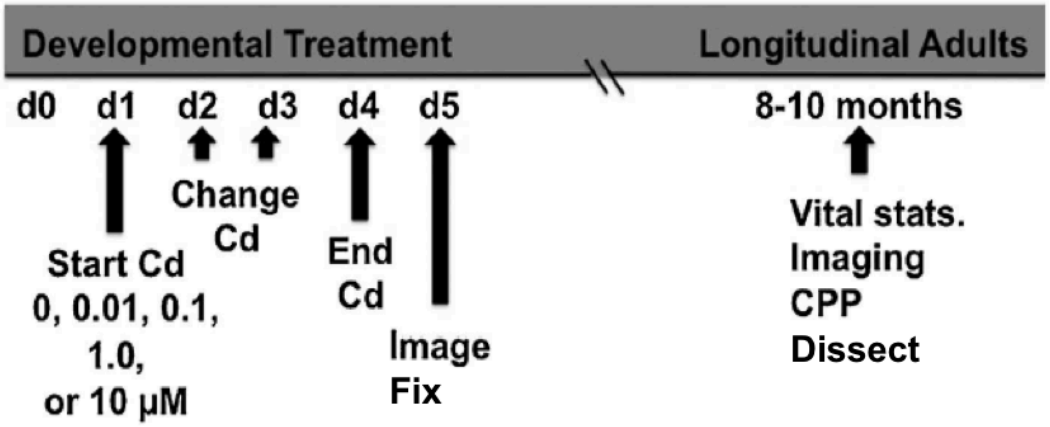

Figure 4. Summary of experimental paradigm, showing timeframe of treatment regimen and experimental measures completed. At d0, shortly after fertilization occurred, eggs were collected and washed. On d1, viable eggs were sorted out and placed in a six well plate, where they received initial exposure to their treatment (Cd only or Cd + Se). The following two days (d2 and d3) saw the aqueous solution changed out before treatment ended on d4, when the larvae were washed three times and placed in fresh fish water. On d5, larvae were either put on the system or fixed in 4% paraformaldehyde and set aside for other experiments. Longitudinal experiments were conducted between 8 and 10 months. Note that Cd + Se experiments were performed the same. Adapted from Wold et al. 2017.

Cd-only concentrations included 0.0 µM, 0.01 µM, 0.1 µM, 1.0 µM, and 10.0 µM Cd (1.124, 11.24, 112.4 and 1124 µg Cd/L). Cd + Se included combinations of 0.0, 1.0, and 10.0 µM Cd with 0.0, 0.1, and 1.0 µM Se (0, 112.4, and 1124 µg Cd/L with 0, 7.896, and 78.96 µg Se/L), for a total of six treatment groups.

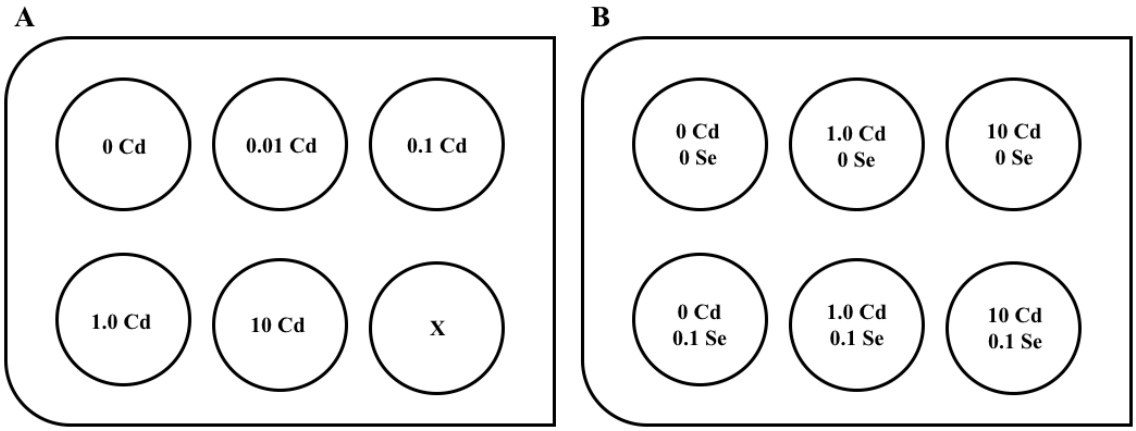

Figure 5. Concentrations used in embryonic exposure. A) shows concentrations for the Cd-only experiment; B) shows concentrations for the Cd + Se experiment. All concentrations are reported in μM.

10 mM stock solutions were made fresh daily, and consisted of either $CdCl_2$ (Sigma Aldrich, St. Louis, MO) or Na_2SeO_3 (Sigma Aldrich, St. Louis, MO) dissolved in RO water. Working solutions were created via serial dilution of the appropriate 10 μM stock solution into fish water. Cd + Se combined treatments were achieved via individual addition of each solution to the appropriate well. At 120 hours (5 dpf) surviving larvae from each well were transferred to 3L tanks and placed on the system to be raised.

Imaging & Measurements

For imaging, 5 dpf larvae were anesthetized with 40μg/L tricaine (MS-222 Sigma) in fish water and transferred to a 3% methylcellulose gel. Imaging was conducted with the aid of a specialized dissecting microscope (M165FC Leica, Heerbrugg, Switzerland), camera (DFC310FX), and imaging software (Leica Application Suite v. 4.1.0, Leica Microsystems, Heerbrugg, Switzerland). Larvae were imaged individually at 32X under bright field and again at 120X under GFP filter to obtain full-body and brain images,

respectively, as outlined in Figure 4. Leica software was used to measure body length, eye size, and interocular distance at 32X and brain region areas (telencephalon, diencephalon, hindbrain) at 120X (Figure 6). This analysis was conducted on 8 larvae per treatment group, performed in quadruplicate.

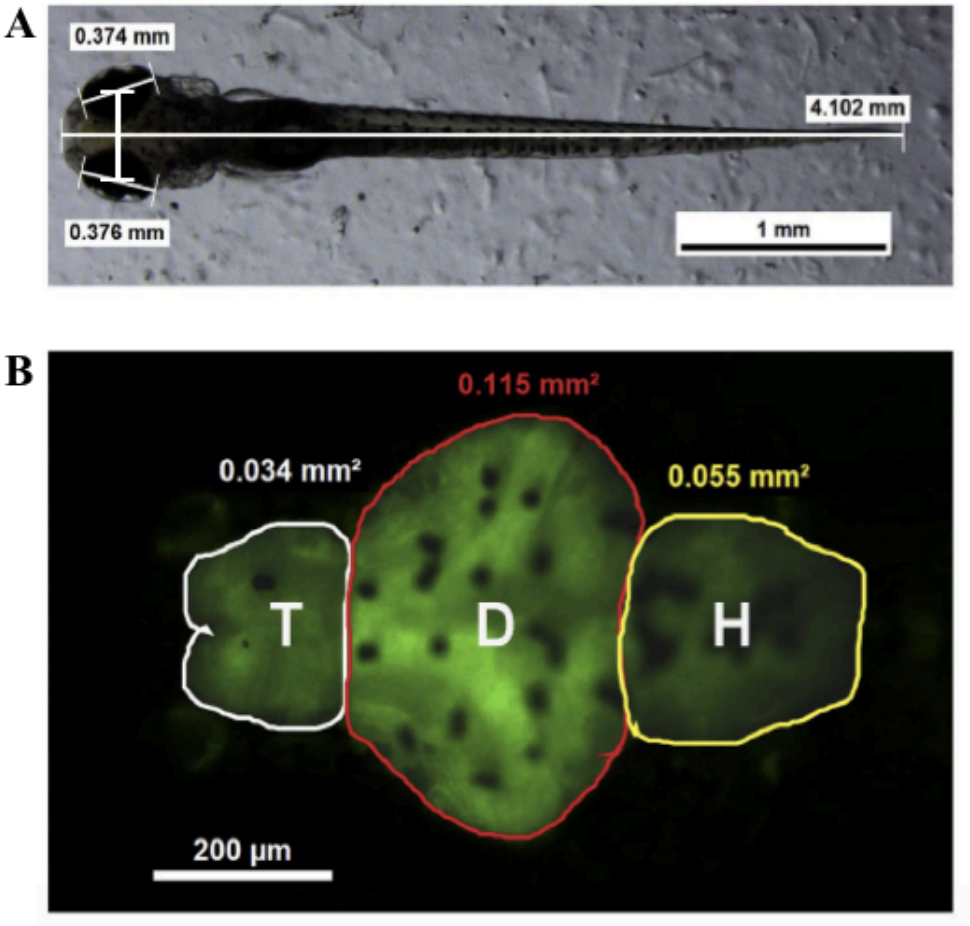

Figure 6. Measurements collected on larval fish. A) shows length measurements at 32X on bright field: length from snout to tail tip, eye length at greatest diameter, and interocular distance from center of left eye to center of right eye. B) shows brain measurements at 120X with GFP filter: telencephalic area (T), diencephalic area (D), and hindbrain area (H). Adapted from Wold et al. 2017.

Biometric Data Collection

Post-behavioral adult fish were anesthetized in 40μg/L tricaine (MS-222 Sigma) and measured for length and weight. Telencephalic area was imaged as shown in Figure 6 and measured; this was possible because the pigmentation and skull overlying the forebrain are transparent, while the rest of the brain is concealed. Survival in each group was documented at six months of age, before behavioral assessment. At about eight months, fish were sexed, weighed, measured for length, and anesthetized in tricaine before being sacrificed via rapid decapitation. Heads were severed just caudal to the pectoral fin and then were fixed in 4% paraformaldehyde. The following day, brains were dissected out and weighed. Note that this weight was used as a metric for comparison rather than as a true reflection of wet weight because of fixation. For brain dissections, a probe was used to pierce the bone just anterior to the telencephalic region, where the teleost skull is at its thinnest. The remainder of the skull was then peeled away to expose the dorsal aspect of the brain. The spinal cord was severed just caudal to where it meets the hindbrain to ensure collection of the entire hindbrain, and the whole brain was then flipped up rostrally to expose the optic chiasm. The optic nerves were severed, and surrounding tissue was peeled away to expose the entire ventral aspect of the brain. From there, the rostral flipping continued until no further connection remained between the brain and the rest of the head. The olfactory bulbs were removed and the brain was set aside.

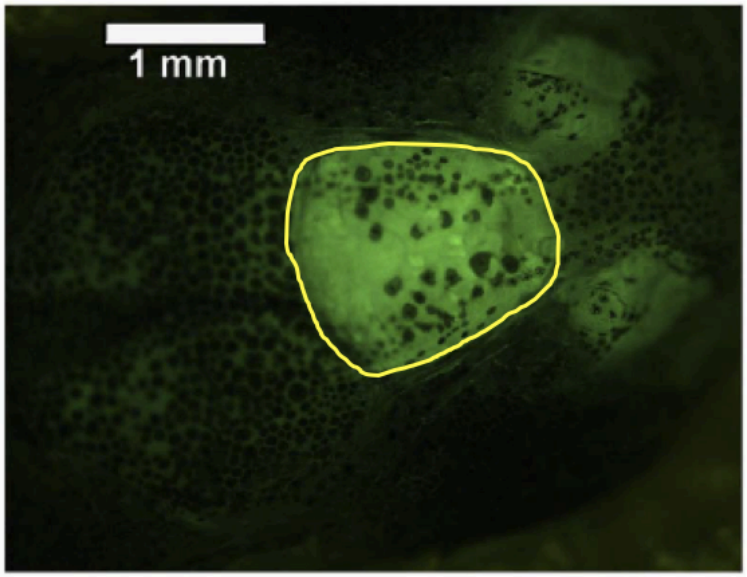

Figure 7. Adult telencephalon measurement. Image showing GFP+ adult brain with telencephalon highlighted. Adapted from Wold et al 2017.

Bioaccumulation

Larval bioaccumulation was measured via mass spectrometry. 5 dpf larvae were fixed in 4% paraformaldehyde before being dehydrated. Whole body samples were collected in triplicate for each of the following concentrations (presented in micromoles as Cd concentration/Se concentration): 0/0, 0/0.1, 0/1.0, 1.0/0, 10.0/0, 1.0/0.1, 1.0/1.0, 10.0/0.1, and 10.0/1.0. To determine tissue-specific bioaccumulation, 100 larvae from each of three groups (0/0, 10.0/0, and 10.0/1.0) were dissected for eyes, brains, and trunks. This experiment was later repeated in triplicate with one alteration – rather than "trunk" including heart, liver, pancreas, gut and swim bladder, trunk dissection was refined to collection of only heart and liver. Embryonic dissections were conducted under the Leica dissecting microscope (M165FC Leica, Heerbrugg, Switzerland). Larvae were transferred from paraformaldehyde into a shallow dish containing RNAlater (Thermo Fisher

Scientific, Waltham, MA, USA). Dissections were performed as summarized in Figure 8. The larvae were first turned on one side and a small microdissection probe was inserted into the eye socket to pry the eye out. The fish were then flipped and the procedure repeated on the other eye. This exposed the brain, which was then excised along with as little surrounding tissue as possible. Finally, the probe was inserted just rostral to the swim bladder to allow removal of the heart and liver. Tissue samples were transferred back to a 4% paraformaldehyde solution as they were collected. Once tissue samples had reached an acceptable wet weight, samples were spun down in a vacufuge at 45°C and 1400rpm (Eppendorf EG, Hamburg, Germany). For mass spectrophotometric analysis, samples were subjected to an acid digest containing HCl and HNO_3 (Fischer Scientific) before being microwaved to complete the process. Cd and Se standards were obtained from Inorganic Ventures (Christiansburg, VA). Samples were then analyzed using a Thermo Scientific iCAP Qc inductively coupled plasma mass spectrometer (Bremen, Germany).

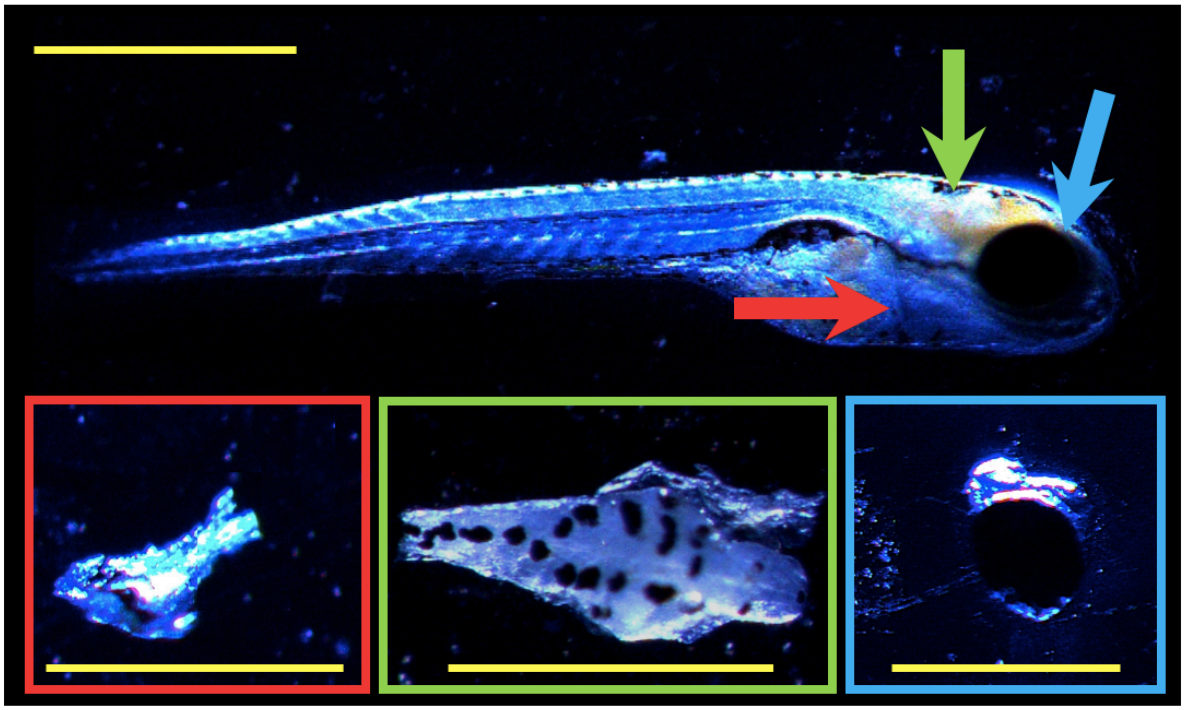

Figure 8. Larval dissection schematic. Arrows indicate different tissue samples; arrow color corresponds to box color around the organ dissected out. Yellow bars are for scale and represent a length of 500 microns. Red = heart/liver, green=brain, and blue = eyes.

Behavioral Assay

Conditioned place preference assay was conducted as described previously (Wold et al., 2017; Mersereau et al., 2016) using adults aged 8 months. This assessment was performed as a measurement of learning response based on monoaminergic reward pathways, utilizing 5mg/L cocaine to simulate the reward obtained via foraging behavior. The cocaine reward works by blocking monoaminergic transporters, especially the dopamine transporter (DAT). This raises extracellular DA levels, mimicking dopaminergic neuronal firing and activating the reward pathway (Volkow & Morales, 2015; Darland et al., 2012; Pierce & Kumaresan 2006). This is the same pathway activated with successful foraging behavior, allowing us in essence to measure environmental responsiveness (Baudonnat et al., 2013; Miller et al., 2013). Fish were

housed in individual 3L tanks for the one-week duration of the assay, and were daily transferred to lanes in a specialized compartmentalized tank (shown in Figure 9) for the assay itself. Compartments were separated by perforated walls that could be swapped out for solid walls in the instance of confinement, and water volume of the three compartments had a 1:2:1 ratio, with the largest compartment being the middle. For free swim, the dividers between compartments contained a 1" diameter hole at the center. When fish were confined to a single compartment for isolations, this was changed out for a solid barrier. On day 1, fish were allowed a 45 minute free swim to acclimatize to the lane. Day 2 involved a 10 minute free swim, during which baseline preference for each chamber was recorded, followed by 30 minute isolation in both the front and back compartments. Day 3 repeated this procedure, with the exception that the order of isolation was reversed, with fish being confined to first the back compartment, then the front. Day 4 included a final baseline, which was then used to determine the preferred compartment. There was a 30 minute isolation in the preferred compartment without drug, followed by a 30 minute isolation in the less-preferred compartment. On this day, the drug was introduced. Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in fish water to a concentration of 5 mg/L and then added to the less-preferred compartment during the second isolation. Water exchange between compartments is not a concern as it was previously determined to be minimal based on flow experiments conducted with phenol red (Darland et al., 2012). This procedure was repeated on day 5, and the first CPP was recorded. Recording occurred one of two ways: movements were tracked using TopScan 3.00 (TopScan 3.00, Clever System Inc., 2011) or via assessment with a ternary code. This code used '1' to indicate presence in the front compartment, '2'

to denote the center compartment, and ‘3’ to mark the rear compartment. Fish that did not move received a ‘0’ to indicate freezing behavior associated with the stress response. Compartments were numbered 1-3, and time spent in each was documented. On day 6 a final baseline measurement was recorded to check for any change in preference as a result of the conditioning. Fish were then returned to their home tank to await further analysis. Change in preference was determined via comparison of the two CPP recordings to the baseline measurement. Fish observed to exhibit a stress response such as freezing for more than half of the reading were excluded from the final analysis. An untreated control group where the fish did not receive any cocaine reward was included in the analysis as a null.

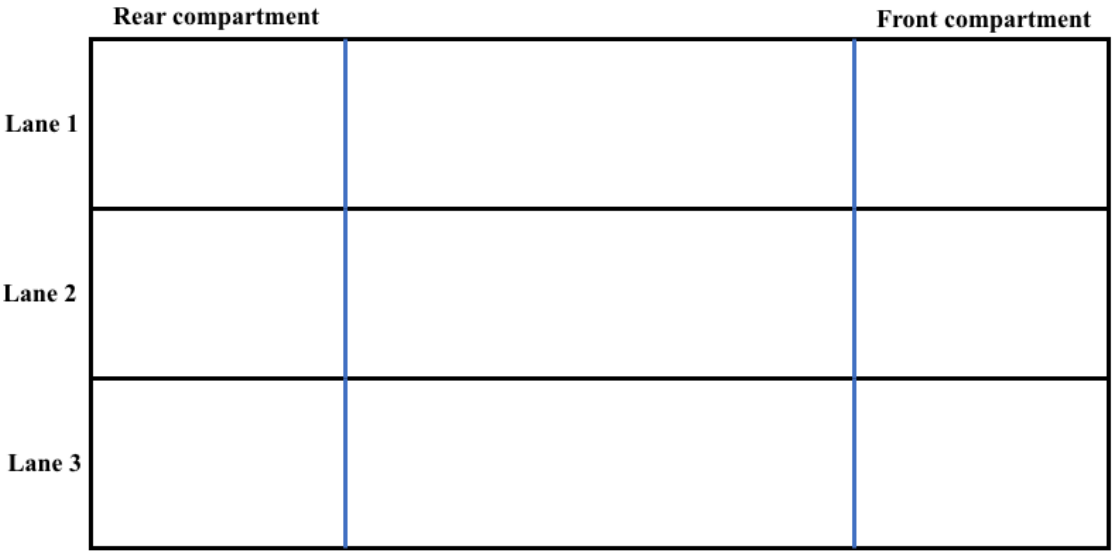

Figure 9. Schematic of tank used for behavioral assay. The front and rear compartments contain 250 ml water; the middle compartment contains 500 ml for a total of 1L water per lane. Blue lines represent removable compartment dividers which may be solid to enable isolation within a compartment or contain a 1” diameter hole to allow movement between compartments.

Statistical Analysis

Relatedness between treatment groups was assessed using one-way ANOVA (R software v. 3.2.2, R Foundation, Vienna, Austria). Tukey's HSD test was used to determine significant differences between groups, with α set to 0.05. Groups with moderate to high variance were further tested for bimodal distribution as well as the presence of any outliers. For adult biometric measurements, Pearson's correlational analysis was used to identify trends between variables such as weight and length. Sexes were compared using a series of unpaired two-tailed t-tests. For groupwise comparisons, variables of interest (i.e. brain weight and telencephalic area) were normalized to body length. Percentages were graphed as such, though they were arcsin transformed for statistical analysis. For behavioral testing, paired t-tests were used to compare baseline preference to post-conditioned preference for each group.

RESULTS

Cadmium Affects Development & Behavior

Early cadmium exposure has been shown to produce detrimental effects in the developing aquatic organism (Sfakianakis et al., 2015; Jezierska et al., 2009a). With this in mind, we sought to describe the impact of cadmium exposure on larval zebrafish, particularly as pertains to neural development. We hypothesized that increasing the concentration of cadmium the larvae were exposed to would produce developmental defects of similarly increasing severity. When exposed to concentrations ranging from 0 μM to 10 μM $CdCl_2$, larval brains displayed a marked decrease in size, most notably in the telencephalic region (Figure 9). All brain regions had a significant decrease in size at the 10 μM concentration, with the diencephalon also being significantly affected at the 1.0 μM concentration (Table 1). We also described an effect on eye size and body length, with significant decreases being present in eye diameter at the 1.0 and 10 μM concentrations and a decrease in body length evident at the 10 μM concentration (Table 1).

Table 1. Summary of cadmium effects on larval metrics. Mean and SEM for 5dpf larval telencephalon, diencephalon, and hindbrain areas (in mm^2), eye diameter (mm), and body length (mm) at each concentration of cadmium. Significant difference from control denoted in bold italics.

	0 µM	**0.01 µM**	**0.1 µM**	**1.0 µM**	**10 µM**
Tel	0.0305 ± 0.00059	0.0316 ± 0.00067	0.0295 ± 0.00049	0.0291 ± 0.00037	***0.0267 ± 0.00053***
Di	0.1210 ± 0.00156	0.1193 ± 0.00208	0.1186 ± 0.00155	***0.1149 ± 0.00212***	***0.1125 ± 0.00165***
Hind	0.0429 ± 0.00088	0.0450 ± 0.00011	0.0659 ± 0.01697	0.0431 ± 0.00151	***0.0399 ± 0.00123***
Eyes	0.3437 ± 0.00228	0.3452 ± 0.03214	0.3409 ± 0.00280	***0.3354 ± 0.00224***	***0.3332 ± 0.00260***
Body	3.962 ± 0.014	3.934 ± 0.017	3.925 ± 0.024	3.930 ± 0.026	***3.815 ± 0.017***

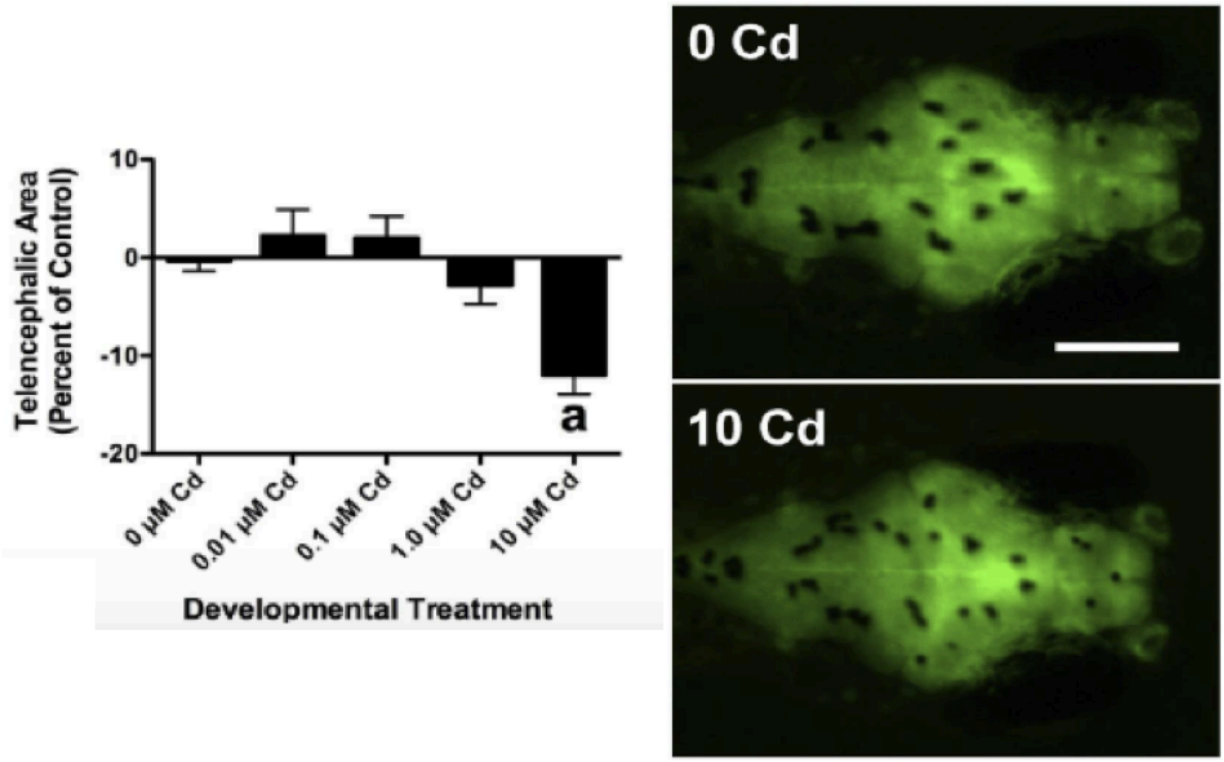

Figure 10. Effects of cadmium exposure on larval brains. Telencephalic area is shown graphically as a percent of the control (left), while overall difference in brain morphology is demonstrated with images taken from GFP+ larvae (right). Treatment groups had n=23-28, with the exception of 0 µM Cd, which had n=73. Different letters indicate significantly different means relative to the control. Scale bar represents 200 microns. Adapted from Wold et al., 2017.

One interesting morphological difference seen in cadmium-treated larvae was the presence of a spinal arch of approximately 10 degrees (Figure 11). This occurred most frequently at the 10 µM concentration, but was also observed at lower concentrations. Spinal abnormalities in aquatic species exposed to heavy metals have been previously documented, and may present as scoliosis, kyphosis, or lordosis, as in the present case (Sfakianakis et al., 2015). Another anomaly we observed was a hatching delay, particularly in larvae exposed to higher concentrations. The delay was most noticeable at 2 dpf, and persisted until 4 dpf when nearly all hatching was complete (Table 2). This

effect has been described previously in other species, especially *Cyprinus carpio* (Jezierska et al., 2009a; Messaoudi et al., 2009).

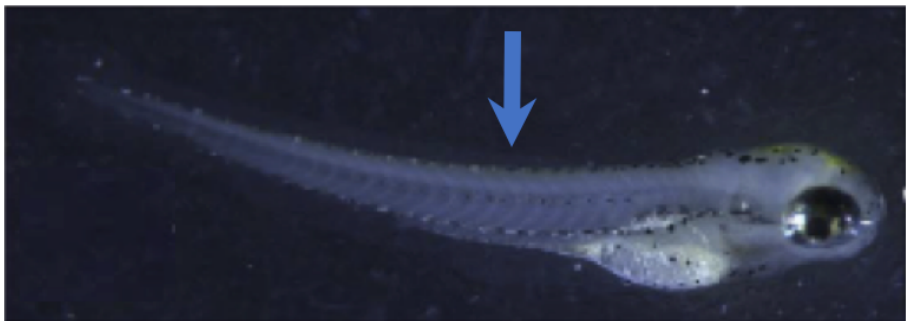

Figure 11. Morphological defect observed in some larvae. Arched spine in 5dpf larval zebrafish treated with 10.0 µM Cd.

Table 2. Summary of hatching data. Average percent of larvae hatched per treatment by days post fertilization. Bold italics denote significant difference from control ($p<0.05$).

Concen. (Se/Cd)	**2dpf**	**3dpf**	**4dpf**
0/0	0.503	0.909	0.988
0/0.01	0.421	0.929	0.966
0/0.1	0.419	0.947	0.965
0/1	0.606	0.968	0.979
0/10	0.328	0.845	0.985
0.01/0	0.442	0.889	1
0.1/0	0.609	***0.972***	1
0.1/1	***0.702***	***0.98***	0.998
0.1/10	0.38	0.824	1
1.0/0	0.677	0.79	1
1.0/1	***0.912***	***1***	1
1.0/10	***0.72***	***1***	1
10/0	0.639	0.908	1
10/10	0.14	0.967	1

Survival was also impacted, with 10 μM treated fish displaying the lowest survival rates at less than 50% of control at 6 months. Longitudinal survival has not been much reported elsewhere, as most studies are concerned with immediate survival and so describe acute effects of metal exposure within a much smaller window. Because we used a series of sublethal concentrations, the majority of our fish survived to be placed on the system and raised. Therefore, our survival counts were taken much later, when they would more accurately reflect fitness via survival to adulthood.

The decrease in brain size we noted in larval fish did not persist into adulthood, with the ratio of brain weight to body length actually showing a significant increase from the control across treatments. However, this trend did not correspond to an increase in learning behavior. Fish showed a steady decrease in percent preference with increasing larval cadmium exposure, with the exception of the 10 μM concentration, which bounced back somewhat, though not to control levels. Overall, we were able to describe a significant impact of cadmium treatment on development and behavior.

Cadmium Affects Larval Development, Morphology, Hatching Rate, & Bioaccumulation

We observed decreases in larval brain size across all brain regions, though this result was most notable in the telencephalon. Our interest in telencephalic development is based on its role in reward pathways related to learning; if we note a decrease in telencephalic size, this may correspond to a decrease in learning behavior. Telencephalic size was significantly reduced by exposure to cadmium (shown in Figure 12 and summarized in Table 1). One-way ANOVA for the effects of cadmium concentration on telencephalic area produced significant results ($F_{4,143} = 10.16$, $p < 0.001$), with the most marked decrease present at the 10 μM concentration.

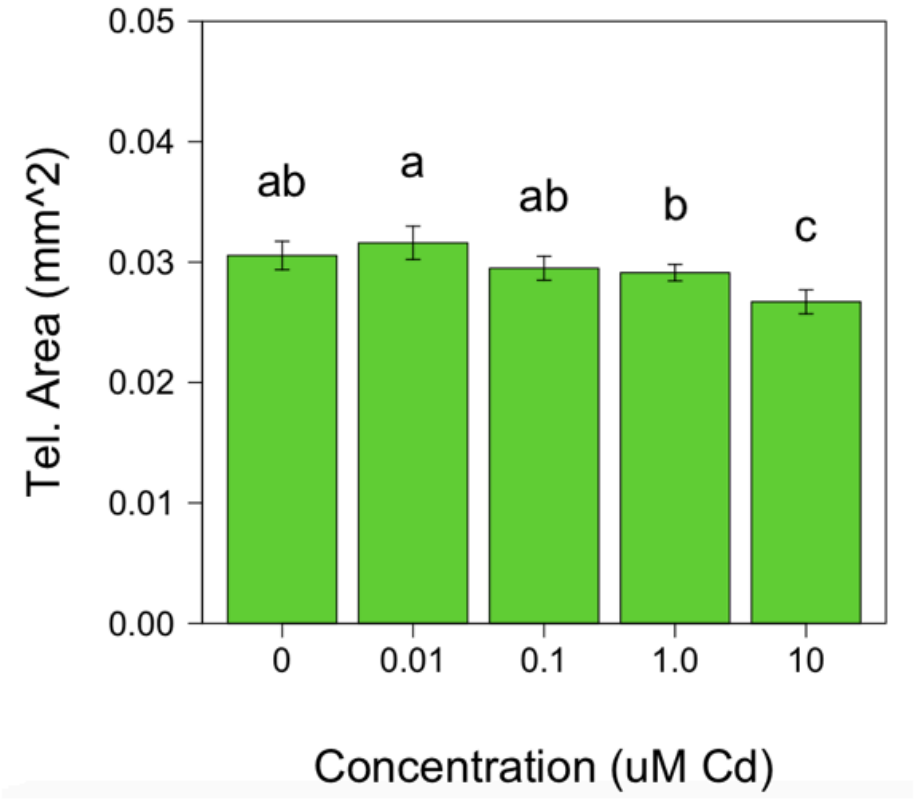

Figure 12. Cadmium affects larval telencephalic size. Shown here are the effects of varied cadmium treatments on telencephalic area. Treatment groups range from n = 32-47, with the exception of 0 μM Cd, which had n = 79; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

We observed a similar decrease in diencephalic area, with the larvae receiving 10 µM Cd treatment showing a significant difference from the control (Figure 13). Larvae treated with 1.0 µM Cd also showed a significant decrease from the control. Diencephalic size was significantly reduced with exposure to cadmium (summarized in Table 1). One-way ANOVA for diencephalons was significant ($F_{4,140} = 4.00$, $p < 0.001$), and followed a similar trend to that of telencephalic area.

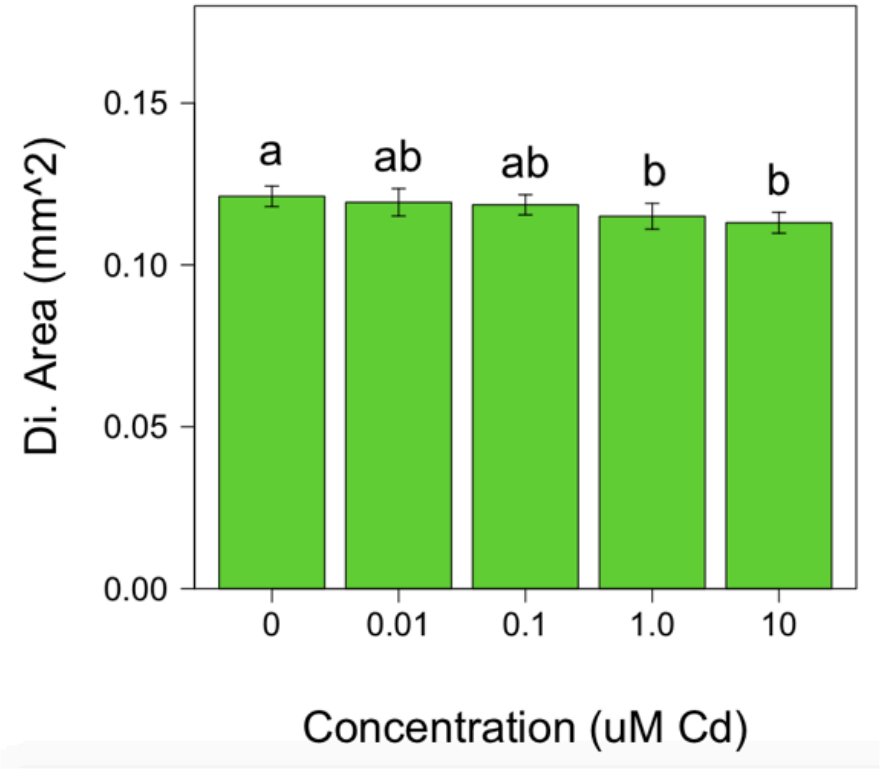

Figure 13. Cadmium affects larval diencephalic size. Shown here are the effects of varied cadmium treatments on diencephalic area. Treatment groups range from n = 32-47, with the exception of 0 µM Cd, which had n = 79; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

Hindbrain size was also affected by cadmium exposure, most significantly again at the 10 µM concentration (Figure 14). Hindbrain area was significantly decreased with exposure to cadmium (summarized in Table 1). A one-way ANOVA of hindbrain treatment produced significant results ($F_{4,140} = 2.96$, $p = 0.022$).

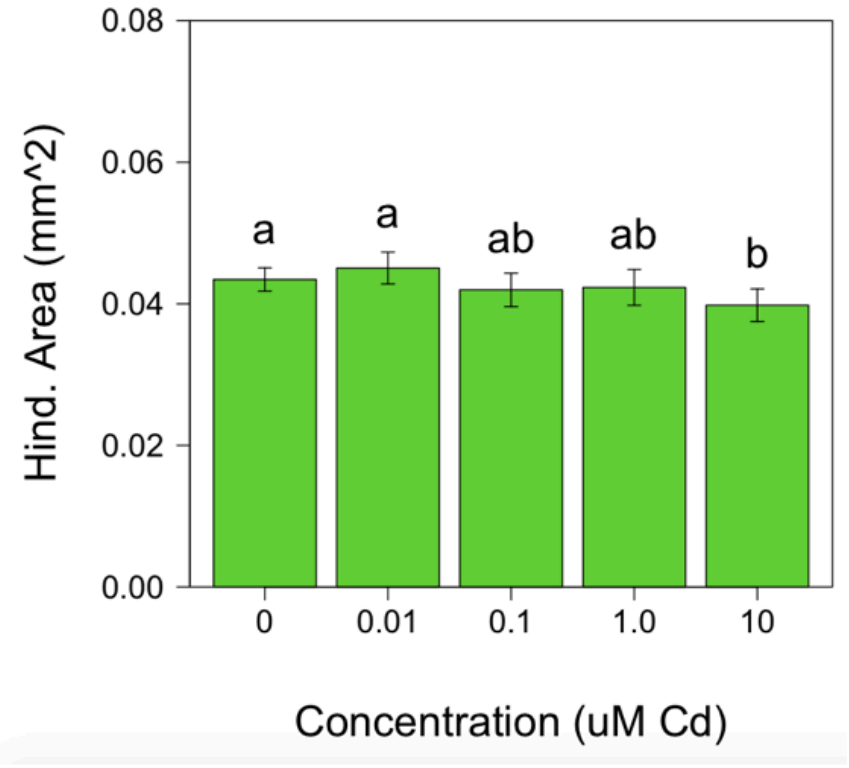

Figure 14. Cadmium affects larval hindbrain size. Shown here are the effects of varied cadmium treatments on hindbrain area. Treatment groups range from n = 32-47, with the exception of 0 µM Cd, which had n = 79; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

As stated above, eye measurements were also affected. We reported a decrease in eye diameter at the 1.0 μM and 10 μM Cd concentrations. As shown in Figure 15, there was a significant decrease in eye diameter with exposure to cadmium (summarized in Table 1). One-way ANOVA on eye diameter produced significant results ($F_{4,102} = 4.082$, $p < 0.001$).

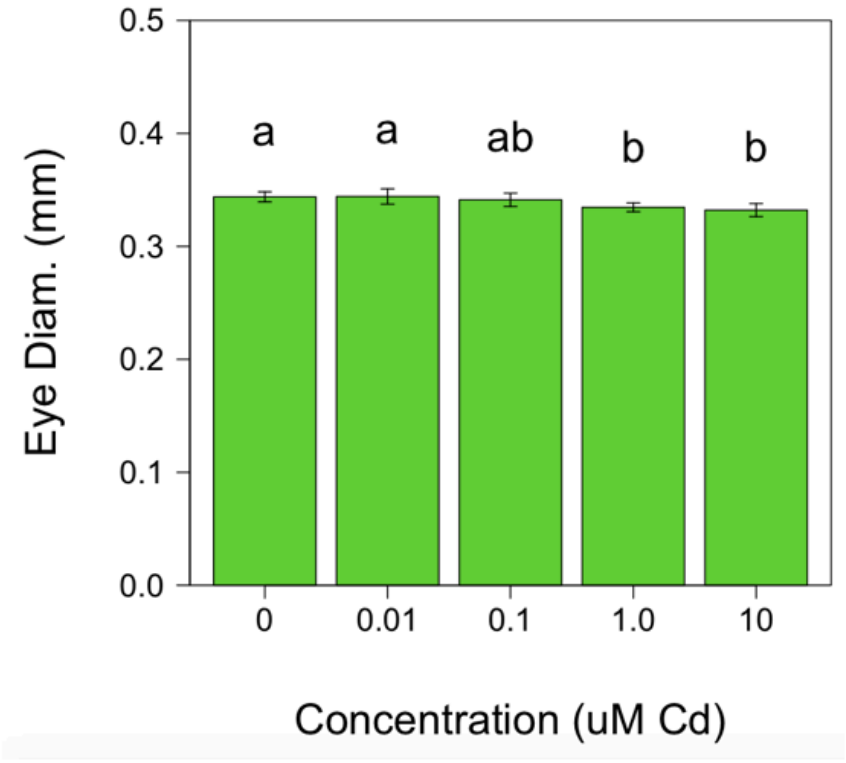

Figure 15. Cadmium affects larval eye diameter. Shown here are the effects of varied cadmium treatments on eye length. Treatment groups range from n = 26-30, with the exception of 0 μM Cd, which had n = 66; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

We reported a decrease in body length at the 10 μM Cd concentration (Figure 16). Exposure to cadmium significantly decreased larval body length, with the average dropping from 3.962 mm in the control to 3.815 mm at 10 μM Cd, representing a 4% decrease in overall body size (summarized in Table 1). The one-way ANOVA for body length of cadmium-exposed larvae was significant ($F_{4,102} = 4.082$, $p < 0.001$).

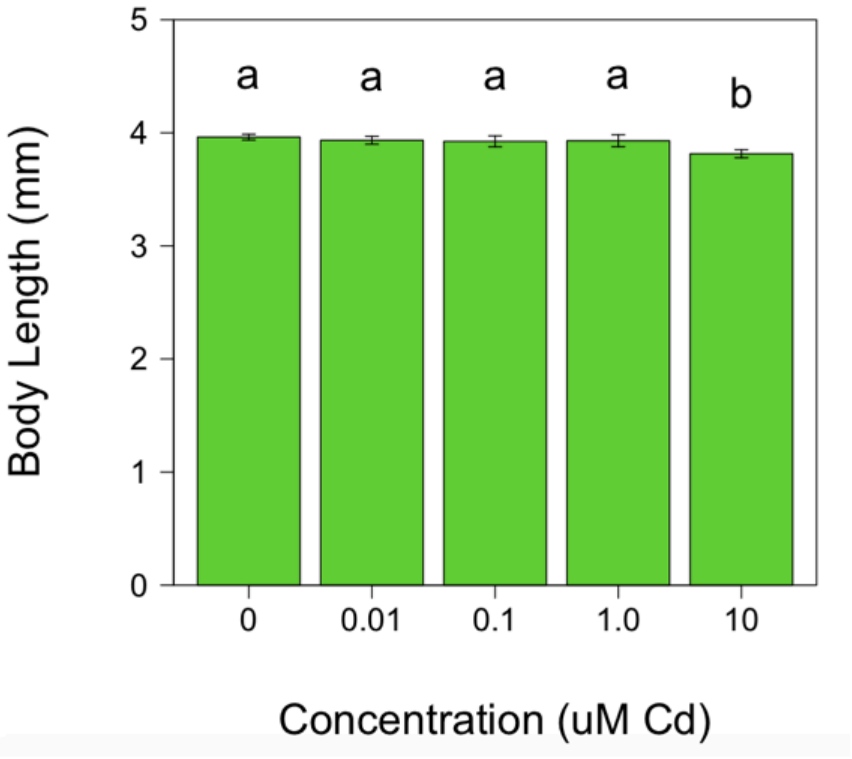

Figure 16. Cadmium affects larval body size. Shown here are the effects of varied cadmium treatments on body length. Treatment groups range from n = 30-35, with the exception of 0 μM Cd, which had n = 90; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

The curved spines seen in cadmium-treated larvae were most prevalent at the higher concentrations, as shown in Figure 17. A one-way ANOVA assessing their prevalence as a percent of the control was significant ($F_{4,51} = 11.62$, $p < 0.001$), with the most significant differences observed at 1.0 µM and 10 µM Cd concentrations.

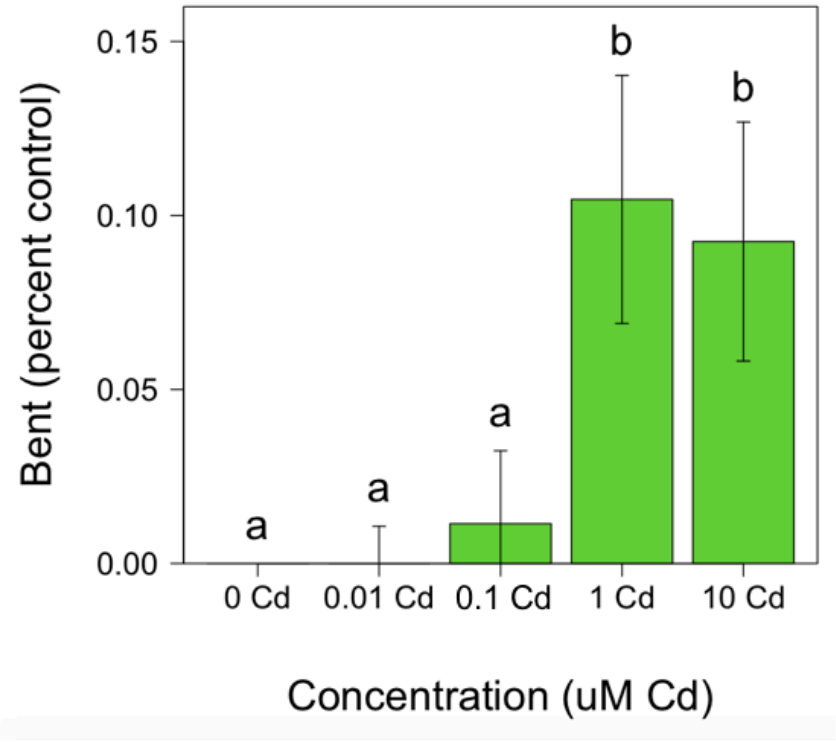

Figure 17. Cadmium affects incidence of spinal curvature. Shown here are the effects of varied cadmium treatments on frequency of spinal arch as a percent of the control. Treatment groups range from n = 7-15; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

There was a delay observed in hatching time with cadmium exposure; this effect was more pronounced at the highest concentration (shown in Figure 18; summarized in Table 3). The effect was most pronounced at 2dpf, and dampened by 3dpf as most larvae were hatched by this time. At 2dpf, fish at low concentrations showed a slight decrease in proportion of eggs hatched when compared to controls (about 50% hatched). At 1.0 µM Cd, hatching rate increased to nearly 70%, and then at 10 µM Cd there was a dramatic decrease to about 33% (Figure 18, data summarized in Table 2). This pattern did not persist to 3dpf; there was a slight increase in percent hatched until the 1.0 µM concentration, peaking at 97% hatched, followed by a decline at the highest concentration to about 85%. One-way ANOVA on cadmium-only treatments did not produce any significant difference at 2dpf ($F_{4,102} = 1.75$, $p = 0.145$) or 3dpf ($F_{4,102} = 1.55$, $p = 0.195$), though there is a trend of slight increase until 1.0 µM with a dropoff at 10 µM.

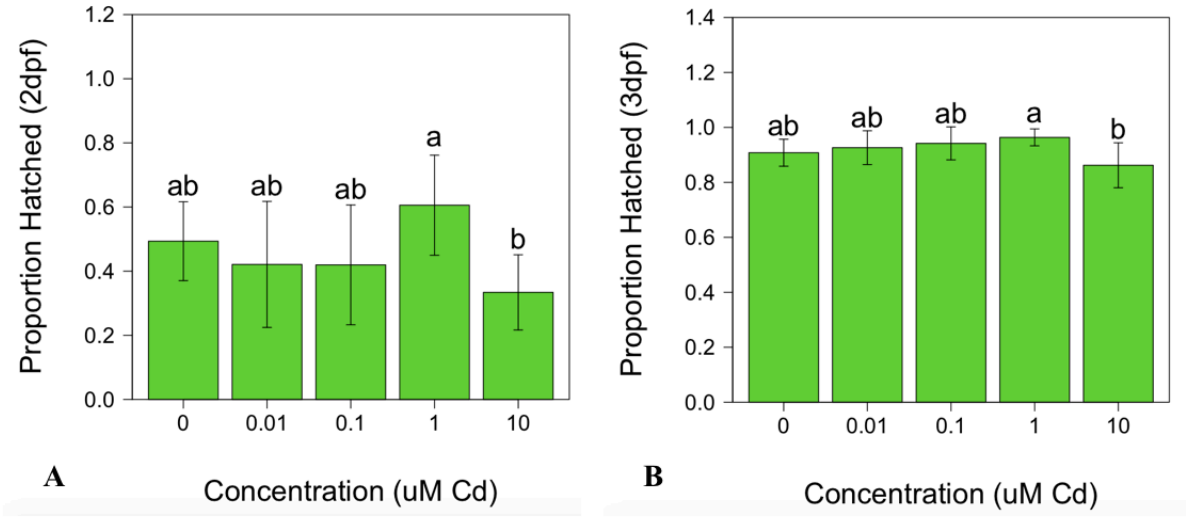

Figure 18. Cadmium affects hatching rate. Shown here are the effects of varied cadmium treatments on proportion of larvae hatched at (A) 2 dpf, and at (B) 3 dpf. Treatment groups had n = 12-28; p = 0.145, 0.195. Different letters denote significantly different means as per Tukey's HSD test.

Cadmium is a ready bioaccumulator; because of this we elected to test for levels of accumulation in whole body samples at varying concentrations. Initial mass spectrometric analysis showed an increase in cadmium accumulation with increasing concentration. This is consistent with other reports of cadmium uptake and sequestration (Matz, Treble, & Krone, 2007). ANOVA was significant ($F_{4,19} = 2.991$, $p < 0.05$), with the highest accumulation appearing at the 10 μM concentration (Figure 19). Further analysis showed a general trend of increase, with a slight spike at the 0.1 μM concentration.

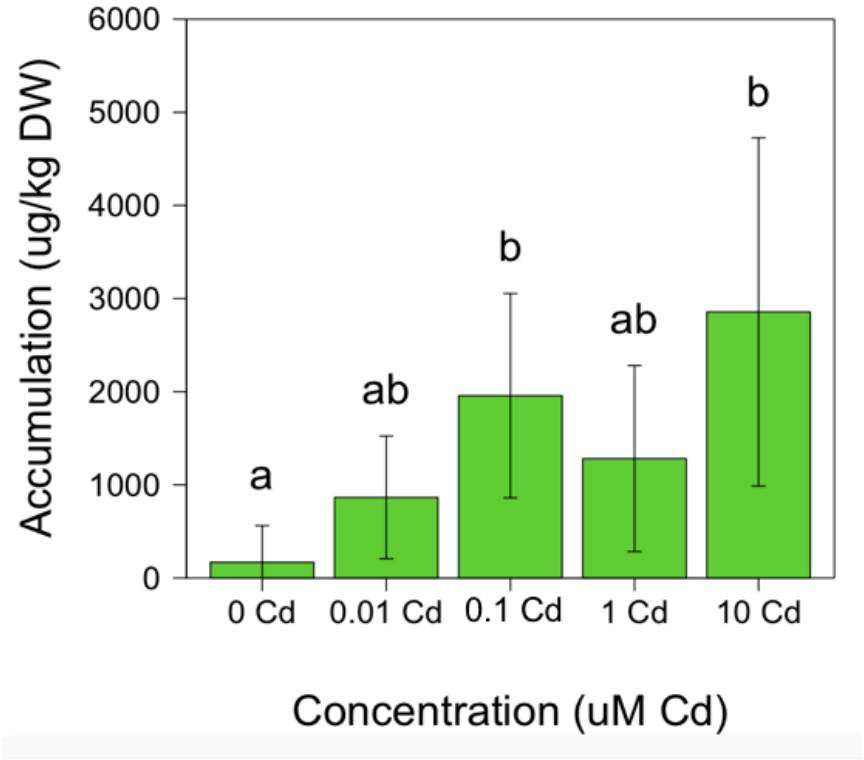

Figure 19. Cadmium concentration affects bioaccumulation. Shown here are the effects of varied cadmium treatments on level of whole tissue cadmium accumulation. Treatment groups range from n = 1-3 tubes of whole body samples, each representing 150-300 larvae; $p < 0.05$. Different letters denote significantly different means as per Tukey's HSD test.

Cadmium Affects Longitudinal Survival, Adult Brain Metrics, & Behavior

Cadmium treatment was found to have an impact on survival to six months, as shown in Figure 20. At the lower concentrations, cadmium-treated fish actually had greater survival than controls, but at 10.0 µM Cd, survival dropped off to about 30%. When taken as a percent of the control, six month survival in 10 µM treated fish dropped to less than 50% of the control. A one-way ANOVA was significant ($F_{4,37} = 4.949$, $p = 0.0027$).

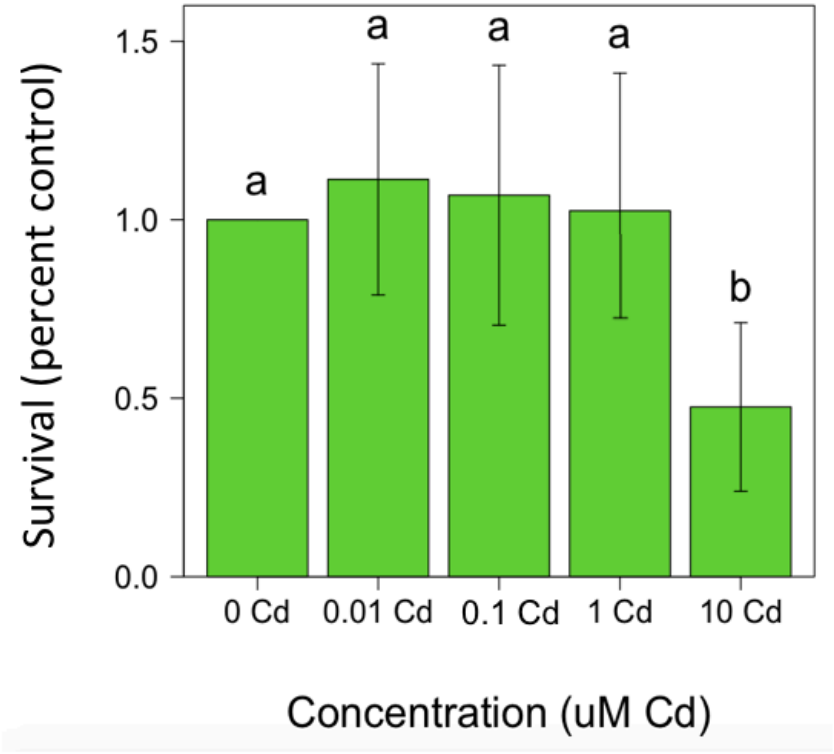

Figure 20. Cadmium affects longitudinal survival. Shown here are the effects of varied cadmium treatments on survival to six months, displayed as a percent of the control. Treatment groups range from n = 50-54, with the exception of 10 µM Cd, which had n = 26; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

The sexes displayed different means across measurements, with females generally tending to be higher, though the difference was not significant. Adult measurements were run through a series of Pearson's correlational tests. Body length and body weight had a strong positive correlation ($r = 0.927$, $r^2 = 0.859$, $p < 0.001$), as did body weight and brain weight ($r = 0.747$, $r^2 = 0.558$, $p < 0.001$), body length and interocular distance ($r = 0.87$, $r^2 = 0.757$, $p < 0.001$), body length and eye diameter ($r = 0.821$, $r^2 = 0.674$, $p < 0.001$), telencephalic area and body length ($r = 0.790$, $r^2 = 0.624$, $p < 0.001$), and telencephalic area and brain weight ($r = 0.794$, $r^2 = 0.630$, $p < 0.001$). Given that fish were not kept in individual tanks, brain and eye measurements were normalized to body length or weight to provide a ratio for more meaningful comparison. Biometric data was assessed by sex with a series of two tailed t-tests (summarized in Table 3).

Table 3. Summary of measurements taken from adults. Weights are in mg, lengths are in mm, and areas are in mm^2, unless otherwise noted. The right half of the table contains ratios, which are unitless. Averages for each sex as well as results of two-tailed t-tests comparing sexes for the variable of interest are reported above. L = body length, W = body weight, BW = brain weight, Eye = eye diameter, Int = interocular distance, Tel = telencephalic area. Sample sizes were n = 44 for males, n = 59 for females. Significant values are indicated with bold italics.

	L (cm)	**W (g)**	**BW**	**Eye**	**Int**	**Tel**	**Wt/L**	**Eye/L**	**Int/L**	**BW/W**	**Tel/L**
M	3.25	0.28	7.4	2.34	2.64	1.99	0.087	0.072	0.081	0.026	0.613
F	3.45	0.39	8.1	2.48	2.86	2.24	0.111	0.072	0.083	0.021	0.648
t	3.37	4.37	2.15	3.11	3.97	2.59	4.67	-0.40	1.32	-4.40	1.12
p	***0.001***	***<0.001***	***0.034***	***0.0025***	***<0.001***	***0.012***	***<0.001***	0.690	0.190	***<0.001***	0.268

Of most interest to us are the ratios of brain weight to body length and telencephalic area to body length. These provide a useful metric for assessing changes in brain size with increasing concentration of larval cadmium exposure. The strong relationship between body length and brain weight as per Pearson's correlational test ($r = 0.747$, $r^2 = 0.558$, $p < 0.001$) provided the basis for our analysis of that ratio by concentration (Figure 21B). When run through a one-way ANOVA to test for effect of cadmium treatment, the results of the brain weight to body length ratio by concentration were significant ($F_{4,98} = 8.868$, $p < 0.001$). There was a steady increase in the proportion of brain weight to body length as the concentration of larval cadmium treatment increased, with a slight decrease at 10 µM Cd. All treatment groups from 0.1 µM Cd displayed a significant increase from the control (Figure 21A).

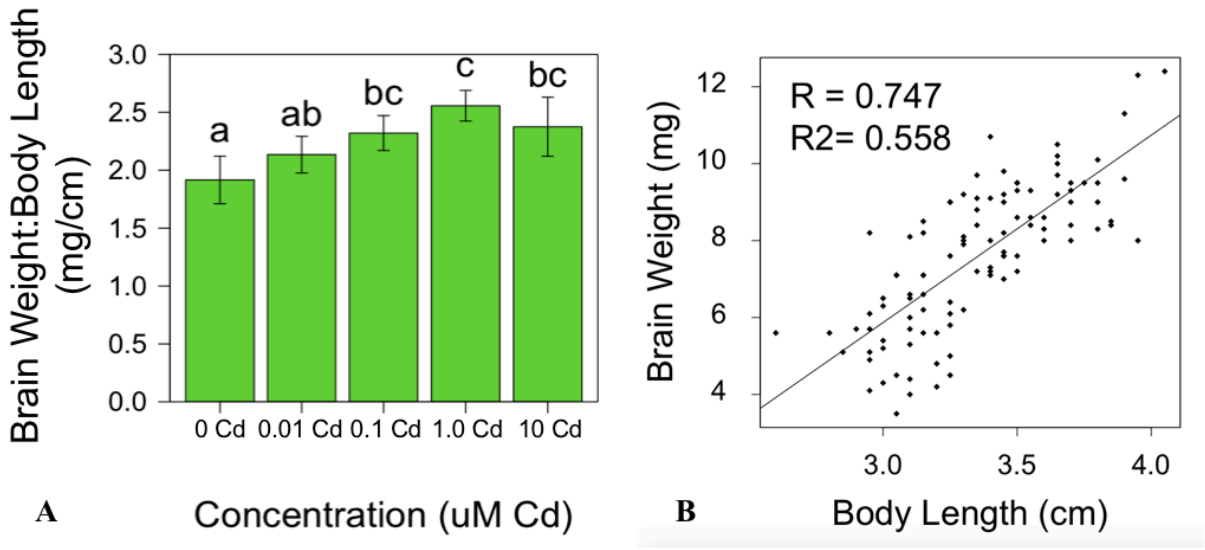

Figure 21. Cadmium affects adult brain weight. Shown here are the effects of varied cadmium treatments on the ratio of brain weight to body length. This effect is displayed (A) by treatment and (B) as a correlation irrespective of concentration. This data represents adult dissections with $n = 103$; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

A similarly strong relationship between telencephalic area and body length ($r = 0.790$, $r^2 = 0.624$, $p < 0.001$) provided the basis for the telencephalic ratio (shown in Figure 22B and summarized in Table 4). A one-way ANOVA assessing the telencephalic area to body length ratio by concentration was not significant ($F_{4,59} = 1.088$, $p = 0.371$), though there was some indication of a slight increase in telencephalic size at the 0.01 µM, 1.0 µM, and 10 µM Cd treatments (Figure 22A).

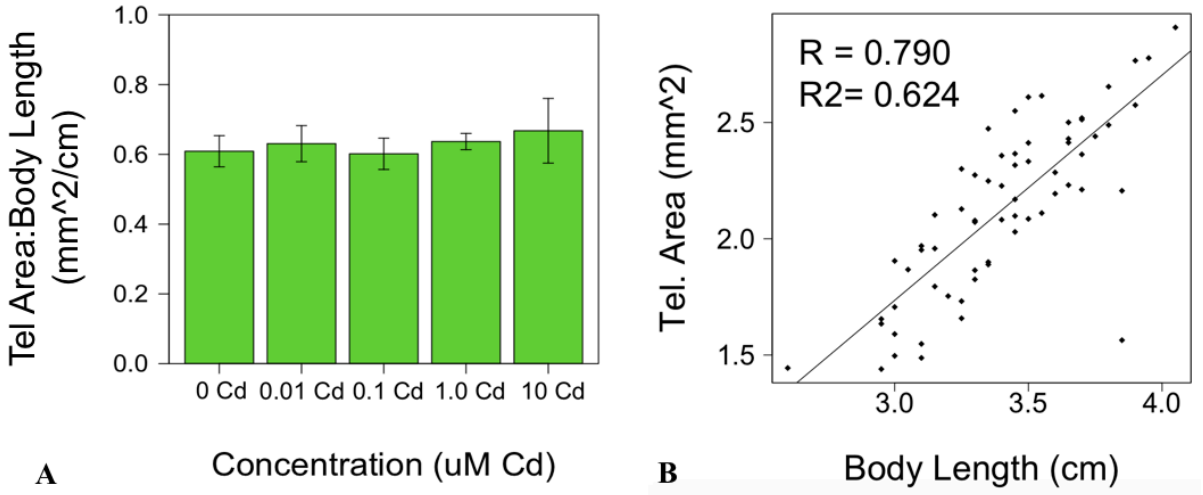

Figure 22. Cadmium affects adult telencephalic area. Shown here are the effects of varied cadmium treatments on the ratio of telencephalic area to body length. This effect is displayed (A) by treatment and (B) as a correlation irrespective of concentration. This data represents adult dissections with $n = 152$; $p = 0.371$. Different letters denote significantly different means as per Tukey's HSD test.

Longitudinal effects of cadmium exposure on aquatic species have not been much studied; adult effects represent a gap in our understanding. As such, we endeavored to determine what effect varying concentrations of cadmium would have on learning behavior in adult fish that were exposed as larvae. At eight to ten months of age, adult fish underwent a conditioned place preference (CPP) behavioral assay to test for

dopaminergic reward response. Test groups included an untreated control that did not receive the cocaine reward used as a behavioral benchmark. There was a significant difference in preference between this group and the control fish. The assay produced a nonlinear effect with a marked decrease in percent preference with increasing concentration of larval cadmium exposure through the 1.0 μM concentration followed by a rebound at the 10 μM concentration (Figure 23). One-way ANOVA was significant ($p < 0.05$).

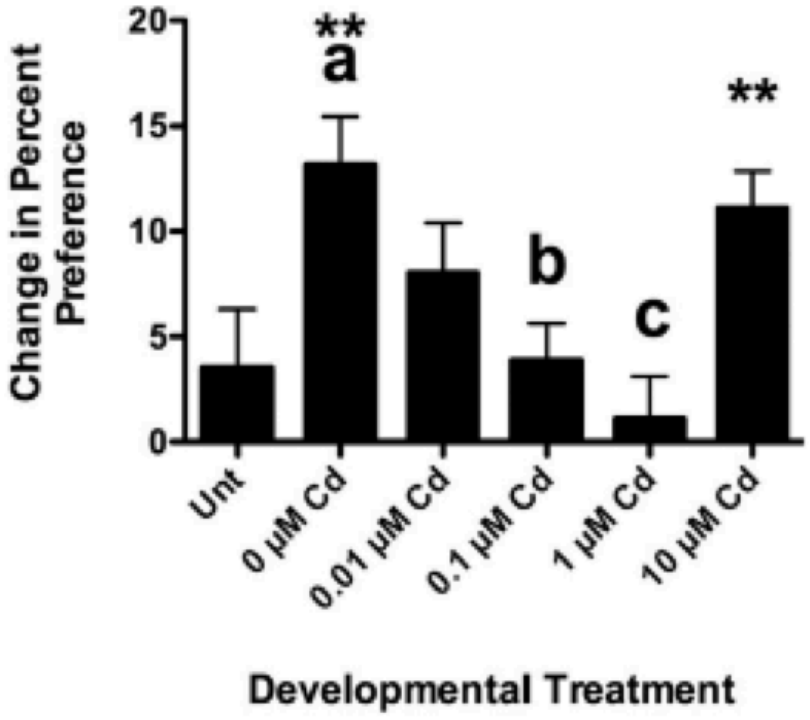

Figure 23. Cadmium affects adult behavior. Shown here are the effects of varied cadmium treatments on reward-based learning as measured by CPP assay. Each treatment group had $n = 30$; $p < 0.05$. Different letters denote significantly different means as per Tukey's HSD test.

Selenium Rescues Cadmium Effects

Metalloids like selenium have been previously observed to dampen the effects of trace metal exposure on a range of organisms (Chen et al., 2017; Lynch et al., 2016; El-Boshy et al., 2015; Liu et al., 2015; Lazarus et al., 2011). Therefore, we sought to examine the impact of co-exposure to selenium on cadmium-treated larvae. Simultaneous exposure to selenium did produce a rescue effect in larval brain size, with even larvae exposed to 10 µM Cd showing a return to nearly baseline, as shown in Figure 24. The same was also true of eye diameter and body length, though the rescue effect seen in the latter was not complete.

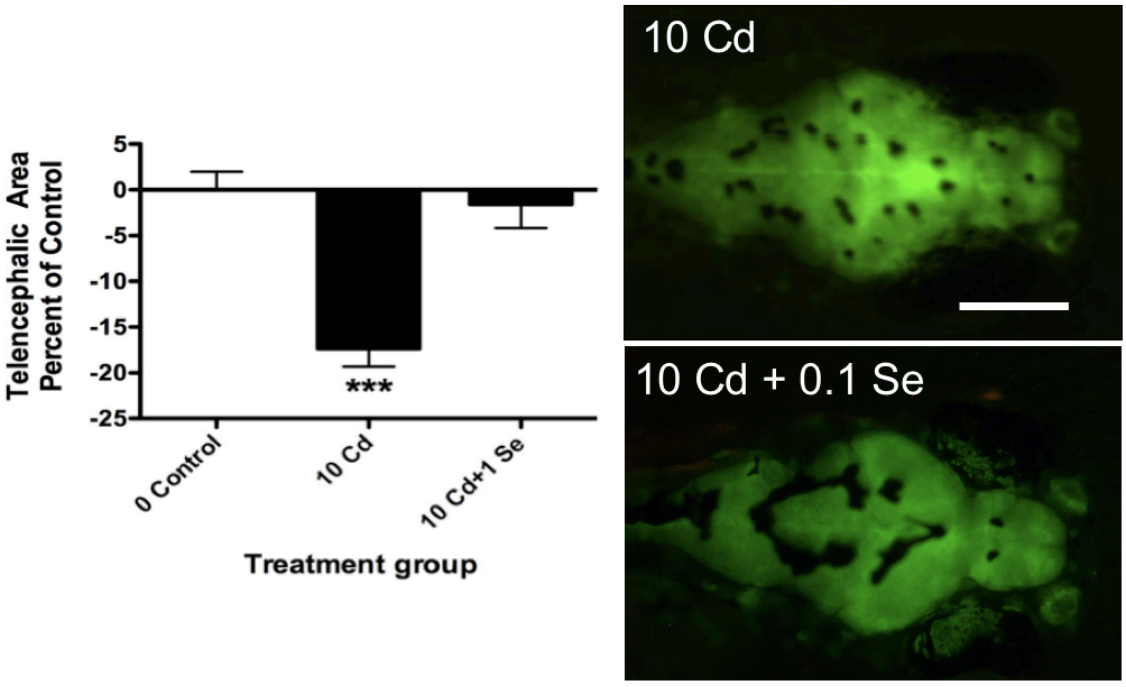

Figure 24. Effects of cadmium plus selenium exposure on larval brains. Telencephalic area is shown graphically as a percent of the control (left), while overall difference in brain morphology is demonstrated with images taken from GFP+ larvae (right). Stars indicate significantly different means. Scale bar represents 200 microns.

Table 4. Summary of larval metrics for combined cadmium and selenium treatments. Mean and SEM for 5dpf larval telencephalon, diencephalon, and hindbrain areas (in mm^2) as well as eye diameter, interocular distance, and body length (mm). Size of the treatment groups varied, with n ranging from 6 (for 5 µM Cd) to 80 (10 µM Cd), though with the exception of 5 µM Cd, group sizes had a minimum n = 32. Bold italics indicate significant difference from control.

0 Cd		***0 Se***	***0.1 Se***
	Tel	0.0298 ± 0.00040	***0.0331 ± 0.00053***
	Di	0.1224 ± 0.00137	***0.1298 ± 0.00166***
	Hind	0.0437 ± 0.00048	0.0460 ± 0.00059
	Eye	0.3383 ± 0.00428	0.3295 ± 0.00679
	Inter	0.1805 ± 0.00503	0.1957 ± 0.00623
	Length	3.8384 ± 0.03213	3.8523 ± 0.49156

1 Cd		***0 Se***	***0.1 Se***
	Tel	0.0315 ± 0.00376	0.0303 ± 0.00045
	Di	0.1293 ± 0.00067	0.1197 ± 0.00156
	Hind	0.0451 ± 0.00124	0.0440 ± 0.00058
	Eye	0.3405 ± 0.00401	0.3440 ± 0.00327
	Inter	0.1943 ± 0.00921	0.2020 ± 0.00433
	Length	3.7753 ± 0.03556	3.5660 ± 0.03923

5 Cd		***0 Se***	***0.1 Se***
	Tel	0.0289 ± 0.00066	0.0309 ± 0.00144
	Di	0.1234 ± 0.00139	0.1279 ± 0.00277
	Hind	0.0421 ± 0.00125	0.0437 ± 0.00094
	Eye	-	-
	Inter	-	-
	Length	-	-

10 Cd		***0 Se***	***0.01 Se***	***0.1 Se***	***1.0 Se***
	Tel	***0.0262 ± 0.00064***	***0.0273 ± 0.00098***	***0.0283 ± 0.00044***	0.0294 ± 0.00087
	Di	0.1171 ± 0.00152	0.1220 ± 0.00188	***0.1207 ± 0.00130***	0.1235 ± 0.00250
	Hind	0.0461 ± 0.00289	0.0452 ± 0.00089	0.0413 ± 0.00051	0.0459 ± 0.00084
	Eye	0.3245 ± 0.00474	-	0.3289 ± 0.00417	-
	Inter	0.1886 ± 0.00459	-	0.1865 ± 0.00636	-
	Length	3.6181 ± 0.05945	-	3.6612 ± 0.04468	-

Selenium did not have a significant impact on the frequency of spinal deformity, though it did appear to produce a slight increase in the incidence of occurrence for the defect. This may be in line with the observation that cadmium sequestration is increased with selenium co-exposure; perhaps increased bioaccumulation exerts a heightened effect on the skeletal system. Treatment with 0.1 μM Se generally accelerated hatching somewhat, with an increase in percent hatched seen even from control fish (Table 3). The 1.0 μM Se treatment may have been overwhelming for the larvae exposed, as it tended to further delay hatching except at the 10 μM Cd treatment.

Selenium did tend to improve survival to 6 months, with the most notable effect observable at the 10 μM Cd concentration. We also noted an increase in behavioral learning when compared with 0 and 1.0 μM Cd, though this effect was not present at the 10 μM Cd treatment. Generally speaking, co-exposure to selenium tended to attenuate the effects of cadmium exposure in zebrafish.

Selenium Affects Larval Development, Morphology, Hatching Rate, & Bioaccumulation

While exposure to cadmium significantly decreased telencephalic brain size, this effect was mitigated by co-exposure to 0.1 µM Se. Telencephalic size was significantly rescued by co-exposure to selenium, as seen in Figure 25 and summarized in Table 4. A one-way ANOVA produced significant results ($F_{7,263} = 11.62$, $p < 0.001$). This included a nearly complete rescue with no significant difference between the control and the 10 µM Cd treatment as confirmed by Tukey's HSD test.

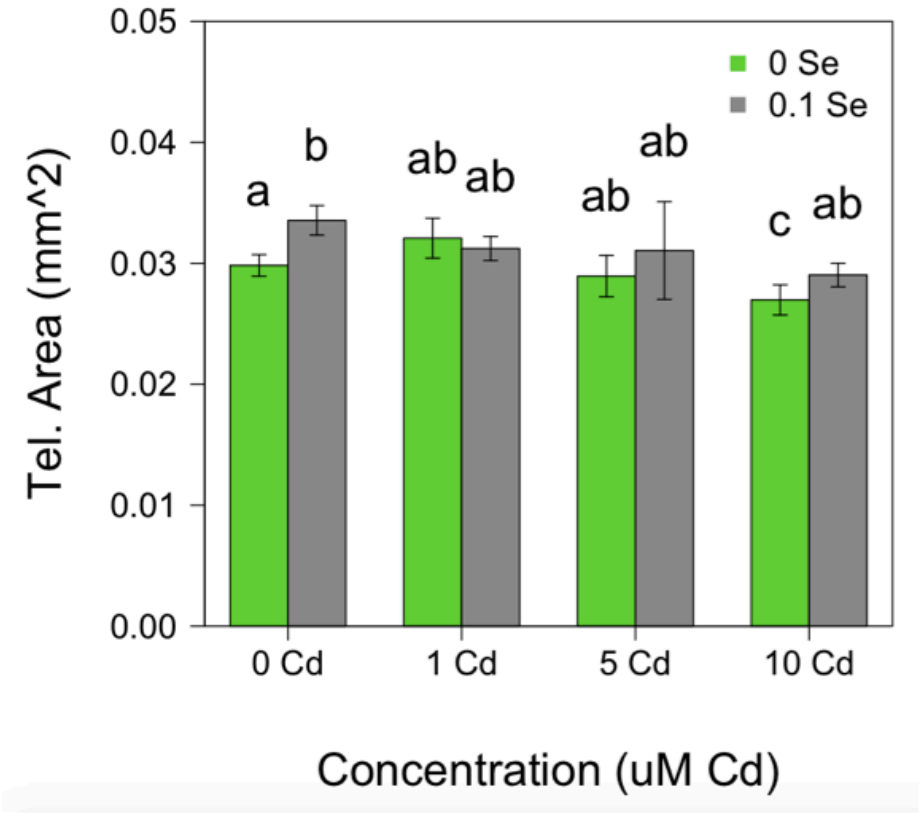

Figure 25. Selenium rescues cadmium-treated larval telencephalic size. Shown here are the effects of cadmium plus selenium treatments on telencephalic area. Size of the treatment groups varied, with n ranging from 6 (for 5 µM Cd) to 80 (10 µM Cd), though with the exception of 5 µM Cd, group sizes had a minimum n = 32 ($p < 0.001$). Different letters denote significantly different means as per Tukey's HSD test.

As in the telencephalon, diencephalic size was significantly rescued with selenium treatment, as shown in Figure 26 (summarized in Table 4). This treatment saw an increase in the means of all selenium-treated subjects when compared with their cadmium-only counterparts. The exception to this was at 1.0 µM Cd, where we saw a slight, though not significant, decrease. The results of the combined treatment ANOVA were significant ($F_{7,253} = 4.82$, $p < 0.001$).

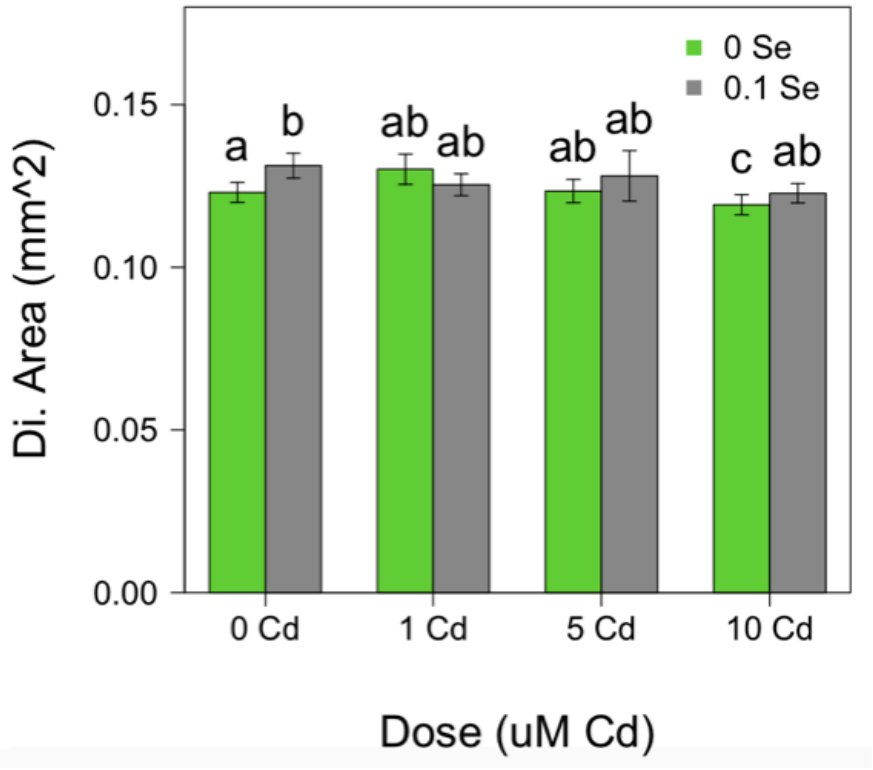

Figure 26. Selenium rescues cadmium-treated larval diencephalic size. Shown here are the effects of cadmium plus selenium treatments on diencephalic area. Size of the treatment groups varied, with n ranging from 6 (for 5 µM Cd) to 80 (10 µM Cd), though with the exception of 5 µM Cd, group sizes had a minimum n = 32 ($p < 0.001$). Different letters denote significantly different means as per Tukey's HSD test.

Although there was a general increase in the hindbrain size of larvae co-treated with selenium, this effect was not significant ($F_{7,253} = 0.68$, $p = 0.692$). Selenium exposure did appear to effect an increase in hindbrain area at 0 µM and 5 µM Cd as compared with controls, though not at 1 µM or 10 µM Cd (Figure 27, Table 4).

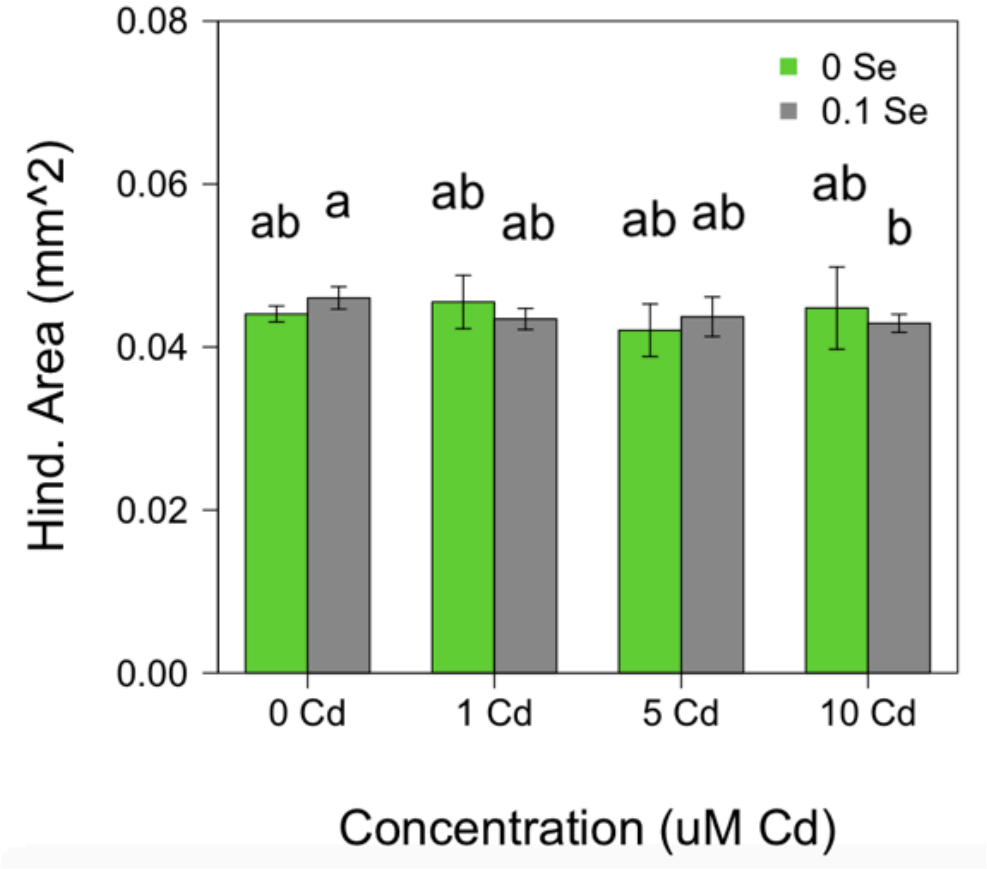

Figure 27. Selenium affects cadmium-treated larval hindbrain size. Shown here are the effects of cadmium plus selenium treatments on hindbrain area. Size of the treatment groups varied, with n ranging from 6 (for 5 µM Cd) to 80 (10 µM Cd), though with the exception of 5 µM Cd, group sizes had a minimum n = 32 ($p < 0.001$). Different letters denote significantly different means as per Tukey's HSD test.

Selenium treatment did have an effect on other larval metrics, including eye diameter. Selenium significantly rescued eye diameter, as seen in Figure 28 (summarized in Table 2). The ANOVA was significant ($F_{5,378} = 7.538$, $p < 0.001$), though the only significant difference present was that of the 10 µM Cd concentration. This treatment group had significantly smaller eyes when compared with the control, but was completely rescued by the addition of 0.1 µM Se.

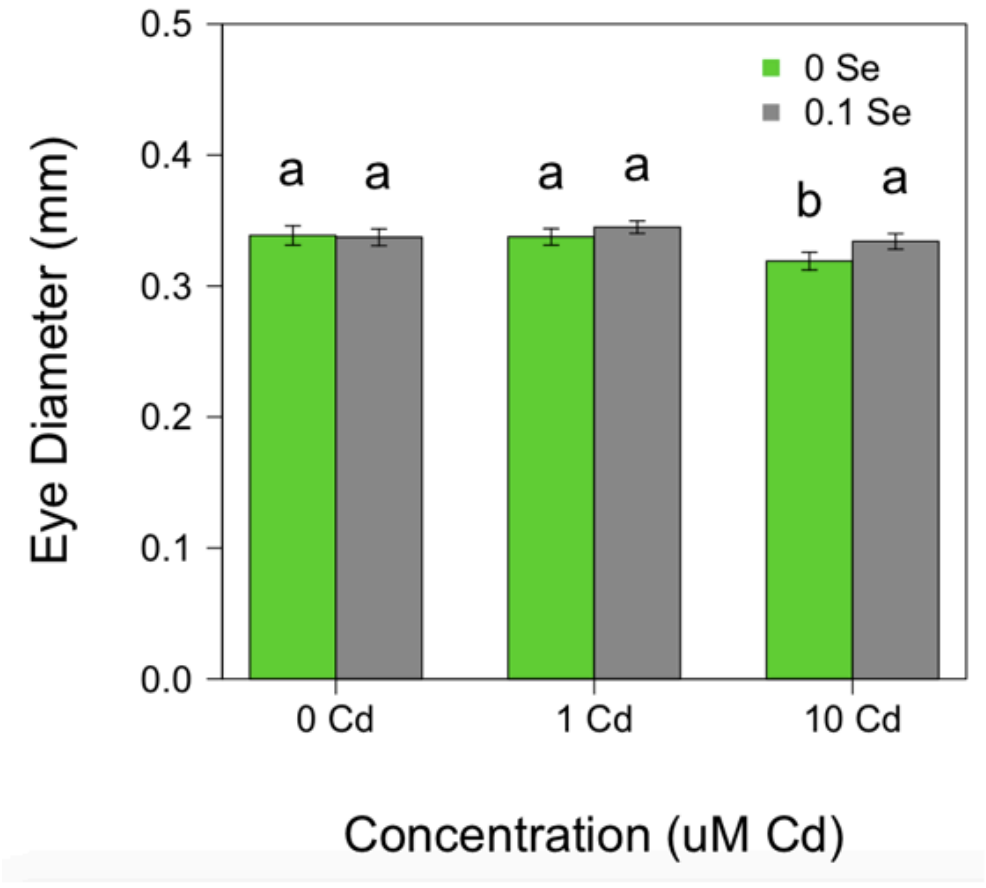

Figure 28. Selenium rescues cadmium-treated larval eye diameter. Shown here are the effects of cadmium and selenium treatments on eye length. Treatment groups each had n = 64; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

Selenium affected body length as well, though selenium treatment did not significantly rescue larval body length (Figure 29, Table 4). There was actually a slight decrease in body length observed with all selenium treated groups. ANOVA was significant ($F_{5,325}$ = 9.477, $p < 0.001$) despite the lack of a rescue effect; 1.0 µM Cd + 0.1 µM Se was significantly smaller than the control, as were both groups treated with 10 µM Cd.

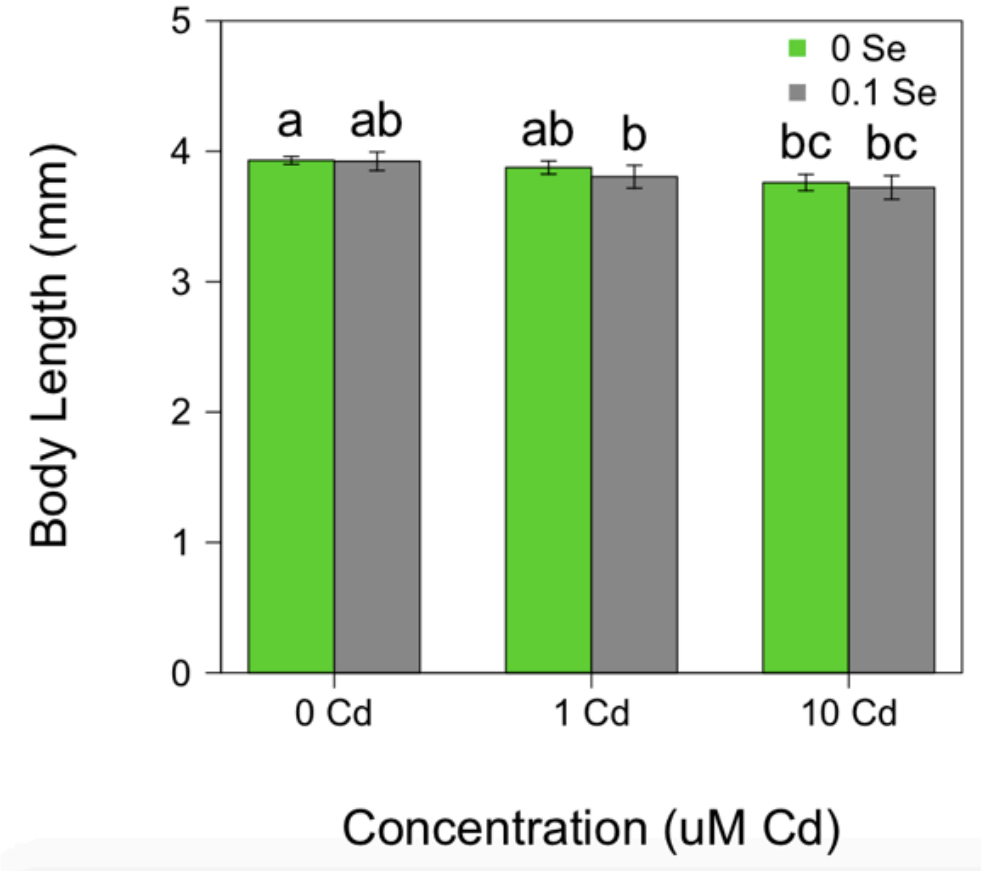

Figure 29. Selenium affects body length of cadmium-treated larvae. Shown here are the effects of cadmium and selenium treatments on body size. Treatment groups each had n = 64; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

The prevalence of spinal curvature was also affected by selenium treatment, as seen in Figure 30. There was a slight decrease in the number of larvae with curved spines, but the effect was not significant. One-way ANOVA did produce significant results ($F_{6,56}$ = 5.042, $p < 0.001$), though the rescue effect was not significant.

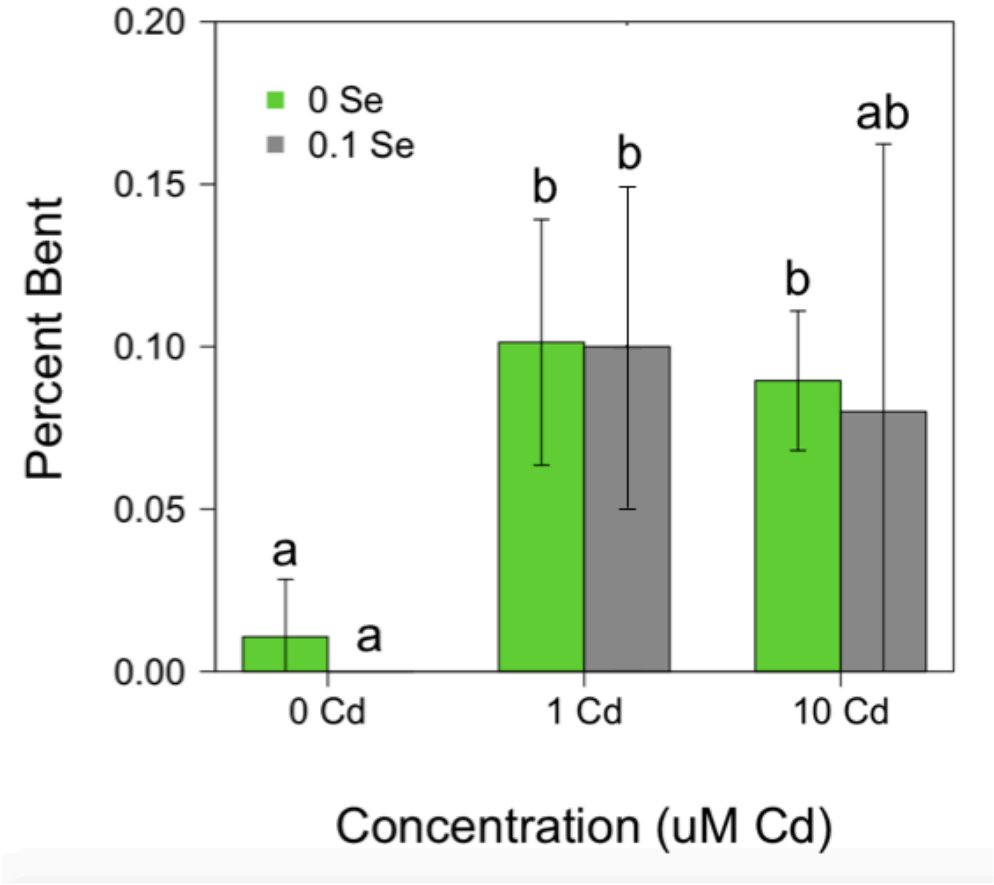

Figure 30. Selenium affects frequency of spinal curvature in cadmium-treated larvae. Shown here are the effects of cadmium plus selenium treatments on frequency of spinal curvature as a percent of the control. Treatment groups had n = 3-6; $p < 0.001$. Different letters denote significantly different means as per Tukey's HSD test.

The hatching delay observed with cadmium exposure was affected by selenium treatment. The addition of selenium produced a trend at 2dpf of increased hatching across treatments at 0.1 µM Se followed by a drop below cadmium-only at 1.0 µM Se. The exception to this is the 10 µM Cd + 1.0 µM Se, which actually remained higher than control with the high concentration of Se (Figure 31A). This trend persisted into 3dpf, but was attenuated due to most larvae being hatched by this point (Figure 31B). ANOVA for combined treatments produced significant results at 2dpf ($F_{8,134} = 3.605$, $p < 0.001$) and at 3dpf ($F_{8,134} = 3.171$, $p = 0.0025$).

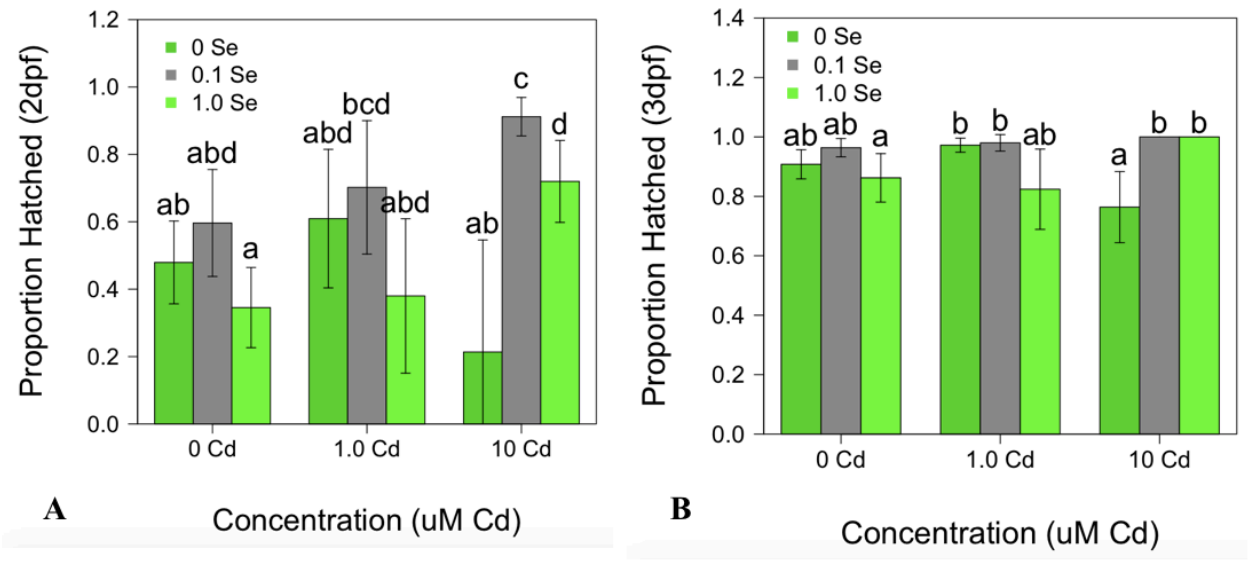

Figure 31. Selenium affects hatching rate of cadmium-treated larvae. Shown here are the effects of varied cadmium and selenium treatments on proportion of larvae hatched at (A) 2 dpf, and at (B) 3 dpf. Treatment groups ranged from n = 8-16, with the exception of 0 µM Cd + 1.0 µM Se (n = 6) and 1.0 µM Cd + 0 µM Se (n = 4); $p < 0.05$. Different letters denote significantly different means as per Tukey's HSD test.

Exposure to cadmium did have a significant impact on bioaccumulation. When we repeated the original whole tissue cadmium experiment with the addition of two different concentrations of selenium (0.1 μM and 1.0 μM), we saw a general increase in absorption, though this effect was not significant (Figure 32). The trend of increased cadmium sequestration with increased selenium concentration is interesting given the prevailing idea that cadmium and selenium interact outside of the biologic system, allowing selenium to interfere with cadmium absorption. However, this effect is not unheard of, and actually aligns with other reports of increased metal accumulation in the presence of a metalloid; Su et al. (2008) described an overall increased body load of mercury in rats also treated with selenium. A one-way ANOVA was significant ($F_{8,49}$ = 5.46, $p < 0.001$).

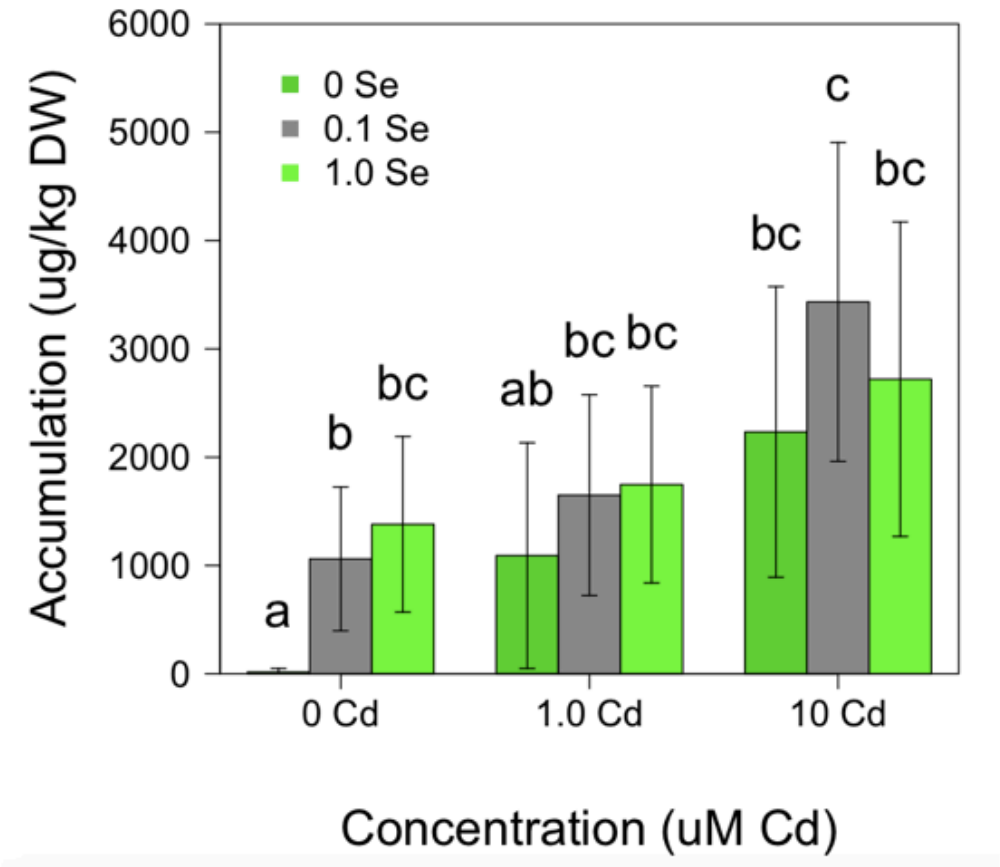

Figure 32. Selenium treatment affects cadmium bioaccumulation. Shown here are the effects of varied cadmium and selenium treatments on level of whole tissue cadmium accumulation. Treatment groups had n = 3, with each sample containing 150-300 whole larvae. Different letters denote significantly different means as per Tukey's HSD test.

An initial assessment of tissue-specific bioaccumulation showed an increase in cadmium absorption with co-exposure to selenium across tissues, with the most notable increase visible in the eyes (Figure 33). This is consistent with other reports of increased metal accumulation with concurrent metalloid treatment (Burger et al., 2012; Can et al., 2011; Su et al., 2008).

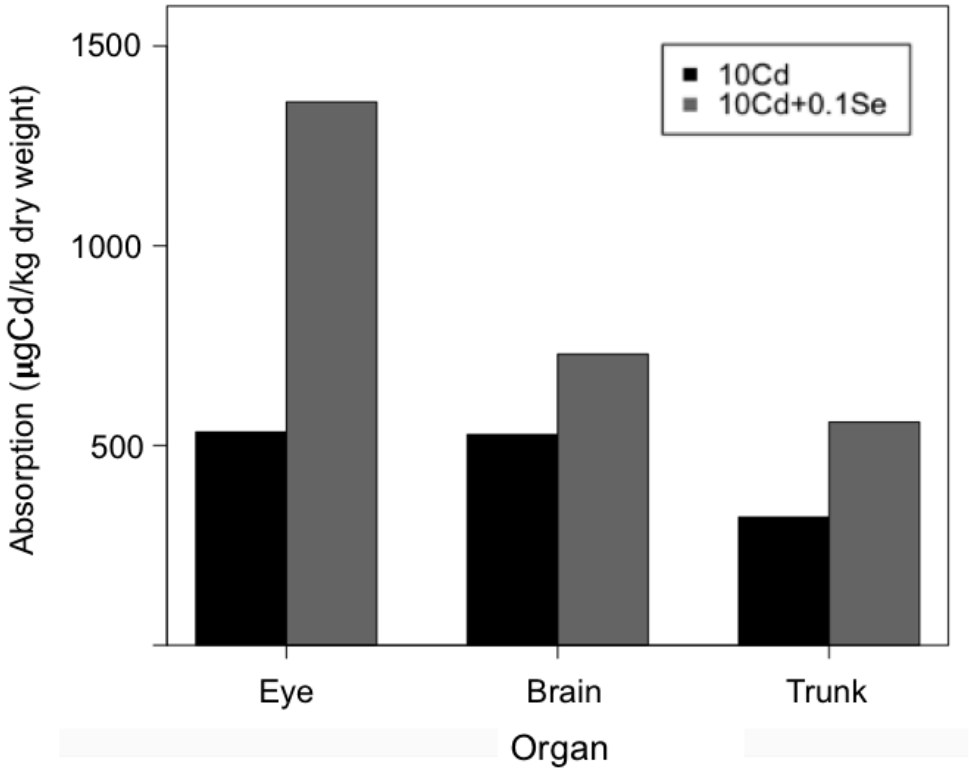

Figure 33. Selenium affects tissue-specific cadmium bioaccumulation. Effects of treatment with cadmium and cadmium plus selenium on assorted tissues.

Selenium Affects Longitudinal Survival & Behavior

Cadmium significantly affected survival to six months; treatment with selenium failed to produce a rescue (Figure 34). Larvae exposed to selenium did experience a slight increase in survival to six months, though Tukey's HSD test did not show a significant difference present between means. The ANOVA was significant ($F_{7,36} = 3.613$, $p = 0.033$), despite the lack of a clear rescue.

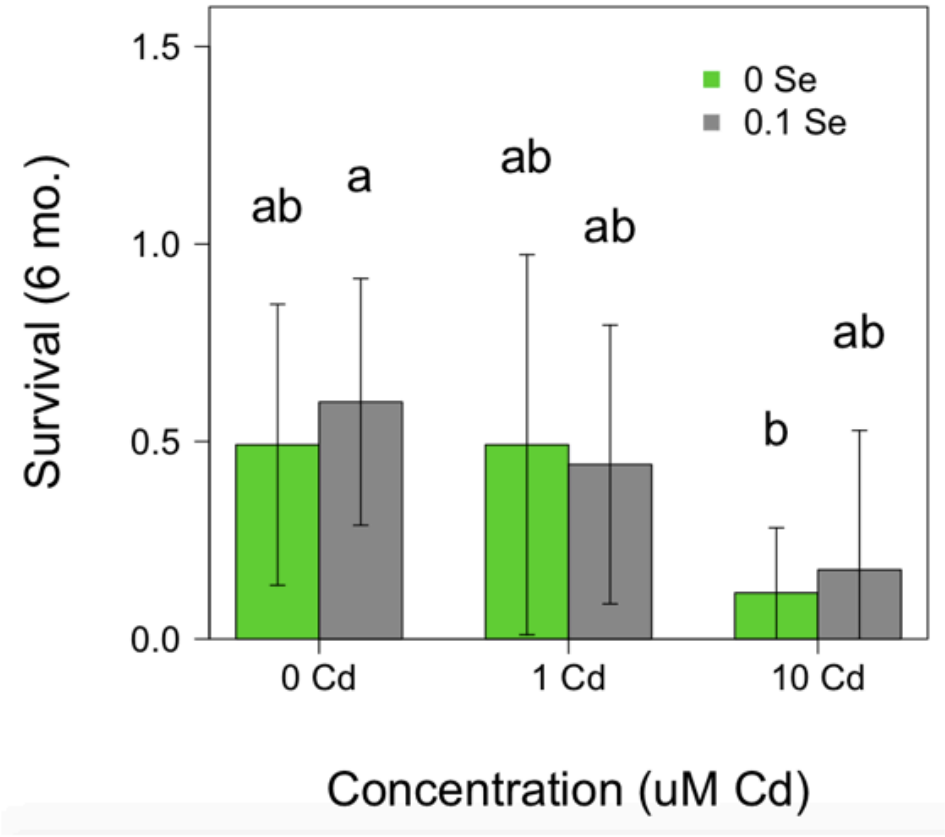

Figure 34. Selenium affects longitudinal survival of cadmium-treated fish. Shown here are the effects of cadmium and selenium treatments on survival to six months. Treatment groups range from n = 53-72, with the exception of 10 µM Cd groups, which had n = 14 (10 µM Cd + 0 µM Se) and n = 21 (10 µM Cd + 0.1 µM Se); $p < 0.05$. Different letters denote significantly different means as per Tukey's HSD test.

Selenium exposure significantly impacted learning behavior in cadmium-treated adults (Figure 35). CPP behavioral assay in adults that received larval exposure to selenium followed the trend observed in the cadmium-only adults, with a decrease in preference at 1.0 μM followed by an increase in the 10 μM treated fish. In the control and 1.0 μM Cd exposed fish, co-treatment with selenium increased percent preference, though not significantly. Interestingly, fish that received 10 μM Cd + 0.1 μM Se as larvae displayed a decrease in preference, though it was not significant. The overall trend was in keeping with the observed sparing effect exerted by selenium treatment. Results of the ANOVA were significant ($F_{6,384} = 4.88$, $p < 0.001$).

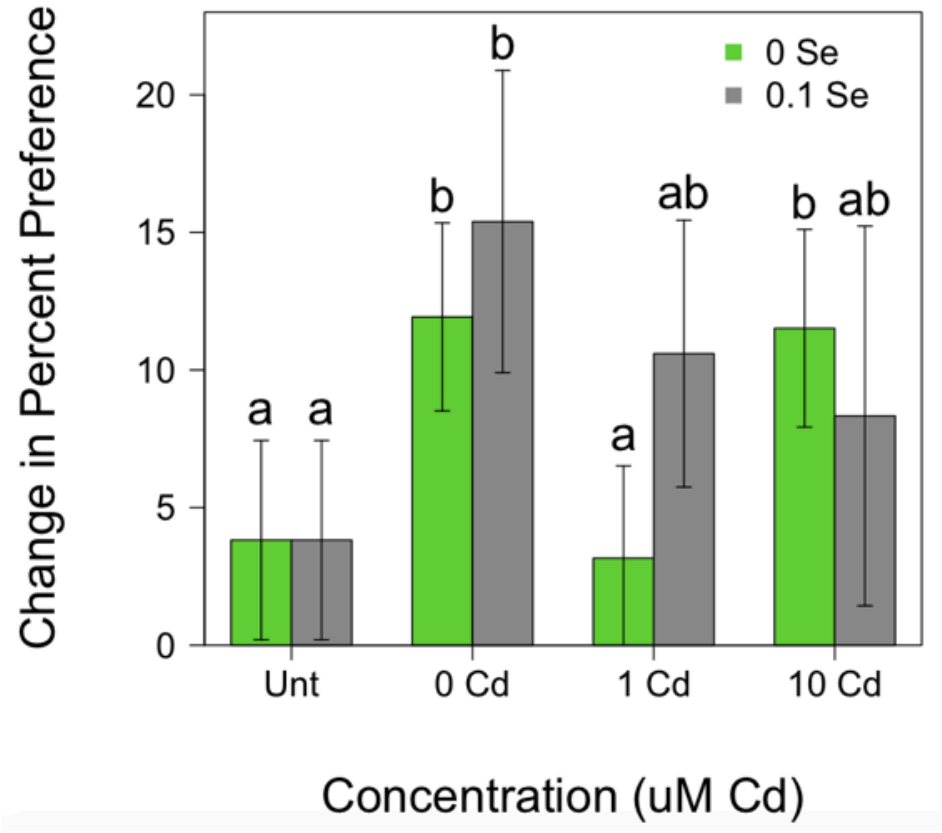

Figure 35. Selenium affects behavior of adult fish treated with cadmium. Shown here are the effects of cadmium and selenium treatments on reward-based learning as measured by CPP assay. Treatment groups had n = 30; $p < 0.01$. Different letters denote significantly different means as per Tukey's HSD test.

DISCUSSION

Aims, Treatment Paradigm, & Water Quality

The overarching aim of this work has been to examine the impact of embryonic cadmium exposure on zebrafish development and behavior, as well as to investigate the potential protective role selenium treatment plays in shielding the organism from the damaging effects of cadmium. In looking at these treatments, we have identified several main areas of influence. First, cadmium exposure has a marked effect on larval brain size, particularly of the telencephalon. Second, the effect of cadmium is also seen in body length, eye diameter, hatching rate, and spinal morphology. Third, bioaccumulation of cadmium depends on concentration, and its uptake varies across tissues. Finally, the effects of cadmium treatment persist into adulthood, affecting survival, biometric measurements, and behavior. All these effects are seen in a dose-dependent manner, with exposure to higher concentrations tending to produce a greater change. Through all of this, combined treatment with selenium plays a critical role in rescuing the organism from these effects.

In recent years, several reviews have described the effect of cadmium exposure on aquatic organisms (Sfakianakis et al., 2015; Sevcikova et al., 2011; Kumar & Singh, 2010; Jezierska et al., 2009a); however, there is a noticeable dearth of information

regarding the impacts cadmium exposure has on the development of the central nervous system as well as any subsequent longitudinal effect on behavior in adults. In light of this, we opted to pay especial attention to larval brain development and any later behavioral impact.

The present work differs from previous studies in several ways with regard to treatment level and timing, as well as analysis window. Specifically, we used lower concentrations than have been previously reported, we set our exposure window to specifically target brain development, and we performed longitudinal analysis on the fish. These experiments utilized lower treatments of cadmium than other studies, with the intent of determining the effects of ecologically-relevant exposure. The maximal concentration we used was 10 μM, though even this little amount is more than a fish would likely experience in nature. This allowed us to observe effects of sublethal concentrations on fish development and physiology. Embryos treated with higher concentrations (≥100 μM) tended to die off (Hallare, 2005; Witeska et al., 1995). Though our treatments were much lower than those used in other studies, they are still much higher than what a fish might encounter naturally, except in cases of acute contamination. Analysis of surface waters in Canada, Ukraine, and Louisiana showed concentrations ranging from 0.05 to 0.65 μg/L, which is much lower than even our lowest concentration (1.124 μg/L) (Zhang et al., 2016; Cremazy et al., 2015; Linnik et al., 2015). However, there is some difference between surface concentrations and sedimentary concentrations, where cadmium may accumulate more readily, though it is unclear how much of this might be bioavailable (Burger, 2008).

Additionally, our treatment paradigm was targeted to impact brain development after several major developmental milestones had been reached. Treatment began at 24 hpf to allow axis formation and patterning, primary neurogenesis, initiation of monoaminergic development, and heart formation (Straudt & Stanier, 2012; Wulliman, 2009; Rink & Wullimann, 2002). Treatment ended at 4 dpf before inflation of the swim bladder and increased swimming behavior, when fish in the wild would normally begin to spend more time higher in the water column and out of contact with the sediment. It was expected that cadmium would affect highly aerobic tissues like the brain, heart, and liver. Finally, longitudinal effects of early cadmium exposure have not been much reported elsewhere. Here, we also present longitudinal effects of early combined exposure to cadmium and selenium to examine a rescue effect.

Water hardness, alkalinity, and temperature may all play a role in metal toxicity. Soft water has been demonstrated to require a ten-fold lower concentration of cadmium to exert the same toxic effect seen in hard water (Alsop & Wood, 2011). Reduced toxicity in hard water is largely due to interactions with and competition between trace metals and other dissolved elements, namely Ca^{2+} and Mg^{2+}, as well as H^{+}; these aqueous minerals interfere with the metal's ability to bind receptors on the gill (Pagenkopf, 1983). When testing the effects of water acidification methods on lead toxicity, Esbaugh et al. (2013) saw a significant drop in the concentration of lead necessary to produce LC50 across methods. Increasing alkalinity also decreased the requisite concentration, but this effect was not nearly as dramatic. The pH is believed affect toxicity by affecting the presence of free metal ions; in an alkaline solution, there is a greater prevalence of carbonate

complexes and thus fewer bioavailable free metal ions. Zebrafish exposed to varying concentrations of cadmium under temperatures outside their ideal range actually required greater concentrations to achieve LC50 (Vergauwen et al., 2012). The authors propose that a heat stress response had a protective effect in hyperthermic conditions. There was increased sensitivity to cadmium at 18ºC, a temperature well below optimal range for zebrafish. This may be due in part to the effect of cadmium exposure on sodium loss (Vergauwen et al., 2012). A later study by the same lab demonstrated increased cadmium toxicity with increasing water temperatures in fish acclimated to 12, 18, 26, or 34ºC for one month (Vergauwen et al., 2013). However, as there is little variability present in these factors for fish raised in our lab, this is not likely to have a major impact on our findings.

It is important to note that we refer to treatment 'concentration' rather than 'dose', because there is some uncertainty as to how much cadmium is actually entering the fish. Fontana (2018) points out that although chemical exposure in the zebrafish model is easy, requiring no more than the creation of an aqueous solution, this is no guarantee of dosage. However, in these experiments, mass spectrometric analysis of tissue samples indicated that cadmium absorption detected in specimens was highly consistent with what we would expect from complete uptake by the larvae. As such, consistent amount of cadmium absorption by the fish is likely not a cause for concern with the present experiment on a broad scale. On the level of the individual, however, there is room for more variation. Our samples were pooled, and therefore reflected the absorption seen in the population, rather than the individual. It is probable there is some individual variation

in gill activity and metal processing affecting uptake and absorption by the individual larva. Other factors that may affect metal uptake, including water temperature, pH, alkalinity, salinity, hardness, and dissolved organic content are not expected to play a significant role in any individual variation that may be present in this study.

Selenium Treatment Exerts a Protective Effect

Recently, selenium has earned much attention for its antioxidative properties, and a good deal of research has been conducted into its ability to rescue the effects of trace metals. Our results confirm this rescue effect, with selenium co-treatment tending to diminish the effects of cadmium exposure in all areas. It is proposed that the mechanism of action for this effect involves both inorganic and organic interactions between the offending trace metal and the protective metalloid. Outside of a biologic system, as in water, cadmium and selenium interact in a primarily mechanical fashion, with selenium providing a kinetic barrier to cadmium binding. Selenium is also expected to provide mechanical interference to cadmium's ability to bind to receptors within the fish, thereby limiting its uptake. This interference has been modeled two ways with regard to metal toxicity. These methods include the biotic ligand model (BLM), which predicts the amount of metal bound to fish gills, and the free ion activity model (FIAM), which is concerned primarily with the amount of metal-binding site interaction, but less so with inorganic interactions of the metals with other molecules in the water (e.g. H^+, Ca^{2+}, Mg^{2+}, etc.). The BLM accounts for both metal speciation in water and competitive binding of transition metal ions and other cations to biotic ligands (Meyer, 1998). The FIAM examines the interaction between a metal and a free surface site but does not account for speciation, though a revised version does account for competition from other cations (Markich et al., 2003; Meyer, 1999; Pagenkopf,

1983). There are two ways to decrease metal bioavailability: by decreasing the amount of free metal ion and thus the potential for it to bind receptor sites, and by increasing the amount of competition from other cations and thus decreasing the amount of metal bound to receptor sites. The latter is the apparent mechanism of selenium interference outside of the system. Once both molecules have gained entry, they begin to interact organically. Free cadmium in the blood complexes with selenide; the two then bind selenoprotein P to form a nonreactive organometal.

Cadmium Exposure Affects Brain Development

Here we have demonstrated a significant developmental impact of cadmium treatment on the central nervous system. Decreased brain size in cadmium-treated larvae tended to follow a nonlinear trend that began with a slight increase from the control followed by a slight decline with increasing concentration. It is possible the slight increase in brain size seen at the lowest concentration of 0.01 μM is the result of a stress response that served to jump-start the system in these larvae. Larvae exposed to 10 μM Cd have smaller brains across regions; the telencephalon, diencephalon, and hindbrain are all significantly smaller as compared with controls. In the telencephalon and diencephalon, this significant decrease is also seen with the 1.0 μM treatment. This may be due in part to the inhibition of neurogenesis reported with developmental cadmium treatment (Chow et al., 2008). There is also an effect of cell death in zebrafish telencephalons, with acridine orange staining revealing a significant increase in apoptotic cells in larvae treated with cadmium (Wold et al., 2017). The telencephalon, in particular, displays a significant decrease from the control at the 1.0 μM concentration, followed by a second significant

decrease from *that* seen at 10 μM for another significantly different mean. This is interesting for its potential to impact behavioral learning and antipredator response if it persists into the adult.

Concurrent exposure to 0.1 μM selenium produced a general rescue effect, such that the significant decreases in brain size seen with cadmium treatment were brought back to control levels. An interesting pattern is observed across brain regions of selenium treatment increasing brain size above the level of cadmium treatment at 0 μM, 5.0 μM, and 10 μM, while actually producing a slight decrease from the cadmium treatment at 1.0 μM. Cadmium control larvae in the combined experiment appeared less susceptible to cadmium damage at the 1.0 μM concentration. This variance in effect could be due in part to the different lines that were used for these experiments. The cadmium-only experiment used line H0189, while the combined-treatment experiments used lines H0229b and H0233. Comparative age of the adults at the time of breeding may have affected the health of the eggs, as could virility of each individual adult. However, it should be noted that sickly-looking clutches were discarded and not used for experimentation. Adult selection for breeding was generally done on the basis of size and apparent robustness, but some of the males in initial experiments were small. It is possible this decrease at the 1.0 μM concentration is due to a similar effect to the one seen in the cadmium-only experiments at the 0.01 μM concentration. Perhaps a lower treatment induces a stress response that spurs additional growth as a compensatory mechanism.

Early Exposure to Cadmium Affects Development

In the present work, we demonstrated a significant developmental effect of cadmium exposure on zebrafish. Larvae exposed to 10 μM of cadmium tended to be smaller, with smaller eyes and brains. Body length and eye diameter of larvae treated with 10 μM were significantly smaller than the control. Eyes were also smaller at 1.0 μM. It is possible the smaller eyes are due to the tendency of cadmium to accumulate there. A greater presence of cadmium within the tissue increases the odds of oxidative damage, and, perhaps as a result, developmental defect. Future tests of these larvae might be conducted to examine visual acuity, foraging, and antipredator behavior.

A rescue effect was seen with the addition of 0.1 μM selenium. Eye diameter showed a significant increase at the 10 μM concentration, making a complete return to baseline. No significant difference existed between the mean of the control group and that of the 10 μM cadmium plus 0.1 μM selenium group. This effect was not seen in body length, where selenium treatment actually decreased length at all concentrations of cadmium. Perhaps this is due to the increased body load of cadmium seen with selenium treatment.

Cadmium Affects Hatching & Spinal Morphology

There were other morphological abnormalities observed during development, the most prevalent of which was the curved spine. Some larvae displayed a lordotic arch of approximately 10 degree curvature (Figure 11). Percentage of fish displaying this morphology significantly increased at the 1.0 and 10 μM concentrations. Spinal deformity has been observed in a number of other instances with embryonic exposure to

heavy metals (Sfakianakis et al., 2015; Jezierska et al., 2009a). There is a possibility that this spinal defect is the result of disruption in calcium uptake and storage seen with cadmium treatment. McGeer (2011) suggests that acute hypocalcemia stemming from cadmium's disruption of calcium absorption is the root cause. Another possibility is that the spinal deformity is not related to bone development at all; spinal curvature may be a result of neural tube defects. Depending on severity, spinal deformity may hinder mobility, which could have a negative impact on antipredator, foraging, and reproductive behaviors.

Selenium co-treatment had only a slight impact on spinal curvature, effecting a small decrease in prevalence but no significant difference between cadmium-treated larvae and selenium-treated larvae. If hypocalcemia is behind the spinal deformity, this would suggest that selenium treatment does not rescue calcium levels. In light of the consideration that selenium actually appears to increase body load of cadmium, perhaps this is not surprising. Supposing that the same amount of cadmium, or perhaps even a greater amount, is gaining entry to the fish, it is to be expected that at least the same level of calcium disruption is occurring.

Another point of note with larvae is hatching rate. There was a delay observed in hatching time with cadmium exposure; this effect was magnified at higher concentrations, with a significant decrease seen at 10 μM cadmium. The effect was most pronounced at 2 dpf, and diminished by 3 dpf when nearly all eggs were hatched. Delayed hatching has been reported in several other instances of metal exposure in fish

exposed to lead, cadmium, or copper (Lugowska et al., 2000; Witeska et al., 1995). Eaton et al. (1978) reported larvae to be consistently more sensitive to cadmium exposure than embryos. Witeska et al. (1995) confirms this, reporting smaller eggs, delayed hatching, and decreased survival. As the egg and chorion are known to exert a protective effect on the developing embryo, shielding it from direct exposure to metals and even storing a large percentage, perhaps the hatching delay is enacted to extend that protection.

Concomitant exposure to selenium generally increased proportion of eggs hatched, especially at 2 dpf. The addition of selenium produced a trend at 2 dpf of increase across treatments at 0.1 μM selenium followed by a drop below baseline at 1.0 μM selenium. The exception to this is the 10 μM Cd + 1.0 μM Se treatment, which actually remained higher than control with the high concentration of selenium. This trend again persisted into 3 dpf, but again was dampened as most eggs hatched by this point. It may be that selenium is reacting inorganically with cadmium in the treatment water, reducing the amount of bioavailable cadmium ions binding to the egg surface. This in turn may indicate to the developing larvae a lesser concentration of cadmium is present, thereby removing the need for the hatching delay.

Tissue Accumulation is Dependent on Treatment

Bioaccumulation assay revealed a logarithmic increase in cadmium absorption with increasing treatment concentration, peaking around 4000 μg Cd per kg dry weight. This suggests that increasing concentration corresponds to increasing tissue sequestration by larval fish at early exposure. In keeping with the free ion activity model, increased

concentration of cadmium in the water led to increased metal species available for binding in the gills, leading to increased overall uptake.

Selenium treatment increased cadmium sequestration in the tissue as compared with cadmium-only groups, though this effect was not significant. This suggests that selenium did not interfere with cadmium uptake. Subsequent tissue-specific dissection of a few highly aerobic organs (incl. eyes, brain, and heart/liver) showed a dramatic increase in cadmium sequestration with selenium treatment. Others have reported a toxicokinetic effect of selenium on cadmium that decreased its overall absorption. Kotyzova et al. (2010) examined rats given adequate and deficient levels of selenium in drinking water, which was also the route of cadmium exposure, and found that rats with adequate selenium had significantly reduced levels of body cadmium upon later examination. Based on di Toro's biotic ligand model (2001), selenium could be acting as a competing cation that provides mechanical interference for cadmium-ligand binding.

It is possible that cadmium has been stored away in these particular organs, especially the eyes, to spare other tissues. Sormo et al. (2011) reported selenium sequestration of mercury in trout that reduced its biological availability to the organism. Su et al. (2008) exposed rat pups to mercury and selenium *in utero* and reported increased absorption of mercury in blood, liver, and kidney also exposed to selenium, proposing this to be the result of formation of a neutral Se-Hg-SelP complex in the blood. A similar complex with cadmium has been proposed, with cadmium interacting with selenide before binding to selenoprotein P to form Se-Cd-SelP (Sasakura & Suzuki, 1998). Preliminary data

suggests that selenium may have an effect on the localization of cadmium accumulation within tissue. Some of the most commonly documented sites for increased accumulation include the liver, kidney, and gill (Burger et al., 2012; Can et al., 2011; Su et al., 2008).

Cadmium & Selenium have Longitudinal Effects

There was significantly decreased longitudinal survival at 10 μM cadmium, where survival to six months dropped below fifty percent of the control. There was a slight increase in survival seen at all lower concentrations of cadmium, with the greatest increase seen at 0.01 μM. This again may be the result of a stress response that ultimately improved survivability. The addition of selenium produced an increase in survival at the 0 and 10 μM cadmium concentrations, but a slight decrease at 1.0 μM. It may be that the combined stress of 1.0 μM cadmium plus 0.1 μM selenium was greater than that of cadmium alone, thereby producing the observed decrease in survival.

We next examined adult metrics, to determine what morphological effects of cadmium persisted into adulthood. Adult brains were normalized to body size, as lower survival at higher concentrations corresponded to more available space per fish, which allowed these more sparsely populated groups to grow larger than their counterparts. Somewhat counterintuitively, there was a steady increase in the ratio of brain weight to body length across concentrations, with 0.1, 1.0, and 10 μM cadmium all displaying a significant increase from the control, with the highest brain weight to body length ratio being observed at the 1.0 μM concentration. There is also a mild increase in telencephalic area to body length at 10 μM Cd. It is possible that this effect is the result of reorganization of neuronal cells and processes in the optic tectum in response to early cadmium disruption

(Favorito et al., 2011). Others have reported disruption of cellular organization and a lack of clear boundaries between brain regions, so although there is greater overall brain weight, there is not necessarily the same level of functionality as seen in controls (Chow et al., 2008; Chan & Cheng, 2003). There is also the possibility of a hypertrophic response by neural stem cells to cadmium damage resulting in an increased population of neurons. This increase in brain mass and telencephalic area did not correspond with increased learning behavior as demonstrated by CPP behavioral assay. This assessment was performed as a measurement of learning response based on monoaminergic reward pathways, utilizing 5mg/L cocaine to simulate the reward obtained via foraging behavior. The cocaine reward works by blocking monoaminergic transporters, especially the dopamine transporter (DAT). This raises extracellular DA levels, mimicking dopaminergic neuronal firing and activating the reward pathway (Volkow & Morales, 2015; Darland et al., 2012; Pierce & Kumaresan, 2004; Rink & Wullimann, 2002). This is the same pathway activated with successful foraging behavior, allowing us to in essence measure environmental responsiveness. Similar work with rats exposed to cadmium in utero demonstrated a decrease in percent place preference and in cocaine self-administration (Cardon et al., 2004; Smith & Nation, 2003). Decreased preference is indicative of decreased learning behavior, which affects foraging success and antipredator behavior.

There was a significant decrease in percent place preference at 0.1 and 1.0 μM cadmium, with a rebound at 10 μM, suggesting that the observed changes in brain mass and telencephalic area do not correspond to increased functionality. The observed increase in behavioral learning at the highest concentration may be an indirect result of decreased

survival. There may be a bias effect exerted by the survival of more robust fish; in other words, the fish who received larval exposure to 10 μM cadmium and survived it did so because they were overall more hardy.

Fish co-exposed to 0.1 μM selenium displayed slightly higher preference at 0 μM cadmium, dramatically increased preference at 1.0 μM cadmium, and slightly decreased preference at 10 μM cadmium. The general trend of increased learning seen with selenium exposure suggests that selenium treatment may have a beneficial effect of its own.

Conclusions & Future Directions

Overall, our work demonstrates the impact of early cadmium exposure on zebrafish development as well as provides evidence for a rescue by selenium. We were able to show a significant effect of cadmium exposure on neural development, spinal morphology, and hatching rate. We demonstrated a selenium rescue in each of these areas that was significant for most measures. In addition, we described a trend of cadmium accumulation that was affected by co-exposure to selenium and displayed regionally-specific sequestration. We also examined longitudinal effects of both cadmium exposure and selenium treatment, reporting significant changes in survival and behavior. In the future, we might look into the effects of cadmium exposure via embryonic contact with sediment to provide a more complete picture of the mechanisms of cadmium exposure and uptake in natural populations. We will also perform tests for visual acuity to further examine the effects of cadmium treatment on eye development as well as any additional impact of cadmium sequestration in the eye. Some preliminary data suggests that

cadmium sequestration is having a detrimental impact on eye function, with cadmium-treated larvae subjected to an optokinetic response (OKR) test exhibiting decreased tracking. This is amplified in the selenium plus cadmium-treated larvae; these have shown an even lesser response to visual stimuli, which would seem to confirm what we saw with increased sequestration. We would also like to test gene expression, particularly in adults treated with 10 μM Cd, to look for something that might explain their resistance to cadmium treatment as demonstrated by behavioral assay. This might include increased expression of cytoprotective genes, particularly those coding for antioxidant enzymes. Another thing we would like to check for is a possible gliosis response that might help explain the increase in proportional brain size of the cadmium-treated fish. We would like to use histology to look for neuronal prevalence and perhaps any evidence of macrophage infiltration.

REFERENCES

Acosta, I. B., Junior, A. S. V., EF, E. S., Cardoso, T. F., Caldas, J. S., Jardim, R. D., & Corcini, C. D. (2016). Effects of exposure to cadmium in sperm cells of zebrafish, Danio rerio. *Toxicol Rep, 3*, 696-700. doi:10.1016/j.toxrep.2016.08.002

Agnihotri, S. K., Agrawal, U., & Ghosh, I. (2015). Brain most susceptible to cadmium induced oxidative stress in mice. *Journal of Trace Elements in Medicine and Biology, 30*, 184-193. doi:10.1016/j.jtemb.2014.12.008

Ahsan, U., Kamran, Z., Raza, I., Ahmad, S., Babar, W., Riaz, M. H., & Iqbal, Z. (2014). Role of selenium in male reproduction - a review. *Anim Reprod Sci, 146*(1-2), 55-62. doi:10.1016/j.anireprosci.2014.01.009

Ali, M. M., Murthy, R. C., & Chandra, S. V. (1986). Developmental and longterm neurobehavioral toxicity of low level in-utero cadmium exposure in rats. *Neurobehav Toxicol Teratol, 8*(5), 463-468.

Alsop, D., & Wood, C. M. (2011). Metal uptake and acute toxicity in zebrafish: common mechanisms across multiple metals. *Aquat Toxicol, 105*(3-4), 385-393. doi:10.1016/j.aquatox.2011.07.010

Antonio, M. T., Peinado, V., Gonzalez, J. C., & Leret, M. L. (2010). Effects of maternal cadmium administration on development of monoaminergic, GABAergic and glutamatergic systems. *Environ Toxicol Pharmacol, 29*(1), 87-90. doi:10.1016/j.etap.2009.11.003

Arner, E., Holmgren, A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem, 267*, 6102-6109.

Arnold, M. C., Lindberg, T.T., Liu, Y.T., Porter, K.A., Hsu-Kim, H., Hinton, D.E., Di Giulio, R.T. (2014). Bioaccumulation and speciation of selenium in fish and insects collected from a mountaintop removal coal mining-impacted stream in West Virginia. *Ecotoxicology, 23*, 929-938. doi:10.1007/s10646-014-1236-4)

Agency for Toxic Substances and Disease Registry (ATSDR). (2012a). *Toxicological Profile for Cadmium*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Agency for Toxic Substances and Disease Registry (ATSDR). (2012b). *Public Health Statement: Cadmium.* Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Banfalvi. (2000). Effects of cadmium on the relationship between replicative and repair DNA synthesis in synchronized CHO cells.

Banni, M., Chouchene, L., Said, K., Kerkeni, A., & Messaoudi, I. (2011). Mechanisms underlying the protective effect of zinc and selenium against cadmium-induced oxidative stress in zebrafish Danio rerio. *Biometals, 24*(6), 981-992. doi:10.1007/s10534-011-9456-z

Bansal, M. P., and Kaur, P. (2005). Selenium, a versatile trace element: Current research implications. *Indian J Exp Biol, 43*, 1119-1129.

Bauer. (1997). Selenium and soils in the Western United States.

Bellinger, F. P., Raman, A. V., Reeves, M. A., & Berry, M. J. (2009). Regulation and function of selenoproteins in human disease. *Biochem J, 422*(1), 11-22. doi:10.1042/BJ20090219

Bernard, A. (2008). Cadmium & its adverse effects on human health. *Indian J Med Res* 128, 557-564.

Bertin, G., & Averbeck, D. (2006). Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie, 88*(11), 1549-1559. doi:10.1016/j.biochi.2006.10.001

Boehmler, W., Obrecht-Pflumio, S., Canfield, V., Thisse, C., Thisse, B., & Levenson, R. (2004). Evolution and expression of D2 and D3 dopamine receptor genes in zebrafish. *Dev Dyn, 230*(3), 481-493. doi:10.1002/dvdy.20075

Bolam, J. P., & Pissadaki, E. K. (2012). Living on the edge with too many mouths to feed: why dopamine neurons die. *Mov Disord, 27*(12), 1478-1483. doi:10.1002/mds.25135

Brown, P., Markich, S. (2000). Evaluation of the free ion actitivy model of metal-organism interaction: extensions of the conceptual model. *Aquatic Toxicology, 51*, 177-194.

Bulteau, A. L., & Chavatte, L. (2015). Update on selenoprotein biosynthesis. *Antioxid Redox Signal, 23*(10), 775-794. doi:10.1089/ars.2015.6391

Burger, J., Jeitner, C., Donio, M., Pittfield, T., & Gochfeld, M. (2013). Mercury and selenium levels, and selenium:mercury molar ratios of brain, muscle and other tissues in bluefish (Pomatomus saltatrix) from New Jersey, USA. *Sci Total Environ, 443*, 278-286. doi:10.1016/j.scitotenv.2012.10.040

Cambier, S., Gonzalez, P., Durrieu, G., & Bourdineaud, J. P. (2010). Cadmium-induced genotoxicity in zebrafish at environmentally relevant doses. *Ecotoxicol Environ Saf, 73*(3), 312-319. doi:10.1016/j.ecoenv.2009.10.012

Can, E., Yabanli, M., Kehayias, G., Aksu, O., Kocabas, M., Demir, V., . . . Seker, S. (2012). Determination of bioaccumulation of heavy metals and selenium in tissues of brown trout Salmo trutta macrostigma (Dumeril, 1858) from Munzur Stream, Tunceli, Turkey. *Bull Environ Contam Toxicol, 89*(6), 1186-1189. doi:10.1007/s00128-012-0824-3

Carageorgiou, H. (2004). *In vivo* and *in vitro* effects of cadmium on adult rat brain total antioxidant status, acetycholinesterase, (Na+, K+)-ATPase and Mg2+-ATPase activities: protection by L-cysteine. *Basic Clin Pharma Tox, 94*, 112-118.

Carageorgiou, H., Tzotzes, V, Sideris, A., Zarros, A., Tsakiris, S. (2005). Cadmium effects on brain acetylcholinesterase activity and antioxidant status of adult rats: modulation by zinc, calcium and L-cysteine co-administration. *Basic & Clinical Pharmacol & Toxicol, 97*, 320-324.

Cardon, A. L., Rocha, A., Valles, R., Bratton, G. R., & Nation, J. R. (2004). Exposure to cadmium during gestation and lactation decreases cocaine self-administration in rats. *Neurotoxicology, 25*(5), 869-875. doi:10.1016/j.neuro.2004.01.001

Casalino, E., Sblano, C., Landriscina, C. (1997). Enzyme activity alteration by cadmium administration to rats: The possibility of iron involvement in lipid peroxidation. *Biochem Biophys, 346*(2), 171-179.

Cavas, T., Garanko, N. N., & Arkhipchuk, V. V. (2005). Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. *Food Chem Toxicol, 43*(4), 569-574. doi:10.1016/j.fct.2004.12.014

Chan, K. M., Ku, L. L., Chan, P. C., & Cheuk, W. K. (2006). Metallothionein gene expression in zebrafish embryo-larvae and ZFL cell-line exposed to heavy metal ions. *Mar Environ Res, 62 Suppl*, S83-87. doi:10.1016/j.marenvres.2006.04.012

Chan, P. K., & Cheng, S. H. (2003). Cadmium-induced ectopic apoptosis in zebrafish embryos. *Arch Toxicol, 77*(2), 69-79. doi:10.1007/s00204-002-0411-1

Chatterjee, D., & Gerlai, R. (2009). High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. *Behavioural Brain Research, 200*(1), 208-213. doi:10.1016/j.bbr.2009.01.016

Chauhan, A., Gu, F., Essa, M. M., Wegiel, J., Kaur, K., Brown, W. T., & Chauhan, V. (2011). Brain region-specific deficit in mitochondrial electron transport chain complexes in children with autism. *J Neurochem, 117*(2), 209-220. doi:10.1111/j.1471-4159.2011.07189.x

Chen, J., & Berry, M. J. (2004). Selenium and selenoproteins in the brain and brain diseases. *Journal of Neurochemistry, 86*(1), 1-12. doi:10.1046/j.1471-4159.2003.01854.x

Chen, J., Pan, T., Wan, N., Sun, Z., Zhang, Z., & Li, S. (2017). Cadmium-induced endoplasmic reticulum stress in chicken neutrophils is alleviated by selenium. *J Inorg Biochem, 170*, 169-177. doi:10.1016/j.jinorgbio.2017.02.022

Chen, M., Li, X., Fan, R., Cao, C., Yao, H., & Xu, S. (2017). Selenium antagonizes cadmium-induced apoptosis in chicken spleen but not involving Nrf2-regulated antioxidant response. *Ecotoxicol Environ Saf, 145*, 503-510. doi:10.1016/j.ecoenv.2017.08.001

Chen, R., Whanger, P., Weswig, P. (1975). Selenium - induced redistribution of cadmium binding tissue proteins: a possible mechanism of protection against cadmium toxicity. *Bioinorganic Chemistry, 4*(2), 125-133.

Chen, W., John, J., Lin, C., Lin, H., Wu, S., Lin, C., & Chang, C. (2004). Expression of metallothionein gene during embryo and early larval development in zebrafish. *Aquat Toxicol* 69, 215-227.

Chen, W. (2007). Expression pattern of metallothionein, MTF-1 nuclear translocation, and its DNA-binding activity in zebrafish (*Danio rerio*) induced by zinc and cadmium.

Cheng, S. H. W., AWK; So, CH; Wu, RSS. (2000). Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos. *Environ Toxicol Chem, 19*(12), 3025-3031.

Cheuk, W. K., Chan, P. C., & Chan, K. M. (2008). Cytotoxicities and induction of metallothionein (MT) and metal regulatory element (MRE)-binding transcription factor-1 (MTF-1) messenger RNA levels in the zebrafish (Danio

rerio) ZFL and SJD cell lines after exposure to various metal ions. *Aquat Toxicol, 89*(2), 103-112. doi:10.1016/j.aquatox.2008.06.006

Chouchene, L., Pellegrini, E., Gueguen, M. M., Hinfray, N., Brion, F., Piccini, B., . . . Pakdel, F. (2016). Inhibitory effect of cadmium on estrogen signaling in zebrafish brain and protection by zinc. *J Appl Toxicol, 36*(6), 863-871. doi:10.1002/jat.3285

Chow, E. S., Hui, M. N., Cheng, C. W., & Cheng, S. H. (2009). Cadmium affects retinogenesis during zebrafish embryonic development. *Toxicol Appl Pharmacol, 235*(1), 68-76. doi:10.1016/j.taap.2008.11.013

Chow, E. S., Hui, M. N., Lin, C. C., & Cheng, S. H. (2008). Cadmium inhibits neurogenesis in zebrafish embryonic brain development. *Aquat Toxicol, 87*(3), 157-169. doi:10.1016/j.aquatox.2008.01.019

Citta, A., Folda, A., Bindoli, A., Pigeon, P., Top, S., Vessieres, A., . . . Rigobello, M. P. (2014). Evidence for targeting thioredoxin reductases with ferrocenyl quinone methides. A possible molecular basis for the antiproliferative effect of hydroxyferrocifens on cancer cells. *J Med Chem, 57*(21), 8849-8859. doi:10.1021/jm5013165

Cooper, G., Manalis, R. (1984). Cadmium: Effects on transmitter release at the frog neuromuscular junction. *Eur J Pharma, 99*(4), 251-256.

Cooper, C., Handy, R., Bury, N. (2006). The effects of dietary iron concentration on gastrointestinal and branchial assimilation of both iron and cadmium in zebrafish (Danio rerio). *Aquat Toxicol* 79, 2:167-175.

Costa-Silva, D. N., M., Wullau, G., Martins, I., Zemolin, A., Cruz, L., Rodrigues, N., Lopes, A., Posser, T., Franco, J. (2015). Oxidative stress markers in fish (*Astyanax* sp. and *Danio rerio* exposed to urban and agricultural effluents in the Brazilian Pampa biome. *Environ Sci Pollut Res Int, 22*, 15526-15535. doi:10.1007/s11356-015-4737-7)

Cremazy, A., Leclair, S., Mueller, K.K., Vigneault, B., Campbell, P.G.C., Fortin, C. (2015).
Development of an in situ ion-exchange technique for the determination of free Cd,
Co, Ni, and Zn concentrations in freshwaters. *Aquat. Geochem*. 21, 259–279.

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., . . . Smeets, K. (2010). Cadmium stress: an oxidative challenge. *Biometals, 23*(5), 927-940. doi:10.1007/s10534-010-9329-x

Darbre, P. (2006). Metalloestrogens: an emerging class of inorganic xenoestrogens with the potential to add to the oestrogenic burden of the human breast. *J Appl Toxicol, 26*, 191-197.

Darland, T., Mauch, J. T., Meier, E. M., Hagan, S. J., Dowling, J. E., & Darland, D. C. (2012). Sulpiride, but not SCH23390, modifies cocaine-induced conditioned place preference and expression of tyrosine hydroxylase and elongation factor 1alpha in zebrafish. *Pharmacol Biochem Behav, 103*(2), 157-167. doi:10.1016/j.pbb.2012.07.017

Denier, X., Hill, E. M., Rotchell, J., & Minier, C. (2009). Estrogenic activity of cadmium, copper and zinc in the yeast estrogen screen. *Toxicol In Vitro, 23*(4), 569-573. doi:10.1016/j.tiv.2009.01.006

Dentice, M., Marsili, A., Zavacki, A., Larsen, P. R., & Salvatore, D. (2013). The deiodinases and the control of intracellular thyroid hormone signaling during cellular differentiation. *Biochim Biophys Acta, 1830*(7), 3937-3945. doi:10.1016/j.bbagen.2012.05.007

Di Toro, D. M., Allen, H. E., Bergman, H. L., Meyer, J. S., Paquin, P. R., & Santore, R. C. (2001). Biotic ligand model of the acute toxicity of metals. 1. Technical Basis. *Environ Toxicol Chem, 20*(10), 2382-2396.

Driscoll, D. M., & Copeland, P. R. (2003). Mechanism and regulation of selenoprotein synthesis. *Annu Rev Nutr, 23*, 17-40. doi:10.1146/annurev.nutr.23.011702.073318

Du, Y., Guo, Q., Shan, M., Wu, Y., Huang, S., Zhao, H., . . . Su, B. (2016). Spatial and Temporal Distribution of Dopaminergic Neurons during Development in Zebrafish. *Front Neuroanat, 10*, 115. doi:10.3389/fnana.2016.00115

Eaton, J. G., McKim, J.M., Holcombe, G.W. (1978). Metal toxicity to embryos and larvae of seven freshwater fish species - i. cadmium. *Bull Environ Contam Toxicol, 19*(1), 95-103.

El-Boshy, M. E., Risha, E. F., Abdelhamid, F. M., Mubarak, M. S., & Hadda, T. B. (2015). Protective effects of selenium against cadmium induced hematological disturbances, immunosuppressive, oxidative stress and hepatorenal damage in rats. *J Trace Elem Med Biol, 29*, 104-110. doi:10.1016/j.jtemb.2014.05.009

El-Ramady, H. R., Domokos-Szabolcsy, É., Abdalla, N. A., Alshaal, T. A., Shalaby, T. A., Sztrik, A., . . . Fári, M. (2014). Selenium and nano-selenium in agroecosystems. *Environmental Chemistry Letters, 12*(4), 495-510. doi:10.1007/s10311-014-0476-0

Esbaugh, A. J., Mager, E. M., Brix, K. V., Santore, R., & Grosell, M. (2013). Implications of pH manipulation methods for metal toxicity: not all acidic environments are created equal. *Aquat Toxicol, 130-131*, 27-30. doi:10.1016/j.aquatox.2012.12.012

Esteban-Vasallo, M. D., Aragones, N., Pollan, M., Lopez-Abente, G., & Perez-Gomez, B. (2012). Mercury, cadmium, and lead levels in human placenta: a systematic review. *Environ Health Perspect, 120*(10), 1369-1377. doi:10.1289/ehp.1204952

European Food Safety Authority (EFSA). (2009). Cadmium in food. *EFSA Journal. 980* 1-139.

European Food Safety Authority (EFSA). (2012). Cadmium dietary exposure in the European population. *EFSA Journal, 10*(1).

Evans, P., & Halliwell, B. (2007). Micronutrients: oxidant/antioxidant status. *British Journal of Nutrition, 85*(S2), S67. doi:10.1079/bjn2000296

Favorito, R., Chiarelli, G., Grimaldi, M. C., De Bonis, S., Lancieri, M., & Ferrandino, I. (2011). Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain. *Chemistry and Ecology, 27*(sup2), 39-46. doi:10.1080/02757540.2011.625937

Feroci, G., Badiello, R., & Fini, A. (2005). Interactions between different selenium compounds and zinc, cadmium and mercury. *J Trace Elem Med Biol, 18*(3), 227-234. doi:10.1016/j.jtemb.2004.09.005

Filipic, M. (2012). Mechanisms of cadmium induced genomic instability. *Mutation Res. 733*, 69-77.

Fontana, B. D., Mezzomo, N. J., Kalueff, A. V., & Rosemberg, D. B. (2018). The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review. *Exp Neurol, 299*(Pt A), 157-171. doi:10.1016/j.expneurol.2017.10.004

Fu, Y., Paxinos, G., Watson, C., & Halliday, G. M. (2016). The substantia nigra and ventral tegmental dopaminergic neurons from development to degeneration. *J Chem Neuroanat, 76*(Pt B), 98-107. doi:10.1016/j.jchemneu.2016.02.001

Fukai, T., & Ushio-Fukai, M. (2011). Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid Redox Signal, 15*(6), 1583-1606. doi:10.1089/ars.2011.3999

Galton, V. A., de Waard, E., Parlow, A. F., St Germain, D. L., & Hernandez, A. (2014). Life without the iodothyronine deiodinases. *Endocrinology, 155*(10), 4081-4087. doi:10.1210/en.2014-1184

Gerlai, R. (2017). Zebrafish and relational memory: Could a simple fish be useful for the analysis of biological mechanisms of complex vertebrate learning? *Behav Processes, 141*(Pt 2), 242-250. doi:10.1016/j.beproc.2017.01.016

Giaginis, C., Gatzidou, E., & Theocharis, S. (2006). DNA repair systems as targets of cadmium toxicity. *Toxicol Appl Pharmacol, 213*(3), 282-290. doi:10.1016/j.taap.2006.03.008

Gilbert, S. F. B., M.J.F. (2016). *Developmental Biology* (11th ed.). Sunderland, Massachusetts: Sinauer Associates, Inc.

Goldman, D., Hankin, M., Li, Z., Dai, X., Ding, J. (2001). Transgenic zebrafish for studying
nervous system development and regeneration. *Transgenic Res.* 10, 21–33.

Gonick, H. (2008). Nephrotoxicity of cadmium & lead. *Indian J Med Res* 128, 335 - 352.

Green, A. J., & Planchart, A. (2018). The neurological toxicity of heavy metals: A fish perspective. *Comp Biochem Physiol C Toxicol Pharmacol, 208*, 12-19. doi:10.1016/j.cbpc.2017.11.008

Guo, S., Wilson, S., Cooke, S., Chitnis, A., Driever, W., & Rosenthal, A. (1999). Mutations in the zebrafish unmask shared regulatory pathways controlling the development of catecholaminergic neurons. *Dev Biol* 208, 473-487.

Guo, S. (2004). Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? *Genes Brain Behav, 3*(2), 63-74.

Hallare, A. V., Schirling, M., Luckenbach, T., Köhler, H. R., & Triebskorn, R. (2005). Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (Danio rerio) embryos. *Journal of Thermal Biology, 30*(1), 7-17. doi:10.1016/j.jtherbio.2004.06.002

Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? *J Neurochem* 97, 1634-1658.

Hanson, G. T., Aggeler, R., Oglesbee, D., Cannon, M., Capaldi, R. A., Tsien, R. Y., & Remington, S. J. (2004). Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *J Biol Chem, 279*(13), 13044-13053. doi:10.1074/jbc.M312846200

Hegarty, S. V., Sullivan, A. M., & O'Keeffe, G. W. (2013). Midbrain dopaminergic neurons: a review of the molecular circuitry that regulates their development. *Dev Biol, 379*(2), 123-138. doi:10.1016/j.ydbio.2013.04.014

Held. (2015). An introduction to reactive oxygen species. *White Paper*.

Hill, A. J. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol. Sci.*(86), 6-19.

Holm. (2005). Developmental effects of bioaccumulated selenium in eggs and larvae of two salmonid species. *Env Tox Chem*.

Holzschuh, J., Ryu, S., Aberger, F., Driever, W. (2001). Dopamine transporter expression distinguishes dopaminergic neurons from other catecholaminergic neurons in the developing fish embryo. *Mechanisms of Development, 101*, 237-243.

Hontela, A., Daniel, D., Ricard, A. (1996). Effects of acute and subacute exposures to cadmium on the interrenal and thyroid function in the rainbow trout, *Oncorhynchus mykiss*. *Aquat Toxicol, 35*(3-4), 171-182.

Hsu, T., Huang, K. M., Tsai, H. T., Sung, S. T., & Ho, T. N. (2013). Cadmium(Cd)-induced oxidative stress down-regulates the gene expression of DNA mismatch recognition proteins MutS homolog 2 (MSH2) and MSH6 in zebrafish (Danio rerio) embryos. *Aquat Toxicol, 126*, 9-16. doi:10.1016/j.aquatox.2012.09.020

Hsu, T., Tsai, H. T., Huang, K. M., Luan, M. C., & Hsieh, C. R. (2010). Sublethal levels of cadmium down-regulate the gene expression of DNA mismatch recognition protein MutS homolog 6 (MSH6) in zebrafish (Danio rerio) embryos. *Chemosphere, 81*(6), 748-754. doi:10.1016/j.chemosphere.2010.07.014

Hu, X., Chen, Q., Jiang, L., Yu, Z., Jiang, D., & Yin, D. (2011). Combined effects of titanium dioxide and humic acid on the bioaccumulation of cadmium in Zebrafish. *Environ Pollut, 159*(5), 1151-1158. doi:10.1016/j.envpol.2011.02.011

Ikemoto, S. (2007). Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res Rev, 56*(1), 27-78. doi:10.1016/j.brainresrev.2007.05.004

Jabeen, F., & Chaudhry, A. S. (2011). Effects of cadmium chloride and sodium selenite alone or in combination on the liver of male Sprague-Dawley rats assessed by different assays. *Biol Trace Elem Res, 143*(2), 1077-1090. doi:10.1007/s12011-010-8946-0

Jamba, L., Nehru, B., Bansal, M. (1997). Redox modulation of selenium binding proteins by cadmium exposures in mice.

Jamba, L., Nehru, B., Bansal, M. (2000). Effect of selenium supplementation on the influence of cadmium on glutathione and glutathione peroxidase system in mouse liver. *J Trace Elem Exp Med, 13*(3), 299-304.

Jarup, L., & Akesson, A. (2009). Current status of cadmium as an environmental health problem. *Toxicol Appl Pharmacol, 238*(3), 201-208. doi:10.1016/j.taap.2009.04.020

Jarup, L., Berglund, M., C.G., E., Nordberg, G. F., & Vahter, M. (1998). Health effects of cadmium exposure - a review of the literature and a risk estimate. *Scand J Work Environ Health, 24*(Suppl 1), 1-51.

Jeong, J., Einhorn, Z., Mercurio, S., Lee, S., Lau, B., Mione, M., Wilson, S., Guo, S. (2006). Neurogenin1 is a determinant of zebrafish basal forebrain dopaminergic

neurons and is regulated by the conserved zinc finger protein Tof/Fezl. *PNAS, 103*(13), 5143-5148.
Jezierska, B., Lugowska, K., & Witeska, M. (2009). The effects of heavy metals on embryonic development of fish (a review). *Fish Physiol Biochem, 35*(4), 625-640. doi:10.1007/s10695-008-9284-4
Jiang, J. H., Ge, G., Gao, K., Pang, Y., Chai, R. C., Jia, X. H., . . . Yu, A. C. (2015). Calcium Signaling Involvement in Cadmium-Induced Astrocyte Cytotoxicity and Cell Death Through Activation of MAPK and PI3K/Akt Signaling Pathways. *Neurochem Res, 40*(9), 1929-1944. doi:10.1007/s11064-015-1686-y
Jihen el, H., Imed, M., Fatima, H., & Abdelhamid, K. (2008). Protective effects of selenium (Se) and zinc (Zn) on cadmium (Cd) toxicity in the liver and kidney of the rat: histology and Cd accumulation. *Food Chem Toxicol, 46*(11), 3522-3527. doi:10.1016/j.fct.2008.08.037
Jin, Y., Liu, Z., Liu, F., Ye, Y., Peng, T., & Fu, Z. (2015). Embryonic exposure to cadmium (II) and chromium (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio rerio). *Neurotoxicol Teratol, 48*, 9-17. doi:10.1016/j.ntt.2015.01.002
Joint FAO/WHO Expert Committee on Food Additives (JEFCA). (2011). Evaluation of certain food additives and contaminants: seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives. (960). India: WHO Library.
Joksimovic, M., Yun, B. A., Kittappa, R., Anderegg, A. M., Chang, W. W., Taketo, M. M., . . . Awatramani, R. B. (2009). Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. *Nat Neurosci, 12*(2), 125-131. doi:10.1038/nn.2243
Jones, I., Kille, P., Sweeney, G. (2005). Cadmium delays growth hormone expression during rainbow trout development. *J Fish Biol, 59*(4), 1015-1022.
Jyoti, V., Saini-Eldukat, B., Hopkins, D., DeSutter, T. (2015). Naturally elevated metal contents of soild in northeastern North Dakota, USA, with a focus on cadmium. *J Soils Sediments, 15*, 1571-1583. doi:10.1007/s11368-015-1122-6)
Kalman, J., Riba, I., Angel DelValls, T., & Blasco, J. (2010). Comparative toxicity of cadmium in the commercial fish species Sparus aurata and Solea senegalensis. *Ecotoxicol Environ Saf, 73*(3), 306-311. doi:10.1016/j.ecoenv.2009.10.013
Kalueff, A. V., Stewart, A. M., & Gerlai, R. (2014). Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol Sci, 35*(2), 63-75. doi:10.1016/j.tips.2013.12.002
Klinck, J., Dunbar, M., Brown, S., Nichols, J., Winter, A., Hughes, C., & Playle, R. C. (2005). Influence of water chemistry and natural organic matter on active and passive uptake of inorganic mercury by gills of rainbow trout (Oncorhynchus mykiss). *Aquat Toxicol, 72*(1-2), 161-175. doi:10.1016/j.aquatox.2004.11.013
Ko, H. A., Chen, S. Y., Chen, H. Y., Hao, H. J., & Liu, F. C. (2013). Cell type-selective expression of the zinc finger-containing gene Nolz-1/Zfp503 in the developing mouse striatum. *Neurosci Lett, 548*, 44-49. doi:10.1016/j.neulet.2013.05.020
Koedrith, P., Kim, H., Weon, J. I., & Seo, Y. R. (2013). Toxicogenomic approaches for understanding molecular mechanisms of heavy metal mutagenicity and carcinogenicity. *Int J Hyg Environ Health, 216*(5), 587-598. doi:10.1016/j.ijheh.2013.02.010

Komjarova, I., & Bury, N. R. (2014). Evidence of common cadmium and copper uptake routes in zebrafish Danio rerio. *Environ Sci Technol, 48*(21), 12946-12951. doi:10.1021/es5032272

Kotyzova, D., Cerna, P., Leseticky, L., & Eybl, V. (2010). Trace elements status in selenium-deficient rats--interaction with cadmium. *Biol Trace Elem Res, 136*(3), 287-293. doi:10.1007/s12011-009-8541-4

Krone, M. (2007). Cell death, stress-responsive transgene activation, and deficits in the olfactory system of larval zebrafish following cadmium exposure.

Kurokawa, S., & Berry, M. J. (2013). Selenium. Role of the essential metalloid in health. *Met Ions Life Sci, 13*, 499-534. doi:10.1007/978-94-007-7500-8_16

Kusch, R. C. K., P.H.; Chivers, D.P. (2008). Chronic Exposure to Low Concentrations of Waterborne Cadmium During Embryonic and Larval Development Results in the Long-Term Hindrance of Antipredator Behavior in Zebrafish. *Environmental Toxicology and Chemistry, 27*(3), 705-710.

Lawrence, C. (2007). The husbandry of zebrafish (Danio rerio): A review. *Aquaculture, 269*(1-4), 1-20. doi:10.1016/j.aquaculture.2007.04.077

Lazarus, M., Orct, T., Aladrovic, J., Ljubic, B. B., Jurasovic, J., & Blanusa, M. (2011). Effect of selenium pre-treatment on antioxidative enzymes and lipid peroxidation in Cd-exposed suckling rats. *Biol Trace Elem Res, 142*(3), 611-622. doi:10.1007/s12011-010-8775-1

Lazarus, M., Orct, T., Jurasoviae, J., & Blanusa, M. (2009). The effect of dietary selenium supplementation on cadmium absorption and retention in suckling rats. *Biometals, 22*(6), 973-983. doi:10.1007/s10534-009-9249-9

Le Guevel, R., Petit, F.G., Le Goff, P., Metivier, R., Valotaire, Y., Pakdel, F. (2000). Inhibition of rainbow trout (*Onchorhynchus mykiss*) estrogen receptor activity by cadmium. *Biology of Reproduction, 63*(1), 259-266.

Letavayova, L., Vlckova, V., & Brozmanova, J. (2006). Selenium: from cancer prevention to DNA damage. *Toxicology, 227*(1-2), 1-14. doi:10.1016/j.tox.2006.07.017

Ley-Quinonez, C. P., Zavala-Norzagaray, A. A., Rendon-Maldonado, J. G., Espinosa-Carreon, T. L., Canizales-Roman, A., Escobedo-Urias, D. C., . . . Aguirre, A. A. (2013). Selected heavy metals and selenium in the blood of black sea turtle (Chelonia mydas agasiizzi) from Sonora, Mexico. *Bull Environ Contam Toxicol, 91*(6), 645-651. doi:10.1007/s00128-013-1114-4

Li, X., Lv, Y., Yu, S., Zhao, H., & Yao, L. (2012). The effect of cadmium on Abeta levels in APP/PS1 transgenic mice. *Exp Ther Med, 4*(1), 125-130. doi:10.3892/etm.2012.562

Li, X., Xing, M., Chen, M., Zhao, J., Fan, R., Zhao, X., . . . Xu, S. (2017). Effects of selenium-lead interaction on the gene expression of inflammatory factors and selenoproteins in chicken neutrophils. *Ecotoxicol Environ Saf, 139*, 447-453. doi:10.1016/j.ecoenv.2017.02.017

Liao, C. M., Ju, Y. R., Chen, W. Y., & Chen, B. C. (2011). Assessing the impact of waterborne and dietborne cadmium toxicity on susceptibility risk for rainbow trout. *Sci Total Environ, 409*(3), 503-513. doi:10.1016/j.scitotenv.2010.10.044

Ling, X. P., Lu, Y. H., & Huang, H. Q. (2012). Differential protein profile in zebrafish (Danio rerio) brain under the joint exposure of methyl parathion and cadmium. *Environ Sci Pollut Res Int, 19*(9), 3925-3941. doi:10.1007/s11356-012-1037-3

Linney, E., Upchurch, L., Donerly, S. (2004). Zebrafish as a neurotoxicological model. *Neurotoxicol. Teratol.*(26), 709-718.

Linnik, P.N., Zhezherya, V.A., Linnik, R.P., Ignatenko, I.I., Zubenko, I.B. (2015). Metals in surface water of Ukraine: the migration forms, feature of distribution between the abiotic component of aquatic ecosystems, and potential bioavailability. *Russ. J. Gen. Chem*. 85, 2965–2984.

Lionetto, M. G., ME; Vilella, S; Schettino, T. (2000). Inhibition of eel enzymatic activities by cadmium. *Aquat Toxicol, 48*, 561-571.

Liu, L., Yang, B., Cheng, Y., & Lin, H. (2015). Ameliorative Effects of Selenium on Cadmium-Induced Oxidative Stress and Endoplasmic Reticulum Stress in the Chicken Kidney. *Biol Trace Elem Res, 167*(2), 308-319. doi:10.1007/s12011-015-0314-7

Liu, L. L., Li, C. M., Zhang, Z. W., Zhang, J. L., Yao, H. D., & Xu, S. W. (2014). Protective effects of selenium on cadmium-induced brain damage in chickens. *Biol Trace Elem Res, 158*(2), 176-185. doi:10.1007/s12011-014-9919-5

Lopez, E., Arce, C., Oset-Gasque, M. J., Canadas, S., & Gonzalez, M. P. (2006). Cadmium induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture. *Free Radic Biol Med, 40*(6), 940-951. doi:10.1016/j.freeradbiomed.2005.10.062

Lugowska, K., Jezierska, B. (2000). Effect of copper and lead on common carp embryos and larvae at two temperatures. *Piscaria, 26*, 29-38.

Luo, S. X., & Huang, E. J. (2016). Dopaminergic Neurons and Brain Reward Pathways: From Neurogenesis to Circuit Assembly. *Am J Pathol, 186*(3), 478-488. doi:10.1016/j.ajpath.2015.09.023

Lynch, S. J., Horgan, K. A., White, B., & Walls, D. (2017). Selenium Source Impacts Protection of Porcine Jejunal Epithelial Cells from Cadmium-Induced DNA Damage, with Maximum Protection Exhibited with Yeast-Derived Selenium Compounds. *Biol Trace Elem Res, 176*(2), 311-320. doi:10.1007/s12011-016-0828-7

MacDonald, T. (2015). Effects of inorganic mercury on developing zebrafish (*Danio rerio*) larvae.

Markich, S. J., Brown, P. L., Jeffree, R. A., & Lim, R. P. (2003). The Effects of pH and Dissolved Organic Carbon on the Toxicity of Cadmium and Copper to a Freshwater Bivalve: Further Support for the Extended Free Ion Activity Model. *Archives of Environmental Contamination and Toxicology, 45*(4), 479-491. doi:10.1007/s00244-003-2175-x

Matz, C. J., Treble, R. G., & Krone, P. H. (2007). Accumulation and elimination of cadmium in larval stage zebrafish following acute exposure. *Ecotoxicol Environ Saf, 66*(1), 44-48. doi:10.1016/j.ecoenv.2005.11.001

McGeer, J., Niyogi, S., Smith, D. (2011). *Homeostasis and Toxicology of Non-Essential Metals*. New York, USA: Academic Press.

Mersereau, E., Boyle, C., Poitra, S., Espinoza, A., Seiler, J., Longie, R., . . . Darland, T. (2016). Longitudinal Effects of Embryonic Exposure to Cocaine on

Morphology, Cardiovascular Physiology, and Behavior in Zebrafish. *International Journal of Molecular Sciences, 17*(6), 847. doi:10.3390/ijms17060847

Messaoudi, I., Deli, T., Kessabi, K., Barhoumi, S., Kerkeni, A., & Said, K. (2009). Association of spinal deformities with heavy metal bioaccumulation in natural populations of grass goby, Zosterisessor ophiocephalus Pallas, 1811 from the Gulf of Gabes (Tunisia). *Environ Monit Assess, 156*(1-4), 551-560. doi:10.1007/s10661-008-0504-2

Messaoudi, I., Hammouda, F., El Heni, J., Baati, T., Saïd, K., & Kerkeni, A. (2010). Reversal of cadmium-induced oxidative stress in rat erythrocytes by selenium, zinc or their combination. *Experimental and Toxicologic Pathology, 62*(3), 281-288. doi:10.1016/j.etp.2009.04.004

Meyer. (1998). Binding of Ni and Cu to fish gills. *Environ Sci Technol*.

Michaelis, M., Gralla, O., Behrends, T., Scharpf, M., Endermann, T., Rijntjes, E., . . . Schomburg, L. (2014). Selenoprotein P in seminal fluid is a novel biomarker of sperm quality. *Biochem Biophys Res Commun, 443*(3), 905-910. doi:10.1016/j.bbrc.2013.12.067

Mikhailova, M., Littlefield, N., Hass, B., Poirier, L., Chou, M. (1997). Cadmium-induced 8-hydroxydeoxyguanosine formation, DNA strand breaks and antioxidant enzyme activities in lymphoblastoid cells. *Cancer Letters, 115*(2), 141-148.

Monaco, A., Grimaldi, M. C., & Ferrandino, I. (2016). Neuroglial alterations in the zebrafish brain exposed to cadmium chloride. *J Appl Toxicol, 36*(12), 1629-1638. doi:10.1002/jat.3328

Nadella, S. R., Fitzpatrick, J. L., Franklin, N., Bucking, C., Smith, S., & Wood, C. M. (2009). Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (Mytilus trossolus) and the protective effect of dissolved organic carbon. *Comp Biochem Physiol C Toxicol Pharmacol, 149*(3), 340-348. doi:10.1016/j.cbpc.2008.09.001

National Institutes of Health (NIH). (2018). Selenium. *NIH Fact Sheets.*

Nair, A. R., Degheselle, O., Smeets, K., Van Kerkhove, E., & Cuypers, A. (2013). Cadmium-Induced Pathologies: Where Is the Oxidative Balance Lost (or Not)? *Int J Mol Sci, 14*(3), 6116-6143. doi:10.3390/ijms14036116

Nawrot, T., Martens, D., Hara, A., Plusquin, M., Vangronsveld, J., Roels, H., & Staessen, J. (2015). Association of total cancer and lung cancer with environmental exposure to cadmium: the meta-analytical evidence. *Cancer Causes Control* 26:1281-1288.

Niethammer, P., Grabher, C., Look, A. T., & Mitchison, T. J. (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature, 459*(7249), 996-999. doi:10.1038/nature08119

Nordberg, G. (2009). Historical perspectives on cadmium toxicology. *Toxicol & Appl Pharmacol* 238, 192-200.

NRCS, U. (2000). Heavy metal soil contamination. *Soil Qualtiy*, 1-7.

Occupational Health and Safety Adminsitration (OSHA). (2017). Cadmium. *Safety and Health Topics.*

O'Donnell, K. C., Vargas, M. E., & Sagasti, A. (2013). WldS and PGC-1alpha regulate mitochondrial transport and oxidation state after axonal injury. *J Neurosci, 33*(37), 14778-14790. doi:10.1523/JNEUROSCI.1331-13.2013

Olm, E., Fernandes, A. P., Hebert, C., Rundlof, A. K., Larsen, E. H., Danielsson, O., & Bjornstedt, M. (2009). Extracellular thiol-assisted selenium uptake dependent on the x(c)- cystine transporter explains the cancer-specific cytotoxicity of selenite. *Proc Natl Acad Sci U S A, 106*(27), 11400-11405. doi:10.1073/pnas.0902204106

Page, A., Chang, C., & El-Amamy, M. (1987). Lead, Mercury, Cadmium and Arsenic in the Environment. *SCOPE. Wiley & Sons* 119-146.

Pagenkopf, G. K. (1983). Gill surface interaction model for trace-metal toxicity to fishes: Role of complexation, pH, and water hardness. *Environ Sci Technol, 17*, 342-47.

Parizek, J. (1978). Interactions between selenium compounds and those of mercury or cadmium. *Env Health Persp, 25*, 53-55.

Park, S. Y., Nair, P. M., & Choi, J. (2012). Characterization and expression of superoxide dismutase genes in Chironomus riparius (Diptera, Chironomidae) larvae as a potential biomarker of ecotoxicity. *Comp Biochem Physiol C Toxicol Pharmacol, 156*(3-4), 187-194. doi:10.1016/j.cbpc.2012.06.003

Patrick, L. (2003). Toxic metals.

Pearson, J. M., & Platt, M. L. (2013). Dopamine: burning the candle at both ends. *Neuron, 79*(5), 831-833. doi:10.1016/j.neuron.2013.08.011

Pedrero. (2008). Protective effect of selenium in broccoli (Brassica oleracea) plants subjected to cadmium exposure.

Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R., & Adam-Guillermin, C. (2013). Comparative genotoxicity of aluminium and cadmium in embryonic zebrafish cells. *Mutat Res, 750*(1-2), 19-26. doi:10.1016/j.mrgentox.2012.07.007

Pierce, R. C., & Kumaresan, V. (2006). The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse? *Neurosci Biobehav Rev, 30*(2), 215-238. doi:10.1016/j.neubiorev.2005.04.016

Pillai, R., Uyehara-Lock, J. H., & Bellinger, F. P. (2014). Selenium and selenoprotein function in brain disorders. *IUBMB Life, 66*(4), 229-239. doi:10.1002/iub.1262

Pollack, A. Z., Schisterman, E. F., Goldman, L. R., Mumford, S. L., Albert, P. S., Jones, R. L., & Wactawski-Wende, J. (2011). Cadmium, lead, and mercury in relation to reproductive hormones and anovulation in premenopausal women. *Environ Health Perspect, 119*(8), 1156-1161. doi:10.1289/ehp.1003284

Ralston, N. V. C. R., L.J. (2016). The "SOS" mechanisms of methylmercury toxicity.

Rayman, M. P. (2012). Selenium and human health. *The Lancet, 379*(9822), 1256-1268. doi:10.1016/s0140-6736(11)61452-9

Richetti, S. K., Rosemberg, D. B., Ventura-Lima, J., Monserrat, J. M., Bogo, M. R., & Bonan, C. D. (2011). Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. *Neurotoxicology, 32*(1), 116-122. doi:10.1016/j.neuro.2010.11.001

Rieger, S., Wang, F., & Sagasti, A. (2011). Time-lapse imaging of neural development: zebrafish lead the way into the fourth dimension. *Genesis, 49*(7), 534-545. doi:10.1002/dvg.20729

Rink, E., & Wullimann, M. (2002a). Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (*Danio rerio*) lead to identification of an ascending dopaminergic system in a teleost. *Brain Res Bull, 57*(3/4), 385-387.

Rink, E., & Wullimann, M. F. (2002b). Development of the catecholaminergic system in the early zebrafish brain: an immunohistochemical study. *Brain Res Dev Brain Res, 137*(1), 89-100.

Rink, E., Wullimann, M. (2001). The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Res, 889*, 316-330.

Rusetskaya, N. Y., & Borodulin, V. B. (2015). Biological activity of organoselenium compounds in heavy metal intoxication. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry, 9*(1), 45-57. doi:10.1134/s1990750815010072

Sadek, K. M., Lebda, M. A., Abouzed, T. K., Nasr, S. M., & Shoukry, M. (2017). Neuro- and nephrotoxicity of subchronic cadmium chloride exposure and the potential chemoprotective effects of selenium nanoparticles. *Metab Brain Dis, 32*(5), 1659-1673. doi:10.1007/s11011-017-0053-x

Saha, U., Fayiga, A., Sonon, L. (2017). Selenium in the soil-plant environment: a review. *Int J Appl Agric Sci*.

Sasakura, C., Suzuki, K. (1998). Biological interactions between transition metals (Ag, Cd and Hg), selenide/sulfide and selenoprotein P. *J Inorg Biochem*, 159-162.

Satarug, S. & Moore, M. (2004). Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. *Environ Health Perspect 112*(10), 1099-1103.

Schmidt. (2013). Neurogenesis in zebrafish - from embryo to adult.

Schutte, R., Nawrot, T. S., Richart, T., Thijs, L., Vanderschueren, D., Kuznetsova, T., . . . Staessen, J. A. (2008). Bone resorption and environmental exposure to cadmium in women: a population study. *Environ Health Perspect, 116*(6), 777-783. doi:10.1289/ehp.11167

Schuyler-Anderson, C. (2015). *Selenium & Tellurium*. US Geological Survey.

Schuyler-Anderson, C. (2017). *Report on Selenium*. US Geological Survey.

Sevcikova, M., Modra, H., Slaninova, A., & Svobodova, Z. (2011). Metals as a cause of oxidative stress in fish: a review. *Veterinarni Medicina, 56*(11), 537-546.

Sfakianakis, D. G., Renieri, E., Kentouri, M., & Tsatsakis, A. M. (2015). Effect of heavy metals on fish larvae deformities: A review. *Environ Res, 137*, 246-255. doi:10.1016/j.envres.2014.12.014

Smith, K. R., & Nation, J. R. (2003). Developmental exposure to cadmium alters responsiveness to cocaine in the rat. *Drug and Alcohol Dependence, 72*(1), 1-11. doi:10.1016/s0376-8716(03)00170-4

Solovyev, N. D. (2015). Importance of selenium and selenoprotein for brain function: From antioxidant protection to neuronal signalling. *J Inorg Biochem, 153*, 1-12. doi:10.1016/j.jinorgbio.2015.09.003

Sormo, E. G., Ciesielski, T. M., Overjordet, I. B., Lierhagen, S., Eggen, G. S., Berg, T., & Jenssen, B. M. (2011). Selenium moderates mercury toxicity in free-ranging freshwater fish. *Environ Sci Technol, 45*(15), 6561-6566. doi:10.1021/es200478b

St Germain, D. L., Galton, V. A., & Hernandez, A. (2009). Minireview: Defining the roles of the iodothyronine deiodinases: current concepts and challenges. *Endocrinology, 150*(3), 1097-1107. doi:10.1210/en.2008-1588
Steinbrenner, H., & Sies, H. (2009). Protection against reactive oxygen species by selenoproteins. *Biochim Biophys Acta, 1790*(11), 1478-1485. doi:10.1016/j.bbagen.2009.02.014
Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R., & Kalueff, A. V. (2014). Zebrafish models for translational neuroscience research: from tank to bedside. *Trends Neurosci, 37*(5), 264-278. doi:10.1016/j.tins.2014.02.011
Strahle, U., & Blader, P. (1994). Early neurogenesis in the zebrafish embryo. *FASEB J, 8*(10), 692-698.
Su, L., Wang, M., Yin, S. T., Wang, H. L., Chen, L., Sun, L. G., & Ruan, D. Y. (2008). The interaction of selenium and mercury in the accumulations and oxidative stress of rat tissues. *Ecotoxicol Environ Saf, 70*(3), 483-489. doi:10.1016/j.ecoenv.2007.05.018
Tashjian. (2006). Selenium absorption, distribution, and excretion in white sturgeon orally dosed with L-selenomethionine. *Env Tox Chem*.
Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy metal toxicity and the environment. *EXS, 101*, 133-164. doi:10.1007/978-3-7643-8340-4_6
Tobe. (2017). Selenium uptake through cysteine transporter mediated by glutathione conjugation. *J Tox Sci*.
Tobwala, S., Wang, H.-J., Carey, J., Banks, W., & Ercal, N. (2014). Effects of Lead and Cadmium on Brain Endothelial Cell Survival, Monolayer Permeability, and Crucial Oxidative Stress Markers in an in Vitro Model of the Blood-Brain Barrier. *Toxics, 2*(2), 258-275. doi:10.3390/toxics2020258
Tolcin, A. C. (2017). *Report on Cadmium* US Geological Survey.
Tomza-Marciniak, A., Pilarczyk, B., Bakowska, M., Pilarczyk, R., Wojcik, J., Marciniak, A., & Hendzel, D. (2011). Relationship between selenium and selected heavy metals concentration in serum of cattle from a non-polluted area. *Biol Trace Elem Res, 144*(1-3), 517-524. doi:10.1007/s12011-011-9075-0
Trevarrow, B., Marks, D. L., & Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron, 4*(5), 669-679.
U.S. Department of Agriculture (USDA). (2000). *Heavy Metal Soil Contamination*. Auburn, AL: Soil Quality Institute.
U.S. Geological Survey (USGS). (1980). Geochemistry of the Pierre Shale and equivalent rocks of late Cretaceous age. Washington, D.C.: U.S. Department of the Interior.
Verbost, P., Flik, G., Lock, R., Bonga, S. (1987). Cadmium inhibition of Ca2+ uptake in rainbow trout gills. *Regulatory, Integrative and Comparative Physiology, 253*(2), 216-221.
Vergauwen, L., Hagenaars, A., Blust, R., & Knapen, D. (2013). Temperature dependence of long-term cadmium toxicity in the zebrafish is not explained by liver oxidative stress: evidence from transcript expression to physiology. *Aquat Toxicol, 126*, 52-62. doi:10.1016/j.aquatox.2012.10.004

Vergauwen, L., Knapen, D., Hagenaars, A., & Blust, R. (2013). Hypothermal and hyperthermal acclimation differentially modulate cadmium accumulation and toxicity in the zebrafish. *Chemosphere, 91*(4), 521-529. doi:10.1016/j.chemosphere.2012.12.028

Volkow, N. D., & Morales, M. (2015). The Brain on Drugs: From Reward to Addiction. *Cell, 162*(4), 712-725. doi:10.1016/j.cell.2015.07.046

Waalkes, M. (2000). Cadmium carcinogenesis in review. *J Inorg Biochem* 79, 241-244.

Weinstein, B., Hemmati-Brivanlou. (1999). Neural induction. *Annu Rev Cell Dev Bio*.

Wen, L., Wei, W., Gu, W., Huang, P., Ren, X., Zhang, Z., . . . Zhang, B. (2008). Visualization of monoaminergic neurons and neurotoxicity of MPTP in live transgenic zebrafish. *Dev Biol, 314*(1), 84-92. doi:10.1016/j.ydbio.2007.11.012

Westerfield, M. (1994). *A guide for the laboratory use of zebrafish Danio (Brachydanio rerio)*. Eugene, OR: University of Oregon Press.

Witeska, M., Jezierska, B., & Chaber, J. (1995). The influence of cadmium on common carp embryos and larvae. *Aquaculture, 129*, 129-132.

Witeska, M., Sarnowski, P., Lugowska, K., & Kowal, E. (2014). The effects of cadmium and copper on embryonic and larval development of ide Leuciscus idus L. *Fish Physiol Biochem, 40*(1), 151-163. doi:10.1007/s10695-013-9832-4

Wold, M., Beckmann, M., Poitra, S., Espinoza, A., Longie, R., Mersereau, E., . . . Darland, T. (2017). The longitudinal effects of early developmental cadmium exposure on conditioned place preference and cardiovascular physiology in zebrafish. *Aquat Toxicol, 191*, 73-84. doi:10.1016/j.aquatox.2017.07.017

Wullimann, M. (2003). Anatomy of neurogenesis in the early zebrafish brain. *Dev Br Res*.

Wullimann, M. F. (2009). Secondary neurogenesis and telencephalic organization in zebrafish and mice: a brief review. *Integr Zool, 4*(1), 123-133. doi:10.1111/j.1749-4877.2008.00140.x

Wullimann, M. F., & Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. *J Comp Neurol, 475*(2), 143-162. doi:10.1002/cne.20183

Xie, Z., Zhang, Y., Li, A., Li, P., Ji, W., & Huang, D. (2010). Cd-induced apoptosis was mediated by the release of Ca2+ from intracellular Ca storage. *Toxicol Lett, 192*(2), 115-118. doi:10.1016/j.toxlet.2009.10.011

Yan, C., & Chan, K. (2004). Cloning of zebrafish metallothionein gene and characterization of its gene promoter region in HepG2 cell line. *Biochem Biophys Acta* 1679, 1:47-58.

Yan, L., Reeves, P. G., & Johnson, L. K. (2010). Assessment of selenium bioavailability from naturally produced high-selenium soy foods in selenium-deficient rats. *J Trace Elem Med Biol, 24*(4), 223-229. doi:10.1016/j.jtemb.2010.04.002

Yu, R. M., Lin, C. C., Chan, P. K., Chow, E. S., Murphy, M. B., Chan, B. P., . . . Cheng, S. H. (2006). Four-dimensional imaging and quantification of gene expression in early developing zebrafish (Danio rerio) embryos. *Toxicol Sci, 90*(2), 529-538. doi:10.1093/toxsci/kfj115

Zeng, H. (2009). Selenium as an essential micronutrient: roles in cell cycle and apoptosis. *Molecules, 14*(3), 1263-1278. doi:10.3390/molecules14031263

Zeng, H., & Combs, G. F., Jr. (2008). Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. *J Nutr Biochem, 19*(1), 1-7. doi:10.1016/j.jnutbio.2007.02.005

Zhang, C., Lin, J., Ge, J., Wang, L.-L., Li, N., Sun, X.-T., . . . Li, J.-L. (2017). Selenium triggers Nrf2-mediated protection against cadmium-induced chicken hepatocyte autophagy and apoptosis. *Toxicology in Vitro, 44*, 349-356. doi:10.1016/j.tiv.2017.07.027

Zhang, R., Wang, L., Zhao, J., Wang, C., Bao, J., & Li, J. (2016). Effects of Selenium and Cadmium on Ion Profiles in the Brains of Chickens. *Biol Trace Elem Res, 174*(1), 218-225. doi:10.1007/s12011-016-0693-4

Zhang, Z., Wang, J.J., Ali, A., DeLaune, R.D. (2016). Heavy metal distribution and water quality characterization of water bodies in Louisianna's Lake Pontchartrain Basin, USA. *Environ. Monit. Assess* 188, 628.

Zhao, W., Liu, W., Chen, X., Zhu, Y., Zhang, Z., Yao, H., & Xu, S. (2014). Four endoplasmic reticulum resident selenoproteins may be related to the protection of selenium against cadmium toxicity in chicken lymphocytes. *Biol Trace Elem Res, 161*(3), 328-333. doi:10.1007/s12011-014-0135-0

Zwolak, I., & Zaporowska, H. (2011). Selenium interactions and toxicity: a review. *Cell Biology and Toxicology, 28*(1), 31-46. doi:10.1007/s10565-011-9203-9