REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF ELEVATED MATERNAL DIETARY SELENIUM IN THE MODEL AMPHIBIAN *XENOPUS LAEVIS*

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ABSTRACT

Selenium (Se) is a contaminant of potential concern in aquatic systems due to its efficient incorporation into food webs, potential for bioaccumulation at higher trophic levels, and role as a developmental toxicant in oviparous vertebrates. While the presence of embryonic/larval deformities due to *in ovo* Se exposure is considered the most sensitive toxicological endpoint, elevated levels of dietary Se have also been associated with alterations to bioenergetic and hormonal status of adult female fishes, which consequently could lead to diminished fitness and impaired reproduction. Adverse reproductive effects in fishes have been the primary focus of Se research thus far, while studies focusing on Se toxicity in amphibians in any regard are severely lacking. The US EPA has recently proposed a new set of criteria for the protection of freshwater aquatic life with regards to acceptable Se tissue threshold levels; however, these values were generated based on effects observed in fishes with negligible existent data on amphibians to assist in this process. Thus, the overall goal of this thesis research was to characterize the reproductive and developmental effects of elevated dietary Se exposure in *Xenopus laevis*, in order to provide a foundation for amphibian related Se research that may assist in establishing effective regulatory guidelines that protect this highly vulnerable and ecologically valuable taxon.

The research presented in this thesis was performed as one large generational bioassay with the analysis of experimental variables divided into three sections in order to evaluate the effects of elevated *in ovo* Se exposure via maternal transfer on early and late stages of larval development in addition to the overall fitness of adult *X. laevis* females after a dietary exposure. Adult *X. laevis* females were fed a diet augmented with L-selenomethionine (SeMet) for 68 days after which they were bred with untreated males. The resultant embryos were incubated up to 5

days post fertilization (dpf) to determine fertilization success, hatchability, mortality and frequency/severity of malformations. Subsamples of 5 dpf tadpoles were selected and raised to completion of metamorphosis for evaluation of mortality, growth and maturation rate. In addition, tissue and blood samples as well as morphometric indices were collected from *X. laevis* females, upon completion of the exposure period and subsequent breeding, to ascertain Se tissue distribution, triglyceride and glycogen levels, cortisol concentrations and the overall health status of SeMet-treated females.

Within the data gathered throughout this research, a foundation of knowledge characterizing Se toxicity in amphibians was established along with the development of an early life stage toxicity threshold for the frequency of teratogenic abnormalities in *X. laevis*. The bioenergetic and stress status in addition to the overall body condition of adult females after a 68 day dietary exposure showed no significant differences among treatment groups. The concentrations of Se measured in the ovary, egg, liver and muscle samples increased with female dietary Se levels with strong positive relationships between egg Se concentrations and the other three tissues being illustrated. Elevated *in ovo* Se exposure had no biologically significant effect on fertilization success, hatchability or mortality within the first 5 dpf; however, the frequency and severity of morphological abnormalities was significantly greater in tadpoles from the highest dose group, with eye lens abnormalities most prominently observed. Late stage larval survival and growth was unaffected by *in ovo* Se exposure; however, the distribution of developmental stages observed at the set time point when 50% of tadpoles completed metamorphosis showed a larger portion of tadpoles at earlier stages of development in the highest dose group despite no overall change in time to metamorphosis. The results of this thesis research in its entirety suggest that amphibians, as represented by *X. laevis*, are potentially more

tolerant to elevated *in ovo* and dietary Se exposures than other oviparous vertebrates studied to date; however, without sufficient data for comparison it is unknown whether *X. laevis* is a tolerant, average or sensitive species among amphibians.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATRF	Aquatic Toxicology Research Facility
CCME	Canadian Council of Ministers of the Environment
cm	Centimetre
CV	Coefficient of variation
d	Day
dpf	Days post-fertilization
d.m.	Dry mass
EC_{10}	Effective concentration in 10 percent of a population
FBSI	Fat body somatic index
g	Gram
GSI	Graduated Severity Index
OSI	Ovarian Somatic Index
h	Hours
hCG	Human chorionic gonadotropin
LSI	Liver Somatic index
ICP-MS	Inductively coupled plasma mass spectrometry
IU	International unit
kg	kilogram
L	Litre
LOQ	Limit of quantification
m	Meter
M	Molar
Met	Methionine
min	Minute
mg	Milligram
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre

mL	Millilitre
mm	Millimetre
MS-222	Ethyl 3-aminobenzoate methanesulfonate
n	Number of replicate
NF	Nieuwkoop and Faber's *Xenopus laevis* developmental stage
ng/g	Nanogram per gram
ng/mL	Nanogram per millilitre
NSERC	Natural Sciences and Engineering Research Council of Canada
p	Probability
s	Seconds
S.E.M.	Standard error of the mean
Se	Selenium
Se^{0}	Solid elemental selenium
Se^{-2}	Inorganic selenide
Se^{+4}	Selenite
Se^{+6}	Selenate
SeCys	Selenocysteine
SeMet	Selenomethionine
SSI	Splenic somatic index
TORT-2	Lobster hepatopancreas
TRAP	Toxicity Relationship Analysis Program
US EPA	United States Environmental Protection Agency
^{o}C	Degrees centigrade
μmol	Micromole
μg/g	Microgram per gram
μg	Microgram
μg/L	Microgram per litre

NOTE TO READERS

The research performed for this thesis involved looking at three different aspects of one large experimental bioassay. The contents have been organized and formatted in a manner that follows the guidelines put forth by the University of Saskatchewan College of Graduate Studies and Research for a manuscript-style thesis. Chapter 1 is a general introduction comprised of a brief literature review along with a description of the project objectives, and Chapter 5 entails a comprehensive discussion and the overall conclusions conveyed by the data. Chapters 2, 3 and 4 of this thesis are written and organized into manuscripts intended for future publication in scientific journals. Therefore, certain sections of each chapter will contain information that has been stated previously in other areas of this thesis. The tables, figures, supporting information and references of published or submitted chapters have been reformatted to create a consistent thesis style. Chapter 2 has been published in *Environmental Science & Technology* (citation listed below) while Chapters 3 and 4 have been submitted to *Bulletin of Environmental Contamination and Toxicology* and *Environmental Toxicology and Chemistry*, respectively.

The content of Chapter 2 was reprinted (adapted) with permission from A.J. Massé, J.R. Muscatello, and D.M. Janz, "Dose-dependent early life stage toxicities in *Xenopus laevis* exposed *in ovo* to selenium." Environmental Science & Technology 49: 13658-13666 (doi: 10.1021/acs.est.5b04303). Copyright (2015) American Chemical Society.

The author contributions for each research chapter include:

> Anita J. Massé (University of Saskatchewan) created the experimental design and performed all aspects of research pertaining to exposure, sample collection, data and statistical analysis as well as composition of manuscript drafts.

Dr. David M. Janz (University of Saskatchewan) provided comments and editorial corrections for manuscripts in addition to scientific guidance, direction and funding for this project.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Properties and sources of selenium

Selenium (atomic number 34, atomic mass 78.96) is an element belonging to group VIA of the periodic table along with oxygen, sulphur, tellurium, and polonium (Reilly, 2006; Young et al., 2010b). The electrochemical properties shared by selenium (Se) and sulphur (S) are the key to their recurrent interaction and substitution in biological, chemical and geological processes (Reilly, 2006). Selenium is also a non-metal, or metalloid, that exists in four oxidation states; thus, the potential for a multitude of inorganic and organic Se compounds to be generated based on the site-specific biogeochemical properties of the receiving environment is substantial (Young et al., 2010b; Janz, 2012).

Selenium is globally distributed with enriched sources naturally found among cretaceous sedimentary deposits of black shale, coal, and phosphate rocks (Maher et al., 2010). The mobilization of Se can transpire through natural biogeochemical processes (e.g. weathering, volcanic activity, wildfires); although, this contribution is minor when compared to the amount released through anthropogenic activities (Maher et al., 2010). The predominant sources responsible for the introduction of Se into the aquatic environment are typically waste by-products from several economically significant industrial processes such as mining, smelting of pyritic ores, oil and gas refining, and irrigation along with the combustion of coal and fossil fuels (Lemly, 2004; Muscatello et al., 2008; Janz, 2012). The extent to which Se contamination could

negatively impact an aquatic ecosystem was initially demonstrated at Belews Lake, North Carolina. In the 1970s, a coal-burning power plant discharged highly concentrated Se-laden wastewater (100-200 µg Se/L) into Belews Lake that resulted in the elimination of over 20 resident fish species with elevated rates of larval deformities in fish for ten years after termination of the source input (Lemly, 1985, 1997b, 2002; Young et al., 2010a).

1.2 Selenium in aquatic environments

1.2.1 Chemical speciation, uptake and bioaccumulation

The biogeochemical cycling of selenium is unique and complex within aquatic environments. Selenides (-II), elemental Se (0), selenites (+IV) and selenates (+VI) are the four oxidative states of Se that provide the foundation for the generation of both inorganic and organic Se species (Young et al., 2010b). Under oxidizing conditions, Se(+VI) and Se(+IV) are hydrolyzed to form the oxyanions, selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), which comprise the majority of dissolved Se present in the water column from anthropogenic sources (Presser and Ohlendorf, 1987; Fan et al., 2002; Maher et al., 2010). The oxyanions of Se typically demonstrate increased solubility and mobility with increasing pH (Young et al., 2010b). Primary producers and microorganisms absorb and biotransform these inorganic forms of Se into a variety of organoselenium species that include the selenoaminoacids selenocysteine (SeCys) and selenomethionine (SeMet) (Fan et al., 2002; Orr et al., 2006). Most notably, SeMet represents 60-80% of the total Se present in contaminated aquatic food webs (Fan et al., 2002; Orr et al., 2006; Maher et al., 2010; Phibbs et al., 2011; Janz et al., 2014); thus, classifying it as the prominent form with the greatest aptitude for inducing dietary toxicity. A substantial enrichment of Se occurs at the base of food webs with the uptake of Se from its aqueous phase by algae. The

potential for a greater than a 100 fold increase in Se concentrations between these two has reported to occur (Luoma and Presser, 2009; Stewart et al., 2010). In this regard, lentic water bodies particularly allow for greater production of SeMet by primary producers due to the longer residence time of excess Se (Orr et al., 2006). Aquatic consumers occupying various trophic levels ingest and incorporate these selenoaminoacids into proteins resulting in the transfer and accumulation of Se through the food web with top predatory species like birds and fish having a greater likelihood of accumulating high concentrations over time (Lemly, 1993a; Hamilton, 2004; Stewart et al., 2010). Ultimately, the degree to which selenium can exert toxicity in an ecosystem is dependent on site-specific characteristics, which pertain to hydrology, water chemistry and food web structure (Simmons and Wallschläger, 2005; Orr et al., 2006).

1.2.2 Guidelines for selenium in the aquatic environment

The regulatory guidelines concerning the extent to which Se may be present in aquatic systems for the protection of organisms is continually evolving with emerging knowledge. The federal water quality guideline for the protection of freshwater aquatic life put forth by the Canadian Council of Ministers of the Environment is 1µg Se/L (CCME, 2007); however, provincial guidelines may vary with British Columbia setting the threshold at 2 µg Se/L (BC MoE, 2014) and Ontario at 100 µg Se/L (MoEE, 1994). The US EPA set a similar guideline at 5 µg Se/L for chronically elevated water concentrations (US EPA, 2004). In 2004, the US EPA drafted a criterion that acknowledged the primary route of Se exposure was through dietary sources by establishing a whole body Se threshold of 7.91 µg Se/g d.m. for the protection of fish populations (US EPA, 2004). With increasing knowledge of Se's biogeochemical behavior and its teratogenic capabilities in aquatic oviparous vertebrates, the US EPA drafted a new set of

criterion for the protection of freshwater organisms in 2014 that is currently undergoing review. With regards to water quality, it proposes the 30 day average of water concentrations in lentic and lotic systems not exceed 1.2 and 3.1 µg Se/L more than once in a three year period (US EPA, 2015). Moreover, the criterion states that fish tissue concentrations should never exceed 15.8, 8.0 and 11.3 µg Se/g d.m. for egg-ovary, whole body, and muscle samples, respectively (US EPA, 2015).

1.3 The essentiality and toxicity of selenium

1.3.1 The essential role of selenium in vertebrate health

Selenium is essential for optimal vertebrate health, and its physiological roles are attributed to its presence in an assortment of selenoproteins in the form of selenocysteine, the 21st amino acid. The size of a selenoproteome varies with species (Papp et al., 2007; Lobanov et al., 2009; Mariotti et al., 2012). Bony fishes have one of the largest selenoproteomes, consisting of 41 selenoprotein subfamilies, while frogs, birds and mammals have 24, 25 and 28, respectively (Lobanov et al., 2009; Janz, 2012; Mariotti et al., 2012). The majority of selenoproteins identified in vertebrates have unknown physiological functions (e.g. selenoprotein V [SelV] or selenoprotein N [SelN]); however, oxidoreductase seleno-enzymes such as glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases are among the few that have been characterized (Papp et al., 2007; Janz et al., 2010; Gladyshev, 2012). Glutathione peroxidases and thioredoxin reductases are enzymes that catalyze oxidation-reduction reactions to effectively minimize oxidative damage and maintain intracellular redox homeostasis. Additionally, thioredoxin reductases are involved in DNA synthesis and protein repair (Papp et al., 2007; Janz et al., 2010; Gladyshev, 2012). Iodothyronine deiodinases are vital to metabolic

processes like thermogenesis, growth, and development by assisting in the conversion of thyroxine (T4) to active triiodothyronine (T3) through the removal of iodine moieties (Papp et al., 2007; Janz et al., 2010; Gladyshev, 2012). Ultimately, the efficiency of these enzymes to perform their catalytic roles relies on sufficient dietary intake of selenium. The nutritional requirements for Se in order to maintain proper physiological functioning of these selenoproteins have been experimentally determined to range between 0.1 and 0.5 μg Se/g d.m. for fishes and between 0.3 to 1.1 μg Se/g d.m. in aquatic birds (NRC, 1993; Puls, 1994; Watanabe et al., 1997; Lin et al., 2005; Janz et al., 2010; Stewart et al., 2010). The optimal dietary Se requirements for amphibians in general, or specifically for *X. laevis*, have not been established although preliminary recommendations suggest 0.3 μg Se/g d.m. for adult amphibians (Ferrie et al., 2014).

Dietary SeCys or SeMet are readily absorbed and metabolized in order to ensure sufficient quantities of Se are available for the synthesis of selenoproteins. Both SeCys and SeMet undergo biotransformation into a common intermediate, selenide (Se^{2-}) (Suzuki and Ogra, 2002; Janz, 2012). Selenide is phosphorylated by selenophosphate synthetase to create SeCys, the vital component of selenoproteins (Janz, 2012). The incorporation of SeCys into a polypeptide chain is guided by selenocysteinyl-tRNA and highly regulated by a UGA codon within a SeCys insertion sequence in order to create the desired selenoprotein (Stadtman, 1996; Janz et al., 2010). Unlike selenocysteine, SeMet may undergo the biotransformation process described above, or avoid it entirely through direct incorporation into any methionine-containing protein structure due to the inability of methionyl-tRNA acylase to discriminate between methionine and SeMet (Young et al., 2010b). Therefore, tissues with high rates of protein synthesis such as those found in skeletal muscle, liver, kidney, pancreas, and erythrocytes will have, in a concentration dependent manner, an increasing proportion of SeMet in their structures

(Schrauzer, 2000). Under normal dietary conditions, this process has the potential to be beneficial by providing a selenium reserve to draw upon when SeCys synthesis is necessary; however, excessive levels of SeMet may compromise cellular redox homeostasis (Suzuki and Ogra, 2002; Janz, 2012).

1.3.2 The toxicological effects of selenium in oviparous vertebrates

Knowledge of the toxicological impact Se could inflict on aquatic biota has been continually expanding over recent decades. The majority of this research has primarily focused on oviparous vertebrates, particularly birds and fish, since they appear to have a greater sensitivity to the negative effects associated with Se-laden environments than other biota with a narrow range between essentiality and toxicity being observed (Janz et al., 2010). Thus far, the most predominant and perhaps most important toxicological effect of Se exhibited in oviparious species are developmental abnormalities. In fish, the maternal transfer of excess dietary Se to the developing oocyte during vitellogenesis results in a substantial source of Se exposure to the larvae during yolk resorption (Janz et al., 2010; Janz, 2012). This route of exposure is considered the cause for the production of a suite of characteristic deformities in fish such as craniofacial deformities, spinal curvatures, missing or deformed fins, and edema (Holm et al., 2005; Muscatello et al., 2006; Janz, 2012). While *in ovo* Se exposure via maternal transfer also occurs in birds, the Se-containing proteins involved as well as the effects produced are quite different when compared to fish. In birds the albumin, not the yolk sac, is where Se is primarily found (Janz et al., 2010). This contrast in Se allocation results in its utilization by the developing chick prior to hatching and yolk sac resorption rather than after. Some characteristic embryonic

deformities produced by Se in birds are malformed bills, reduction/absence of eyes, and limb deformities (Hoffman and Heinz, 1988; Ohlendorf et al., 1988; Janz et al., 2010).

Aside from reproductive and developmental endpoints, recent research has investigated the potential consequences of sublethal Se exposure on stress-induced energy utilization in adult fishes. The link between altered bioenergetic status and physiological stress response to elevated Se exposure has been established, but is still poorly understood. Several field studies have reported alterations in triglyceride, glycogen and cortisol concentrations of fishes and anurans collected from Se-contaminated sites, but a defined physiological pattern or mechanism by which this occurs remains unclear (Hopkins et al., 1999; Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2010; Ward et al., 2006). Increases in basal cortisol levels have been observed in both elevated aqueous and dietary Se exposures in fishes (Miller et al., 2007; Thomas and Janz, 2012); however, the ability to mount a response to a secondary stressor is diminished. Female rainbow trout (*Oncorhynchus mykiss*) chronically exposed to an 8.47 μg Se/g d.m. diet for 126 days showed a marked increase in basal plasma cortisol levels when compared to control fish (Wiseman et al., 2011). However, Se-exposed trout plasma cortisol levels remained similar to their basal levels following a 3 minute handling stressor whereas the control trout initiated a greater response (Wiseman et al., 2011). In addition, dietary Se affected both the accumulation and partitioning of energy stores in liver and muscle of these trout (Wiseman et al., 2011). In an evaluation of triglyceride and glycogen levels prior to and following swimming trials in adult zebrafish (*Danio rerio*), storage and utilization of triglyceride and glycogen levels were altered in the Se-treated fish when compared to fish in the control group (Thomas and Janz, 2011; Thomas et al., 2013). These altered energetic dynamics have been associated with changes to the expression of proteins involved in either the synthesis or

metabolism of these macronutrients such as citrate synthase, HOAD (β-hydroxyacyl coenzyme A dehydrogenase) and SREBP 1 (sterol regulatory element binding protein 1) (Goertzen et al., 2011; Thomas et al., 2013). Further research investigating the connection between energy reserves, stress and sublethal Se toxicity is necessary to understand the potential impact on both the individual and a population within an ecosystem.

1.3.3 The toxicological effects of selenium in amphibians

There is marginal knowledge pertaining to the toxic effects of selenium on amphibians. The majority of existent data related to this research topic has been gathered from coal combustion waste deposition sites with high Se content that have reported a number of associated adverse effects on the inhabiting anuran populations (Rowe et al., 1996; Raimondo and Rowe, 1998; Hopkins et al., 2000; Hopkins et al., 2006; Ward et al., 2006; Metts et al., 2013). However, Se is merely one contaminant existing at these sites among many. Other contaminants (e.g. arsenic, mercury, cadmium) present have the capability to produce detrimental reproductive effects in aquatic vertebrates independently in addition to influencing the bioavailability and toxicokinetics of Se within the organism resulting in either an increase or decrease of toxicological effects (Thompson and Bannigan, 2008; Bergeron et al., 2010; Singha et al., 2014). This makes it very difficult to establish a causal relationship between the presence of Se and its effects in amphibians. Nonetheless there appears to be similarities between the effects observed in amphibians at these sites and those seen in fish exposed to elevated levels of Se.

Amphibians efficiently accumulate and retain Se in their tissues during all stages of their life cycle (Hopkins et al., 2006; Unrine et al., 2007; Rowe et al., 2011; Metts et al., 2012;

Lockard et al., 2013). At contaminated sites, selenium has been found at elevated concentrations in larval and adult tissues as well as eggs. Hopkins et al. (2006) determined female eastern narrow mouth toads (*Gastrophyryne carolinensis*) collected from a site near a coal-burning power plant transferred significant quantities (up to 100 µg/g dry mass) of selenium to their eggs when compared to toads at a reference site. Amphibian larvae exposed to coal fly ash were shown to efficiently accumulate Se in their tissues (Unrine et al., 2007) and retain it through metamorphosis (Snodgrass et al., 2003, 2004, 2005; Rowe et al., 2011). The presence of high Se concentrations in tissues during highly sensitive and critical transitional stages of development (i.e. embryo-tadpole and tadpole-frog) could result in severe consequences to the overall health of an anuran and its ability to successfully shift from an aquatic to a terrestrial life stage.

Elevated Se concentrations in larval amphibian tissues are associated with increased incidences of deformities and mortalities. The larvae of eastern narrow mouth toads collected from coal ash contaminated sites enriched with Se had an 11% reduction in hatching success, 19% reduction in viability, and a 55-58% increase in the frequency of developmental deformities and abnormal swimming when compared to reference larvae (Hopkins et al., 2006). Elevated incidences of mortality among amphibian larvae at contaminated sites containing Se may be related to an inability to efficiently swim or feed due to vertebral and craniofacial malformations (Rowe et al., 1996, 1998a; Burger and Snodgrass, 2000; Janz et al., 2010). This relationship between Se and amphibian malformations is supported through synchrotron X-ray fluorescence analysis detecting high concentrations of Se localized within the malformed areas of bullfrog (*Rana catesbeiana*) tadpoles presenting with oral deformities (Punshon et al., 2005; Janz et al., 2010). Tadpoles with elevated Se levels have also displayed reduced growth (Rowe et al., 1996; Snodgrass et al., 2004, 2005; Metts et al., 2012), elevated maintenance costs (Rowe et al.,

1998b), altered predator avoidance capabilities (Raimondo et al., 1998; Hopkins et al., 2006), reduced survival (Rowe et al., 2001; Snodgrass et al., 2004, 2005; Roe et al., 2006; Lockard et al., 2013), and altered time to metamorphosis (Snodgrass et al., 2004; Janz et al., 2010; Metts et al., 2012).

1.3.3.1 Mechanisms of selenium toxicity

The mechanism by which selenium exerts its toxic effects remains uncertain. Initial investigations of this topic focused on the substitution of Se for S in amino acids. It was argued that the amino acids containing Se in the place of S disrupted the formation of S-S linkages consequently producing a protein that is improperly folded and dysfunctional (Janz et al., 2010). However, this hypothesis has diminishing support for a number of reasons. As discussed previously, the incorporation of SeCys into a protein is highly regulated, so the synthesis of any protein requiring Se for its structure or function will have a specific mRNA sequence and will require a UGA codon for selenocysteinyl-tRNA (Stadtman, 1996; Janz et al., 2010). Moreover, the presence of a terminal methyl group in both the structure of methionine and SeMet prevents the formation of covalent bridges indicating that both structures have very little to no influence on the tertiary structure of a protein. It is therefore unlikely that improper protein structure and function due to the substitution of Se for S in either methionine or cysteine is the mechanism by which toxicity occurs (Janz et al., 2010).

Oxidative stress has been proposed as an alternative mechanism of Se toxicity with glutathione homeostasis playing a key role in its propagation (Palace et al., 2004; Janz et al., 2010; Janz, 2012). Under normal conditions, glutathione peroxidase (GPx) and reduced glutathione (GSH) act together as an intracellular antioxidant (Reddy and Massaro, 1983). At

sufficiently high levels of Se, the antioxidant capacity of this GPx-GSH collaboration become overwhelmed and a depletion of GSH occurs leading to the proliferation of reactive oxygen species (ROS) that damage DNA, protein, and lipids (Spallholz, 1994; Spallholz et al., 2001; Misra and Niyogi, 2009). Selenium assumes many different forms, which can directly or indirectly influence glutathione homeostasis. For example, SeMet has very little interaction with the glutathione antioxidant system, but upon its biotransformation to methylselenol or dimethylselenide, these metabolites react with glutathione to produce ROS such as superoxide anions, hydrogen peroxide, and hydroxyl radicals (Spallholz, 1994; Spallholz et al., 2001). The maternal transfer of SeMet to the yolk sac of eggs, and its subsequent breakdown by developing larvae during yolk sac resorption leads to the production of ROS that damage cellular components, and is hypothesized to be the cause of larval fish deformities in Se rich aquatic environments (Palace et al., 2004; Janz et al., 2010).

1.4 Amphibians

1.4.1 Amphibians as indicator species

Amphibians are ectothermic vertebrates that are categorized into three orders: Anura (toads and frogs), Caudata (salamanders and newts) and Gymnophiona (caecilians, worm-like) (Shi, 2000). They are morphologically and physiological distinct as a vertebrate class. Their renowned attributes include highly permeable skin, lack of cleidoic (shelled) eggs, and a metamorphic stage in development (Vitt and Caldwell, 2009). The largest group of amphibians that also have the broadest geographical distribution are the anurans (Shi, 2000). Over twenty years ago the label of "canaries in a coal mine" was given to amphibians upon realization of their global population declines (Kerby et al., 2010). This analogy proposed that amphibians were

exceptionally sensitive to environmental changes and thus were an excellent indicator species able to alert scientists to ecological distress. There are several factors implicated in the rapid decline of amphibians, one of which is environmental contamination. The reasoning behind their suspected high susceptibility to contaminants over other organisms is generally owing to their permeable eggs, skin and gills, aquatic-terrestrial life cycle and a relatively rudimentary immune system (Bridges et al., 2002; Wake and Vredenburg, 2008; Kerby et al., 2010). Although these deductions are logical to presume, there is insufficient evidence to entirely support the claim that amphibians are the most sensitive species to chemical contamination within an ecosystem.

Research into amphibian sensitivity to environmental contaminants has shown that it may not be as simple as once thought. This leaves their role as an ideal indicator species in question. A review by Birge et al. (2000) compared several amphibian species (e.g., *Rana pipiens* [northern leopard frog], eastern narrowmouth toad) with commonly tested fish (eg. rainbow trout, *Pimephales promelas* [fathead minnow]) to a number of aqueous contaminants. The relative sensitivity of amphibians to fish for the embryo-larval stage was determined by performing 694 comparisons using 50 metals and organic compounds. The conclusion was that 64% of the time amphibians were more sensitive than fish. A subsequent study by Bridges et al. (2002) exposed southern leopard frog (*Rana sphenocephala*) tadpoles to five chemicals (4-nonylphenol, carbaryl, copper, pentochlorophenol, permethrin) each having a different mode of action. The LC50s at 24 and 96 h were then compared to published data on commonly tested fish species (i.e., fathead minnow, bluegill sunfish [*Lepomis macrochirus*], rainbow trout). Overall, the southern leopard frog tadpoles were more tolerant in 48 % of comparisons, while in 22.5% of comparisons there were no significant differences. In addition, the results found that the southern leopard frog tadpoles were very tolerant to chemicals like carbaryl and permethrin while more

sensitive to metals like copper. These two studies demonstrate the inadequacy of using an entire class of vertebrates with very diverse natural histories and physiology as an overall indicator of ecological health. It is a misguided assumption that amphibians will have a greater sensitivity to all contaminants when compared to other species within a community. Furthermore, it is imperative to note then that in both studies the dermal route of contaminant exposure was solely explored, providing a very limited view of a much larger picture.

Nonetheless, amphibian populations play important roles in a community and should not be overlooked or given diminished importance when investigating contaminant effects on an ecosystem. In some habitats, amphibian numbers and biomass exceed that of all other vertebrates (Stebbins and Cohen, 1995), so any drastic changes to their population could have serious repercussions for the entire community. The biphasic nature of their lifecycle allows amphibians to act as vectors for contaminants, transferred from aqueous to terrestrial environments (Unrine et al., 2007). Moreover, many amphibians have an aquatic herbivorous tadpole stage and a terrestrial carnivorous adult stage thus designating them as both an important predator and prey species in the food web (Burger and Snodgrass, 2000; Murphy et al., 2000).

1.4.2 Energetic status and the stress response as gauge of anuran fitness

Physiological stress is a multifaceted response to both anticipated and unexpected environmental stressors that challenge an organism's established homeostatic parameters (Cockrem, 2013; Dantzer et al., 2014). These stressors include contaminant exposure, habitat destruction, reduced food availability, temperature fluctuations as well as preparations for reproduction and migration (Hopkins et al., 1999; Cockrem, 2013; Dantzer et al., 2014). The physiological stress response is facilitated through a series of biochemical events initiated by the

hypothalamic-pituitary-interrenal (HPI) axis in amphibians. Neuroendocrine stimulation initiates the release of corticotropin-releasing hormone from the hypothalamus, which subsequently stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream for transportation to the adrenocortical tissue within the interrenal glands, where stimulation of corticosteroid synthesis and release generates a negative feedback cycle within the HPI axis (Denver, 2009b). The most prevalent corticosteroid for terrestrial amphibians in response to stress is corticosterone; however, cortisol has been reported to be the major corticosteroid in metamorphosing ranid tapdoles and in permanently aquatic anurans (i.e. *Xenopus laevis*) and urodeles indicating a similar role in osmoregulation as in fishes (Herman, 1992; Norris, 2007; Denver, 2009b; Dantzer et al., 2014). The release of cortisol/corticosterone prompts a number of metabolic processes related to the mobilization and utilization of energy stores including glycogenolysis, gluconeogenesis, and lipolysis (Herman, 1992; Denver, 2009b). While the metabolic expense associated with an acute stressor is beneficial to the survival of an individual, prolonged stress could have implications for the bioenergetic status (i.e. triglyceride and glycogen levels) of an amphibian resulting in the inability to effectively reproduce, grow and resist disease (Moore and Miller, 1984; Herman, 1992; Denver, 2009a; Falso et al., 2015).

Triglycerides are the principal lipids contained in amphibian energy reserves. Anuran amphibians primarily store lipids in abdominal fat bodies, but they may also be found in the liver, subcutaneous tissue, muscle, gonads, and tail (Sheridan and Kao, 1998). Lipids provide an excellent source of energy because they have twice the energetic yield of carbohydrates upon oxidation, and can be stored in high concentrations within the body due to their low water solubility (Fitzpatrick, 1976). Triglycerides also play a vital role in anuran physiological processes such as metamorphosis, gonadal maintenance, production of gametes, and metabolic

maintenance during dormancy (Fitzpatrick, 1976). Contaminant exposure may reduce triglyceride storage or utilization in anurans resulting in reduced fitness. For example, larval leopard frogs fed a rationed diet containing a high concentration of vanadium had significantly lower growth rates, delayed metamorphosis, and reduced lipid content at metamorphosis when compared to controls (Rowe et al., 2009).

Glycogen functions as a secondary long-term energy store (Pinder et al., 1992). It is primarily located in the liver, but low concentrations can also be found within muscle tissue. It is an essential form of energy that can be rapidly metabolized to supply energy in response to activities such as predator avoidance/escape, foraging/prey capture, territorial defense, and courtship (i.e., mating calls) (Pough et al., 1992). In addition, freeze-tolerant anurans can rapidly access and convert glycogen to glucose, which acts as a cryoprotectant essential to winter survival (Pinder et al., 1992; Dinsmore and Swanson, 2008). Lowered freeze survival is associated with low hepatic glycogen stores. Ultimately, the depletion of triglyceride or glycogen energy stores can lead to reduced survivorship and impaired reproduction of amphibian species (Dinsmore and Swanson, 2008).

1.4.3 *Xenopus laevis* as a laboratory model

1.4.3.1 Advantages and disadvantages

Xenopus laevis, commonly known as the African clawed frog, belongs to the order Anura and family Pipidae. Their native range includes large parts of sub-Saharan Africa from Uganda and Zaire to South Africa and west to Cameroon (Cannatella and de Sa, 1993). They generally prefer slow moving to stagnant waters (i.e., swamps, irrigation ditches, reservoirs, lakes) in cooler upland areas (Tinsley et al., 1996; Green, 2002). The name, *Xenopus laevis*, describes its

characteristic smooth skin and three clawed toes on each hind foot; *Xenopus* means "strange foot" and *laevis* means "smooth". They are among the most aquatic anurans, which may correspond with their very strange appearance. *X. laevis* have flattened, pear-shaped bodies, small heads, dorsally positioned eyes without eyelids, and lateral line organs that resemble "stitches" on their dorsum. They have fore limbs that are short and small while their hind limbs are long and muscular with toes that are fully webbed with the three inner toes on each foot having black claws (Cannatella and de Sa, 1993). Other unique features include the absence of a tongue, a well-developed but concealed middle ear, and the absence of vocal chords or sacs. The tadpoles are filter feeders that have a pair of sensory tentacles, and orient their heads downward while rapidly moving their tail to maintain position (Cannetella and de Sa, 1993).

The use of *Xenopus laevis* as a model organism has a number of advantages in laboratory research. Probably the most significant reason for the use of *X. laevis* is its ability to reproduce year-round through the use of commercial hormone preparations such as human chorionic gonadotropin (Gurdon, 1996). This is a huge advantage when most other amphibians have a limited breeding season of just a few weeks per year. *X. laevis* also produce an abundance of large sized eggs per spawn and have a relatively short time period between embryo and sexually mature adult (Green, 2002). Compared to other anuran species the husbandry of *X. laevis* is easy. They can be readily obtained through distributors, housed in aquaria, fed pelleted food and have a relatively excellent resistance to disease (Gurdon, 1996; Green, 2002). Moreover, the embryonic development of *X. laevis* has been studied for decades giving a substantial knowledge base with established methods for studying oocyte, egg, and embryo development (Green, 2002).

There are a few disadvantages to using *X. laevis* as a model organism in laboratory studies. Despite the relative ease of husbandry and the extensive laboratory use of *X. laevis* for decades, there is marginal evidence based knowledge on how to maintain a healthy and productive colony and therefore no established standardized husbandry protocol concerning information on the necessary water quality parameters, environmental conditions or nutritional requirements (Green, 2002). In addition, *X. laevis* is an allotetraploid, which potentially makes genetic analysis problematic. With a chromosome set twice that of a diploid organism, researchers have greater difficulty in manipulating its genome to identify the functions of particular genes and their subsequent inheritance through multiple generations (Amaya et al., 1998). *X. laevis* have significant differences in their genetics, life history, physical characteristics, and relative tolerance to environmental contaminants when compared to temperate native species (McDiarmid and Mitchell, 2000; Elinson and del Pino, 2012). For these reasons, researchers utilizing *X. laevis* must be cautious with the extrapolation of their results to other anuran species.

1.4.3.2 *Xenopus laevis* oogenesis

The ovaries are the largest organs in the adult *X. laevis* female. Each ovary has multiple lobes (approx. 24 lobes per ovary) containing hundreds of oocytes at various stages of development, which comprise the majority of the ovarian volume (Rasar and Hammes, 2006). The oocytes contained within an ovary are surrounded by granulosal, thecal and epithelial layers. The germinal epithelial layer forms a continuous layer with the visceral peritoneum and surrounds the underlying granulosal, theca layers as well as follicular cells (Rasar and Hammes, 2006; Linder et al., 2010). The theca layer contains extracellular matrix, blood vessels,

fibroblasts and the developing oocytes (Rasar and Hammes, 2006). Follicular development is initiated by increases in gonadotropins (Linder et al., 2010). With the onset of vitellogenesis, the theca begins by surrounding the granulosal layer then slowly becomes separated from it by the *membrane propria* to form the glandular *theca externa* and the fibrous *theca interna* (Linder et al., 2010). Each oocyte develops a layer of follicular cells and vitelline membrane that surrounds and separates it from the theca layer (Rasar and Hammes, 2006). With further maturation, the follicle cells create gap junctions that cross the vitelline membrane and connect them to the oocyte surface to facilitate yolk accumulation (i.e. vitellogenesis) and regulation of meiosis (Rasar and Hammes,, 2006; Mónaco et al., 2007). A fully developed oocyte primed for ovulation and fertilization is comprised of a well-defined animal pole containing the nucleus, a vegetal pole containing protein rich yolk platelets and an equatorial band that separates the two.

Oocyte yolk formation, also known as vitellogenesis, is a fundamental process that ensures the embryo has adequate nutrition for development. Vitellogenin, a 400-500 kDa phospholipoglycoprotein, is the precursor yolk protein necessary for the development of the oocyte's yolk sac (Linder et al., 2010). Under the regulation of the hypothalmic-pituitary-gonadal-liver endocrine axis, vitellogenin is synthesized in the liver, transported to the ovary through the bloodstream and enters the oocyte. The method of vitellogenin uptake by the maturing oocyte is not completely understood; however, it is hypothesized to occur through pinocytotic and endocytotic processes in addition to facilitated communication between follicular cells and oocytes through gap junctions (Brachet, 1979; Gilbert, 2000; Mónaco et al., 2007). In a mature oocyte, vitellogenin is enzymatically cleaved into two yolk proteins, phosvitin and lipovitellin, and subsequently packaged into yolk platelets with glycogen granules and lipochondrial inclusions storing their respective yolk components (Gilbert, 2000). The process of

vitellogenesis is frequently altered due to contaminant exposure making it a vital biomarker of reproductive fitness in oviparous species (Kime et al., 1999). Moreover, it is hypothesized to be the primary method of *in ovo* Se exposure for embryonic and larval aquatic oviparous vertebrates that is responsible for the production of teratogenic abnormalities (Janz et al., 2010).

Oogenesis in an adult *X. laevis* female is a persistent and asynchronous process with a cycle considered complete when a large proportion of oocytes have become post-vitellogenic (Rasar and Hammes, 2006). Even though oogenesis is continuous and progressive in *X. laevis* with no defined boundaries, there are six stages of oocyte development that have been outlined below by James Dumont (1972). The diameter size of Stage I oocytes range from 50 to 300 µm and Stage VI oocytes from 1200 to 1300 µm demonstrating that oocyte size gradually increases with developmental stage. Stage I and II oocytes are considered pre-vitellogenic while Stage VI oocytes are post-vitellogenic. Stage II oocytes account for the greatest proportion (45%) of the total oocyte population in stages II to VI while the other five stages each contribute only 10 to 15% of the total. Pigmentation and vitellogenesis commences in Stage III oocytes giving them a uniformly light brown (early) to dark brown (late) appearance with no definition between the two poles. Stage IV oocytes experience increased growth due to the rapid vitellogenesis which coincides with the polarization of pigments. Vitellogenesis decreases in Stage V oocytes and two distinct hemispheres outlining animal and vegetal poles are created. Stage VI oocytes have two distinct hemispheres separated by an unpigmented equatorial band and have ceased vitellogenesis.

Oogenesis in *X. laevis* is greatly affected by three factors: temperature, age of the female and nutrition. *X. laevis* naturally prefer warm, calm waters year round, and under agreeable

laboratory conditions (i.e. 12h light:12h dark photoperiod; 19 to 23ºC water temperature) *X. laevis* are capable of efficient oogenesis and adequate egg production year round (Green, 2002). However, a marked reduction in the quality and quantity of eggs produced by laboratory housed *X. laevis* does occur seasonally indicating an innate period of torpor that could be induced under lower water temperatures. However, estrogen production and metabolic processes related to oogenesis may improve with a period of cold exposure in *X. laevis* with some laboratories reporting an improvement in egg quality of cold adapted frogs housed at 16°C who are gradually reintroduced to warmer water prior to egg collection (Green, 2002). Additionally, the age of the female can greatly influence the quality and number eggs produced in each clutch with the poorest fecundity observed in older commercially reared females. In general, *X. laevis* females reach their reproductive peak by 2 to 3 years of age with the capability to produce up to 4 clutches per year each containing approximately 10 to 20 thousand eggs (Green, 2002). Furthermore, oogenesis in *X. laevis* is highly dependent on food supply, but the nutritional qualities inherently required to attain optimal performance in any amphibian is unknown. The best diet for these opportunistic carnivorous feeding anurans is highly debatable due to inconsistencies between laboratories with no critical evaluation of efficacy. For example, laboratories report feeding their *X. laevis* colonies diets consisting of chopped meats, commercially packaged frozen invertebrates, worms, crickets or pelleted foods (Green, 2002). Although *X. laevis* has been the standard laboratory model amphibian for decades, there is a lack of knowledge and remains no universally accepted housing protocols to optimize the health and oogenesis of this species in captivity.

1.4.3.3 Xenopus laevis development

A comprehensive system for the classification of stages in *Xenopus laevis* embryonic and larval development was outlined in the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1994). This invaluable document describes in detail the complex changes an embryo undergoes to complete metamorphosis in 66 distinct steps. The first 15 stages of embryo development include blastulation, gastrulation, and formation of the neural plate. The majority of the early structural and systemic development of the larvae occurs from stages 15 - 38 with intricate later stage larval development continuing through to stage 53. Approximately 2 days after fertilization (dpf), the embryos begin hatching (stages 35-38). The mouth breaks through (stage 40) and the tentacle rudiments begin to appear (stage 44) one day later. At stage 45 (~4 dpf) larvae begin feeding and by stage 48 (~7 dpf) the yolk has completely disappeared. The hind limb bud develops in stages of 46 - 53. The formation of individual toes of the hind legs occurs at stages 54 - 57 followed by rapid hind limb growth in stages 57 - 60. The forelimb bud appears at stage 48, and continues to grow and form articulations until stage 62. Resorption of the tail begins at stage 62 and metamorphosis is complete at stage 66.

The 66 stages of embryo and larval development outlined in The Normal Table of *Xenopus* can be conveniently divided into three metamorphic stages: premetamorphosis, prometamorphosis and metamorphic climax (Shi, 2000; Brown and Cai, 2007). Premetamorphosis (stages 1-53) is the period of early embryo and larval development that occurs in the absence of thyroid hormone. Prometamorphosis (stages 55 - 57) is characterized by hind limb growth and toe differentiation in the presence of a rising concentration of thyroid hormone. However, initial formation of hind limbs occurs towards the end of the prometamorphosis stage

(Shi, 2000; Brown and Cai, 2007). Metamorphic climax (stages 58 - 66) involves rapid morphological changes that are stimulated by peak thyroid hormone levels (Shi, 2000; Brown and Cai, 2007). These changes include complete resorption of the tadpole's tail, development of the fore limbs, and the restructuring of internal organs.

Metamorphosis, like embryonic development, is an extremely vulnerable time for anuran amphibians due to the major biochemical, morphological and physiological changes that occur. Larval amphibians need to transform from an aquatic herbivore to a terrestrial carnivore; this requires complete restructuring of the respiratory system, digestive tract, cranium, jaw, and pelvic girdle (Murphy et al., 2000). The dramatic alterations in gene expression and endocrine actions needed to coordinate these changes creates a multitude of opportunities for toxic chemical interactions, resulting in decreased survivorship and reduced overall fitness of the transformed population. During metamorphic climax, the suppression of the immune system to prevent an autoimmune response to adult cells present in the larval body leaves them susceptible to infections as well as contaminant exposures (Murphy et al., 2000). Moreover, resorption of the larval tail may redistribute stored chemicals thereby increasing their availability for metabolism and the potential production of toxic metabolites (Murphy et al., 2000). The restructuring of epithelial tissues may alter the rate of uptake and transport of chemicals (Henry, 2000), and the mobilization of energy reserves (i.e., triglycerides) along with any lipophilic chemicals contained within will be released into the system (Murphy et al., 2000).

1.5 Research objectives and hypothesis

There is growing concern about the presence of selenium in aquatic systems due to its efficient incorporation into food webs and its role as a developmental toxicant in aquatic

oviparous species like fish and birds. Presently, there is insufficient data on the effects of selenium on amphibians. A number of field studies speculate that selenium is causing a higher incidence of larval amphibian mortality and deformities at industrially contaminated sites. To date, no extensive laboratory research has been performed to isolate and confirm selenium's role without the influence of other commonly found toxicants at these sites. The overall goal of this thesis is to investigate the possible exposure routes, reproductive and developmental effects, and the overall sensitivity of amphibians exposed to Se in order to gauge the effectiveness of regulatory guidelines in protecting declining amphibian populations.

1.5.1 Objectives

Utilizing the model amphibian species *Xenopus laevis*, the three main objectives of this thesis were:

1) To determine the consequences of *in ovo* selenium exposure on early and late stage larval anuran development
2) To determine the consequences of elevated dietary selenium on the fitness of female anurans
3) To determine the sensitivity of anurans to elevated *in ovo* or dietary Se in relation to other aquatic oviparous species

1.5.2 Hypotheses

The overall null hypothesis of this research was:

H_0: Chronically elevated dietary Se exposure in adult female *Xenopus laevis* will produce no adverse effects on their fitness or the development of their progeny.

The specific null hypotheses are:

1) H_0: There will be no adverse effects on egg fertilization success, biometric indices, energetic status or physiological stress response in female *X. laevis* fed elevated levels of dietary Se.
2) H_0: There will be no difference in measured concentrations of Se among female *X. laevis* tissues after chronic dietary exposure to elevated Se.
3) H_0: *In ovo* Se exposure via maternal transfer will have no effect on apical endpoints relating to morphometrics, malformation, maturation, metamorphosis or mortality of developing *X. laevis* larvae.

CHAPTER 2

DOSE-DEPENDENT EARLY LIFE STAGE TOXICITIES IN *XENOPUS LAEVIS* EXPOSED *IN OVO* TO SELENIUM

2.1 Preface

The most prominent and valued portion of this generational bioassay is presented within this chapter. The purpose of this research was to characterize the effects of early life stage Se toxicities that arise through elevated *in ovo* exposure via maternal transfer in amphibians in order to determine their relative sensitivity in comparison to other aquatic oviparous vertebrates. An evaluation of endpoints that included mortality, hatching success, and fertilization success were performed within the first five days of development along with an extensive deformity analysis of 5 day post fertilization tadpoles. This data allowed for the generation of EC_{10} values related to the incidence of teratogenic abnormalities induced by *in ovo* Se exposure in *Xenopus laevis,* a representative amphibian model species, which will potentially assist in establishing more comprehensive regulatory guidelines.

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2.2 Abstract

Selenium (Se) is a developmental toxicant in oviparous vertebrates. The adverse reproductive effects of Se toxicity have been predominantly investigated in fishes and birds with only a few studies focusing on amphibians. The objective of this study was to determine tissue-based Se toxicity thresholds for early life stage Se toxicities in *Xenopus laevis* as a consequence of *in ovo* exposure through maternal transfer of dietary Se. Following a 68-day dietary exposure to food augmented with L-selenomethionine (SeMet) at measured concentrations of 0.7 (control), 10.9, 30.4, or 94.2 µg Se/g dry mass (d.m.), adult female *X. laevis* were bred with untreated males, and resulting embryos were incubated until 5 days post-fertilization (dpf). The measured Se concentrations in eggs were 1.6, 10.8, 28.1 and 81.7 µg Se/g d.m., respectively. No biologically significant effects were observed on fertilization success, hatchability or mortality in offspring. Frequency and severity of morphological abnormalities were significantly greater in 5 dpf tadpoles from the highest exposure group when compared to the control, with eye lens abnormalities being the most prominent of all abnormalities. The estimated EC_{10} value for frequency of total early life stage abnormalities was 44.9 µg Se/g egg d.m., which suggests that this amphibian species is less sensitive to *in ovo* Se exposure than most of the fish species studied to date.

2.3 Introduction

Selenium (Se) is a naturally occurring, globally distributed metalloid that becomes mobilized and released into aquatic environments predominantly through anthropogenic activities related to mining, coal-fired power plants, oil refining and irrigation of seleniferous soils for agricultural purposes (Lemly, 2002; Maher et al., 2010). Inorganic selenate and selenite are typically the forms of Se introduced into receiving aquatic ecosystems by these industrial practices. Both of these Se forms are subsequently absorbed and biotransformed by primary producers and microbes into a variety of organic Se species. Selenomethionine (SeMet) is the most prominent organoselenium species found in Se-contaminated aquatic food webs, representing 60-80% of total Se (Fan et al., 2002; Orr et al., 2006; Maher et al., 2010; Phibbs et al., 2011; Janz et al., 2014). Selenium concentrations increase more than 100 fold between the aqueous phase and algal uptake (Luoma and Presser, 2009; Stewart et al., 2010). This Se enrichment at the base of the food web, in addition to its accumulation and transfer to higher trophic levels via dietary pathways, can result in adverse reproductive and consequently developmental effects in oviparous vertebrates such as birds, fishes and possibly amphibians (Luoma and Presser, 2009; Janz et al., 2010). Although Se is an essential trace element in all animals, it presents a significant toxicological hazard due to the narrow range between essentiality and toxicity, particularly in early life stages of fishes and birds (Luoma and Presser, 2009; Janz et al., 2010).

Selenium exerts its most toxic effects through transfer from the diet of high trophic level oviparous adult females to their oocytes; thus, the primary exposure route of developing embryos occurs through maternal transfer rather through direct aqueous exposure (Stewart et al., 2010).

Vitellogenin, the yolk precursor protein, is synthesized and exported from the liver, incorporated into developing ovarian follicles, and subsequently enzymatically cleaved into the primary yolk proteins lipovitellin and phosvitin (Janz et al., 2010). These yolk proteins typically contain sulfur due to methionine (Met) residues, however unregulated, dose-dependent substitution of SeMet for Met occurs with elevated maternal dietary intake (Janz et al., 2010; Janz, 2012). Greater quantities of SeMet present in the yolk sac/albumin of eggs is hypothesized to initiate a series of biochemical events that lead to early life stage deformities or mortalities that appear in larval fishes and/or embryonic birds inhabiting Se-contaminated aquatic ecosystems. During yolk sac/albumin resorption by developing embryos, SeMet is catabolized into a variety of metabolites (e.g., methylselenol and dimethylselenide) that have been reported to cause generation of reactive oxygen species that damage cellular components such as DNA, proteins and lipids during this extremely sensitive stage of development (Palace et al., 2004; Janz et al., 2010).

The teratogenic effects of Se in oviparous vertebrates have been mainly studied in fish and bird species, with excess dietary Se in adult females causing a greater incidence of morphological abnormalities and embryonic mortality in their progeny, respectively (Janz et al., 2010). The allocation of Se to either the yolk sac for fishes or to the albumin for birds influences the most sensitive toxicity endpoints observed in these two vertebrate taxa (Janz et al., 2010). Craniofacial, vertebral, and fin abnormalities, in addition to other toxicities such as edema, are characteristic abnormalities that are observed in larval fishes collected from Se-contaminated sites upon yolk sac absorption following hatching (Holm et al., 2005; Muscatello et al., 2006; Janz et al., 2010). Comparatively, characteristic embryonic deformities produced by Se in birds that may contribute to mortality before hatching are malformed bills, reduction/absence of eyes,

and limb malformations (Janz et al., 2010). Importantly, data pertaining to the effects of Se in developing amphibians are currently lacking. The majority of studies to date have focused on effects of contaminant mixtures (i.e. coal combustion residues) on amphibian reproduction and development, of which Se was only one component (Hopkins et al., 2006; Janz et al., 2010; Metts et al., 2013). Other toxic contaminants (e.g., arsenic, cadmium, mercury) found within these mixtures may have also influenced larval morphological abnormalities in addition to Se. Thus, there exists uncertainty on whether Se is the major contributor to an assortment of toxicological effects observed in larval stage amphibians exposed to coal combustion residues in field and laboratory studies. These effects include reduced hatching and viability, increased frequency of deformities, reduced growth and survival, elevated energetic maintenance costs, altered predator avoidance capabilities, and altered time to metamorphosis (Rowe et al., 1996; Raimondo et al., 1998; Hopkins et al., 2000; Snodgrass et al., 2003, 2004; Hopkins et al., 2006; Metts et al., 2013). With this valuable knowledge base, it becomes imperative to characterize developmental Se toxicity in amphibians exclusively, without the influence of other toxicants, to establish a reference point for comparison. The purpose of this study was to investigate the potential adverse effects of chronic exposure to elevated concentrations of dietary SeMet by means of *in ovo* maternal transfer on the early development of *Xenopus laevis* progeny in order to gain a greater understanding of the sensitivity of amphibians to Se toxicity.

Recently, the United States Environmental Protection Agency (US EPA) proposed a tissue-based protective criterion for Se in aquatic organisms, assuming that fishes are the most sensitive taxa (US EPA, 2015). However no amphibian data were included in the draft US EPA criterion, and it is unknown whether the criterion will be protective to amphibians in Se-contaminated environments. To assist in filling this knowledge gap, reproductively mature *X.*

laevis females were fed diets augmented with SeMet at concentrations based on environmental relevance as well as published literature from field and laboratory research (Hopkins et al., 2006; Muscatello et al., 2006; Thomas and Janz, 2011; Thomas and Janz, 2014). Upon completion of the 68-day chronic exposure period, the females were then bred with untreated males to determine fertilization success, hatching success, embryo mortality, and the frequency and severity of morphological abnormalities within the first five days of embryo/larval development. The results were analyzed in relation to the concentrations of Se present in subsamples of eggs taken from each female to aid in the characterization of early life stage toxicities and to obtain egg Se-based toxicity thresholds for amphibians that can be potentially utilized in establishing a regulatory criterion for their protection at Se-contaminated sites.

2.4 Materials and methods

2.4.1 Test species and laboratory conditions

Sexually mature adult *Xenopus laevis* breeding pairs were purchased from Xenopus 1 (Dexter, MI, USA) and housed at 17±1°C water temperature and a 12h light:12h dark photoperiod in the Aquatic Toxicology Research Facility (ATRF) located at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK. Adult frogs were fed Nasco™ frog brittle (Newmarket, ON, Canada) *ad libitum* daily. Males and females were housed separately in large Min-O-Cool™ aquaria (84" L X 24" W X 22" D) with a 12" water depth and flow-through conditions (0.75-0.9 L/min). Frogs were acclimated for two months under these laboratory conditions prior to commencing the experiment.

2.4.2 Diet preparation

Seleno-L-methionine (≥ 98% purity; Sigma Aldrich, Oakville, ON, Canada) was dissolved in a fixed volume of deionized water and thoroughly mixed with a predetermined quantity of finely ground *Nasco™ frog brittle* to create the nominal dietary concentrations of 10, 30, and 90 µg Se/g dry mass (d.m.). The control diet was comprised of equivalent quantities of deionized water and ground brittle without the addition of SeMet. The resultant paste for each diet was processed in a meat grinder to form long cylindrical strands, which after freezing at -20°C were broken into small pieces. Diets were stored in airtight containers at -20°C and n=6 representative samples of each diet were taken prior to and during the feeding trial for total Se analysis.

2.4.3 Experimental design

Prior to commencing dietary SeMet exposures, each adult female (n=40) was bred with an individual male in order to release mature (post-vitellogenic and preovulatory) ovarian follicles from the ovary. This initial breeding was performed to ensure that subsequent dietary SeMet exposures maximized maternal transfer of Se to ovarian follicles at early (pre-vitellogenic) stages of oogenesis. In preparation for breeding, the water temperature was slowly increased over one week until it reached 20°C and was subsequently maintained throughout the breeding trial. Human chorionic gonadotropin (hCG; Sigma Aldrich, Oakville, ON, Canada) dissolved in phosphate buffered saline was administered to both males and females to stimulate amplexus and induce spermiation and ovulation. An initial priming dose of 25 IU hCG was injected sub-dermally into the dorsal lymph sacs of all *X. laevis* prior to a second dose (500 IU hCG for females, 250 IU hCG for males) 24 hours later (Sive et al., 2000). Each randomly

selected breeding pair was placed in separate 20 L covered aquaria within a darkened area immediately after the second injection and left to breed overnight. The following day breeding pairs were removed from their aquaria and embryos were collected and discarded.

Eight recently bred *X. laevis* females were subsequently divided evenly into four partitioned sections of a large Min-O-Cool™ tank and maintained under the same laboratory conditions stated previously. Each pair of females was designated a section of the tank according to their treatment group with the control group being situated nearest to the in-flow and the highest dietary exposure group furthest. In total, 5 tanks were organized and managed utilizing the same design and methods, which equated to $n=10$ female *X. laevis* per treatment group. Adult females were fed daily with 2 g of either control or SeMet augmented diets for 68 days. Animals were allowed to feed for 4 hours and any excess food was then siphoned from the tank. Adult males were held under similar conditions and fed the control diet. Water quality was monitored daily for the duration of the exposure period (pH 7-8, total ammonia < 0.25 mg/L, dissolved O_2 > 80%).

Following dietary exposures, *X. laevis* females were immediately bred with untreated males using the same procedure described above, and embryos were collected. These experimental procedures were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol no. 20120070), and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.4.4 Embryo collection and incubation

On the morning following breeding, the gelatinous matrix surrounding fertilized egg masses was removed using a 2% L-cysteine solution (Sigma Aldrich, Oakville, ON, Canada).

Embryos were subsequently rinsed using a Modified Barth's Saline solution and placed into petri dishes containing this same solution for sorting (Sive et al., 2000). A random subsample of embryos (n=100) was collected from each female and stored at -80°C for determination of total Se concentrations. A second random subsample of embryos (n=100) was preserved in 10% phosphate buffered formalin for 48 h and subsequently stored in 70% ethanol to assess fertilization success. The third subsample involved selecting embryos (n=500) from individual females, placing them in 150 mL embryo cups (50 embryos/cup) immersed in facility water contained within a large Min-O-Cool™ tank and incubating them for 5 days at 22±1°C. The number of mortalities and hatchings were recorded daily over the 5-day incubation period.

2.4.5 Quantification of selenium in maternal diet and eggs

Samples of experimental diets (n=6 per diet) were lyophilized using a freeze dryer (Dura-Dry™ MP, FTS systems, Stone Ridge, NY, USA) and 100 mg samples were cold digested in Teflon vials using 5 mL of ultrapure nitric acid and 1.5 mL hydrogen peroxide. Once digested, samples were concentrated on a hot plate (<75°C), reconstituted in 5 mL of 2% ultrapure nitric acid, and stored at 4°C until inductively coupled plasma-mass spectrometry (ICP-MS) analysis was performed to determine total Se concentrations. Pooled embryo samples from individual females underwent the same process except the mass was equivalent to the subsample of 100 embryos collected and freeze dried. A certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada) was used to determine Se recovery. The ICP-MS limit of quantification was ≤ 0.87 ng Se/g for all samples.

2.4.6 Assessment of fertilization, embryo mortality and hatchability

A random subsample of embryos (n=100) collected from each female was used to determine fertilization success. These vials were non-sequentially labeled and examined in a blind fashion using an Olympus model S261 dissecting microscope. The number of eggs fertilized were counted and divided by the total to obtain a percentage. Percent embryo hatching success and mortality was determined by dividing the number of successfully hatched or dead embryos, respectively, from each female (total n=500 embryos per female).

2.4.7 Evaluation of morphological abnormalities in tadpoles

At 5 dpf, a random subsample (n=200) of incubating tadpoles from each female were euthanized with buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate; 700 mg/L), preserved in 10% phosphate buffered formalin for 48 h and stored in 25 mL glass vials (100 tapdoles/vial) containing 70% ethanol. These vials were non-sequentially labeled and examined in a blind fashion using an Olympus model S261 dissecting microscope with Image-Pro Discovery Software. The *Xenopus laevis* Atlas of Abnormalities (Bantle et al., 1991) was used as a reference during the process of characterizing abnormalities and defining the parameters associated with the degree of severity. Representative images of these characteristic abnormalities are included in the appendix of this thesis (Figures C2.S1-C2.S3).

The frequency and severity of early life stage abnormalities were evaluated pertaining to edema as well as craniofacial, vertebral, ocular lens, gut, and tail fin. The frequency was calculated as either the number presenting as malformed, or the number presenting as normal depending on the statistical analysis required, divided by the total observed from that particular female (n=200) to gain a percentage. The severity for each type of abnormality (excluding those

related to the eye lens) observed in an individual tadpole was assigned a numerical value or ranking based on its degree of severity (i.e., 0 = normal, 1 = mild, 2 = moderate, 3 = severe) which then provided the basis for calculating the Graduated Severity Index (GSI) (Formation Environmental, 2012). The number of tadpoles for each severity ranking (excluding those given a score of "0") for a particular abnormality were counted, multiplied by their specific ranking score and summed to obtain a total score. The total scores for each abnormality were summed to create a final score that was divided by the number of categories of abnormalities (of which there were five) then further divided by the total number of tadpoles observed (including those ranked normal or "0") to provide a mean GSI score for the progeny related to individual females (Formation Environmental, 2012).

2.4.8 Statistical analyses

All data were tested for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene Median tests, respectively. If necessary, data were $\log_{10}$ transformed before performing parametric statistical analysis (SigmaPlot 11.0, Systat Software, San Jose, CA, USA). One Way Analysis of Variance (ANOVA) followed by a Holm-Sidak test compared treatment groups to the control group for significant differences. If data did not meet parametric assumptions after transformation, Kruskal-Wallis ANOVA on Ranks followed by Dunn's test was used for statistical comparisons. Data that passed parametric assumptions were presented as mean ± S.E.M., and those that did not were presented as a box indicating the median, 25^{th} and 75^{th} percentiles, whiskers as 10^{th} and 90^{th} percentiles, and black dots as outliers. An alpha value of 0.05 was designated for both parametric and non-parametric ANOVA tests.

The untransformed raw data pertaining to the frequency of both individual and total abnormalities in relation to egg Se concentrations were evaluated using triangular distribution of the maximum likelihood tolerance distribution analysis model to estimate EC_{10} values using the USEPA's Toxicity Relationship Analysis Program (TRAP; version 1.3). If EC_{10} values could not be estimated using TRAP due to inadequate partial effects in the dataset, ToxStat™ (version 3.5; Western Ecosystems Technology, Cheyenne, WY, USA) was used.

2.5 Results

2.5.1 Selenium concentrations in maternal diet and embryos

The Se concentrations measured in embryos collected from each female increased in proportion to concentrations of Se in the maternal diet. The measured concentrations of Se in diets administered to the adult female *X. laevis* over the exposure period were analogous to the nominal concentrations (control, 10, 30 and 90 µg Se/g d.m.), at 0.7 ± 0.01, 10.9 ± 0.20, 30.4 ± 0.43 and 94.2 ± 3.10 µg Se/g d.m. (Table 2.1). Concentrations of total Se in embryos collected from adult females fed the 0.7, 10.9, 30.4 and 94.2 µg Se/g d.m. diets were 1.6 ± 0.07, 10.8 ± 0.68, 28.1 ± 2.91 and 81.7 ± 3.40 µg Se/g d.m., respectively (Table 2.1). A significantly greater accumulation of Se was observed in embryos collected from females exposed to diets augmented with SeMet when compared to those fed the control diet ($p < 0.001$; Table 2.1). The mean concentration of Se quantified in the embryos collected from females assigned the control diet had a 2:1 embryo to diet ratio, while the concentrations in embryos collected from females administered the SeMet augmented diets were observed to have approximately a 1:1 embryo to diet ratio.

Table 2.1: Total selenium (Se) concentrations in diets and *Xenopus laevis* eggs, and fertilization success, hatching success and mortality observed in embryos exposed *in ovo* through maternal transfer of elevated dietary Se concentrations over a 68-day exposure period.

Nominal Se Concentration in Maternal Diet (µg Se/g d.m.[a])	**Measured Se Concentration in Maternal Diet (µg Se/g d.m., *n*=6)**	**Measured Se Concentration in subsamples of 100 eggs (µg Se/g d.m., *n*=9-10)**	**Fertilization Success (%)**	**Hatching Success (%)**	**Mortality within 5 dpf (%)**
Control	0.7 ± 0.01	1.6 ± 0.07	96.0 (91.7, 98.3)	98.8 (98.5, 99.5)	1.4 (0.5, 1.9)
10	10.9 ± 0.20	10.8 ± 0.68***	98.0 (73.8, 99.3)	99.0 (97.0, 99.3)	1.0 (0.7, 3.6)
30	30.4 ± 0.43	28.1 ± 2.91***	89.0 (84.0, 93.0)	96.9* (95.2, 98.0)	3.9* (2.8, 4.8)
90	94.2 ± 3.10	81.7 ± 3.40***	94.0 (92.0, 98.0)	99.0 (96.0, 99.4)	1.1 (0.6, 4.4)

[a], dry mass

[b], days post-fertilization

Data are presented as mean ± SEM, or median (25th percentile, 75th percentile), of *n*=9-10 females if not stated otherwise.

*, Significant difference ($p< 0.05$) in comparison to control using Kruskal-Wallis ANOVA on Ranks followed by Dunn's test.

***, Significant difference ($p< 0.001$) in comparison to control using a one way ANOVA followed by Holm-Sidak test.

2.5.2 Fertilization success, embryo mortality and hatchability

Selenium exposure had minimal to no significant effect on fertilization success, hatching success or embryo/larval mortality within the first 5 dpf. There were no significant differences among treatment groups for fertilization success, with median values of 96.0%, 98.0%, 89.0% and 94.0%, respectively (Table 2.1). The median embryo/larval mortality within the first 5 dpf ranged from 1.0 to 3.9%, and was significantly greater in the 28.1 µg Se/g egg d.m. group when compared to the control ($p < 0.05$; Table 2.1). Hatching of embryos was completed by 3 dpf, and hatching success ranged from 96.9 to 99.0% among treatment groups, which was significantly lesser in the 28.1 µg Se/g egg d.m. group when compared to the control group ($p < 0.05$; Table 2.1).

2.5.3 Frequency and severity of morphological abnormalities

In ovo Se exposure had a significant impact on the frequency of abnormalities. The frequency of total abnormalities (tadpoles exhibiting at least one abnormality) associated with 1.6 (control), 10.8, 28.1 and 81.7 µg Se /g egg d.m. were 18.4 ± 1.7%, 13.1 ± 1.9%, 19.5 ± 2.5% and 80.7 ± 7.6%, respectively (Fig. 2.1A). A significant increase in the percentage of total abnormalities was observed in the highest dose group containing 81.7 µg Se/g egg d.m. when compared to control ($p < 0.001$; Fig. 2.1A).

The presence of specific abnormalities typically reflected the results observed for total abnormalities. With the exception of tail fin abnormalities, the highest dose group of 81.7 µg Se/g egg d.m. had significant increases in the incidence of characteristic Se-induced deformities (abnormal craniofacial, spinal, gut, eye lens structure, and edema) when compared to the control ($p < 0.001$; Figs. C2.S4-C2.S8). Eye lens abnormalities were discovered to be the most sensitive

indicator of *in ovo* Se exposure in 5 dpf tadpoles when compared to the other morphological categories evaluated. The median frequencies of eye lens abnormalities were 1.5%, 1.5%, 2.5% and 74.3% for the 1.6 (control), 10.8, 28.1 and 81.7 µg Se/g egg d.m. treatment groups, respectively, which was significant at the highest dose ($p < 0.001$; Fig. 2.2).

The Graduated Severity Index (GSI), representing the cumulative degree of severity for all abnormalities, demonstrated that there was a significant increase in the combined severity of edema as well as vertebral, craniofacial, gut, and tail fin malformations only in the highest dose group of 81.7 µg Se/g egg d.m. when compared to the control ($p < 0.001$, Fig. 2.1B). The mean GSI scores were 0.12 ± 0.03, 0.06 ± 0.01, 0.10 ± 0.02 and 0.74 ± 0.2 for the 1.6, 10.8, 28.1 and 81.7 µg Se/g egg d.m. treatment groups, respectively. The proportions of tadpoles without any abnormalities, or specifically without eye lens abnormalities, are presented in Figs. 2.3A and 2.3B, respectively, in relation to egg Se concentrations for individual females. At the lowest range of Se concentrations (1.2 to 1.9 µg Se/g egg d.m.), the proportion of tadpoles without any abnormalities ranged from 74.5 to 89.1%, while at the same egg Se concentrations 97.5 to 100% were free of lens abnormalities. The y-intercepts (with 95% confidence limits) for these modeled curves were calculated to be 0.81 (0.82, 0.84) and 0.95 (0.98, 0.99) for Figs. 2.3A and 2.3B, respectively. The egg Se concentrations associated with females from the highest dose group ranged from 65.6 to 97.5 µg Se/g egg d.m. and exhibited a broad effects range of 0 to 80.5% for total abnormalities and 2.0 to 92.5% for eye lens abnormalities (Figs. 2.3A and 2.3B). The estimated EC_{10} values (including 95% confidence limits) based on these data were 44.9 (41.5, 48.2) and 43.4 (41.4, 45.4) µg Se/g egg d.m. for total abnormalities and eye lens abnormalities, respectively. A comparison of estimated EC_{10} values for each specific type of abnormality

revealed that those related to the eye lens were the most sensitive teratogenic indication of *in ovo* Se toxicity (Table C2.S1).

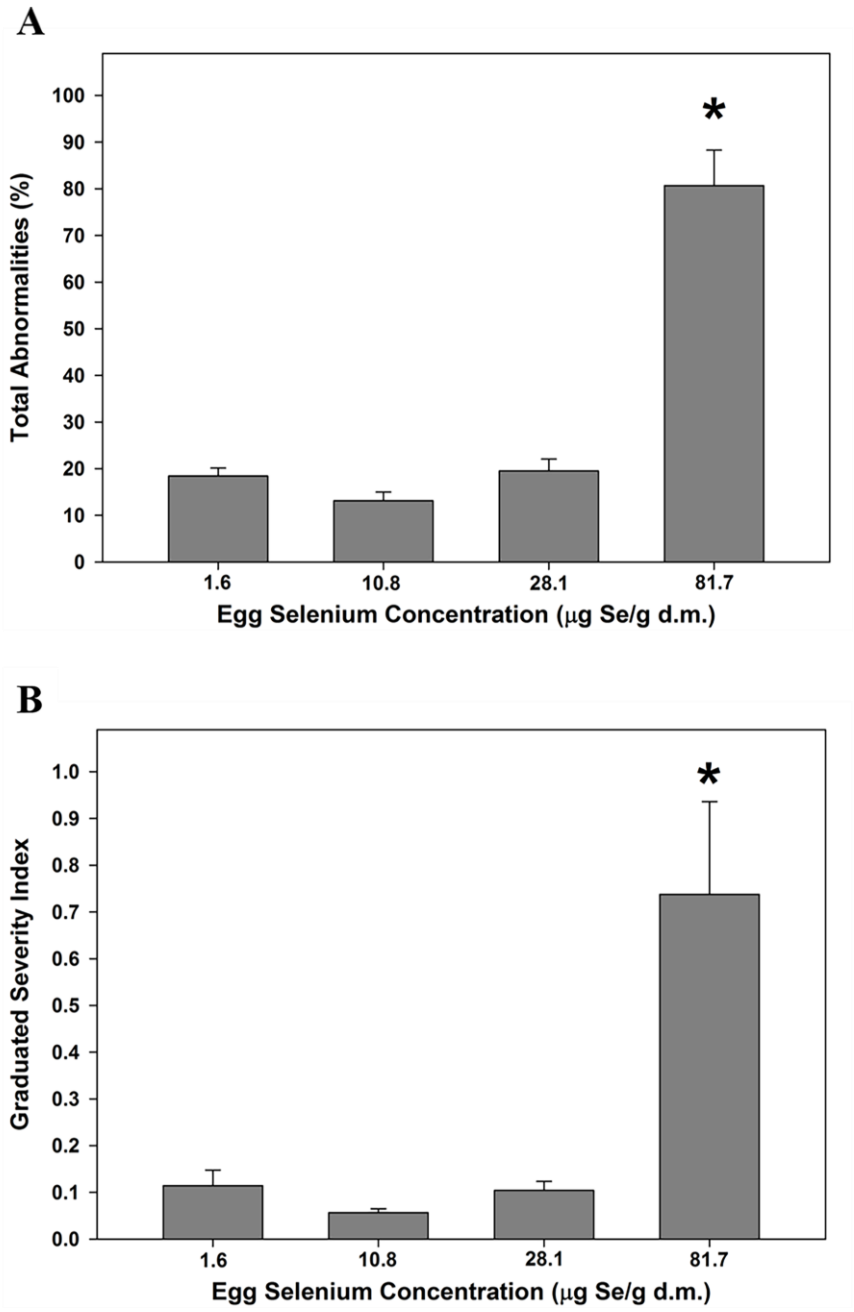

Figure 2.1: Frequency (A) and severity (B) of total morphological abnormalities (sum of abnormal craniofacial, vertebral, gut, fin, and lens structures, and edema) in 5 days post-fertilization (dpf) *Xenopus laevis* tadpoles exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer. *, Significant difference from control group using one way ANOVA followed by Holm-Sidak test ($p < 0.001$; n=9-10 females).

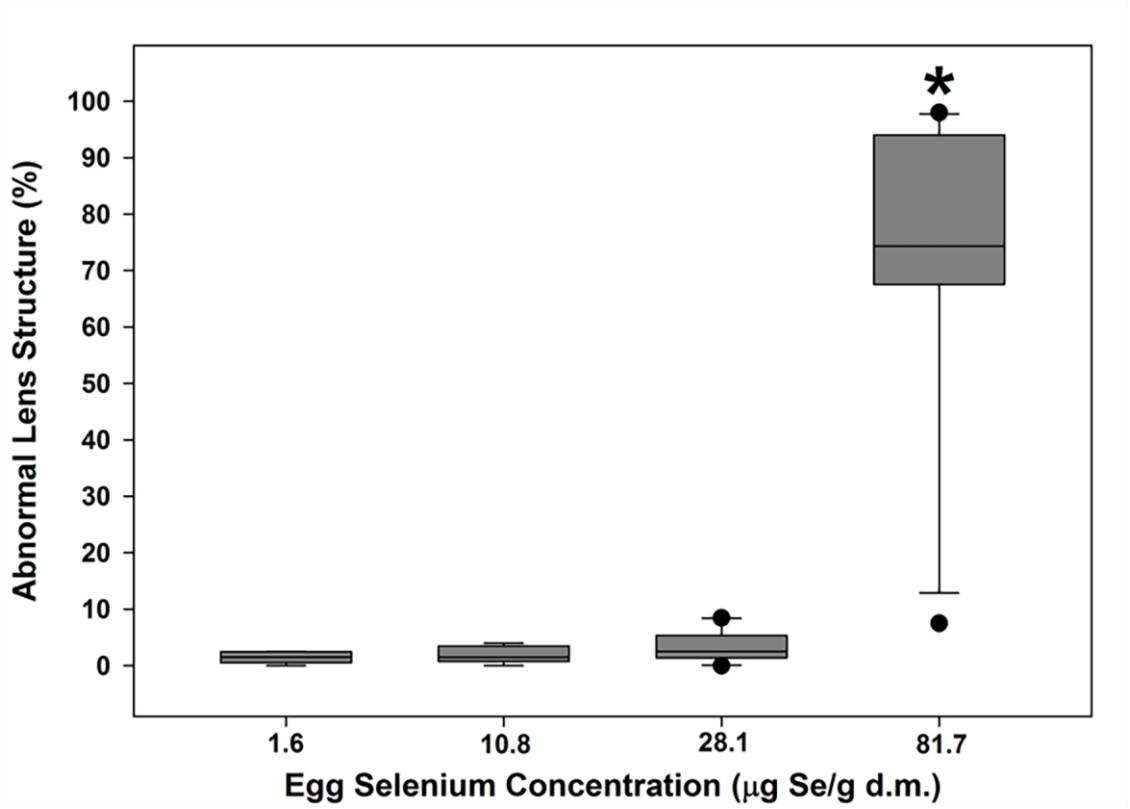

Figure 2.2: Frequency of lens abnormalities detected in 5 days post-fertilization (dpf) *Xenopus laevis* tadpoles exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer. *, Significant difference from control group using Kruskal-Wallis one way ANOVA on Ranks followed by Dunn's test ($p < 0.001$; n=9-10 females).

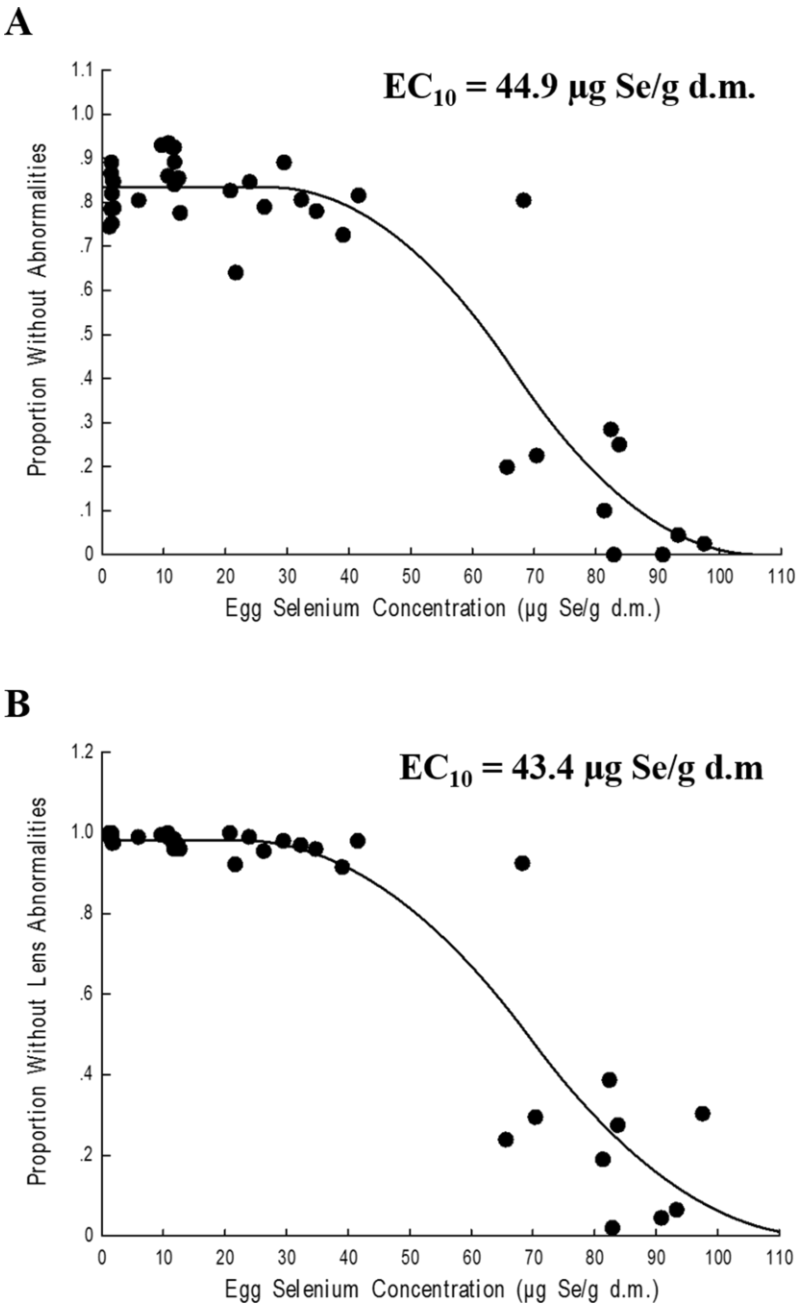

Figure 2.3: Relationships between the proportion of morphologically normal 5 days post-fertilization (dpf) *Xenopus laevis* tadpoles and selenium concentration (µg Se/g egg dry mass [d.m.]) in embryos collected after a 68-day maternal dietary exposure to 0.7, 10.9, 30.4, or 94.2 µg Se/g food d.m.. *X. laevis* tadpoles without: (A) any abnormalities and (B) exclusively lens abnormalities from each female. EC_{10} values were estimated using USEPA's Toxicity Relationship Analysis Program.

2.6 Discussion

2.6.1 Dietary Se requirements, rate of maternal transfer and environmental exposure

Optimal dietary Se requirements for physiological homeostasis have not been established for amphibians in general, or specifically for *X. laevis*, although preliminary recommendations for the dietary requirements for adult amphibians have been suggested to be 0.3 µg Se/g d.m (Ferrie et al., 2014). The nutritional requirements for Se have been experimentally determined to range between 0.1 and 0.5 µg Se/g d.m. for fish and between 0.3 to 1.1 µg Se/g d.m. in aquatic birds (NRC, 1993; Watanabe et al., 1997; Lin et al., 2005; Janz et al., 2010; Stewart et al., 2010). In the current study, the control diet of 0.7 µg Se/g d.m. administered to adult *X. laevis* females produced no observable negative effects on their progeny for any of the early life stage developmental endpoints assessed including fertilization success, mortality, hatching success, and malformations. This indicates dietary Se requirements essential for optimal health outlined previously for other oviparous species are sufficient for amphibians as well in this regard.

The concentrations of Se quantified in eggs produced by *X. laevis* females increased proportionally with the levels present in their diet. A greater transfer of Se from the maternal diet to eggs occurred in the control group (0.7 µg Se/g food d.m.), which suggests that under conditions of adequate dietary Se female *X. laevis* transfer a greater proportion of ingested selenium to their developing oocytes. However, at dietary concentrations equal to or greater than 10.9 µg Se/g d.m. the transfer from maternal diet to oocytes appeared to reach a steady state. Similar trends for the rate of Se transfer between chronic maternal dietary exposure and measured egg Se concentrations have been observed in laboratory studies in fish. For example, adult female zebrafish (*Danio rerio*) fed diets containing 1.3, 3.7, 9.6 or 26.6 µg Se/g d.m.

exhibited a greater rate of Se transfer to their eggs when dietary concentrations were below 9.6 µg Se/g d.m.; comparably, the concentrations of Se in the eggs reached a steady state equivalent to maternal dietary concentrations equal to or above 9.6 µg Se/g d.m. (Thomas and Janz, 2014).

Dietary exposure is the most important pathway for Se accumulation in aquatic vertebrates, with more than 90% of Se body burden derived from diet (Stewart et al., 2010). Hence it becomes critical to generate dose-response data relating dietary and tissue Se concentrations to effects magnitude in order to accurately predict degree of exposure and the associated risk to populations inhabiting Se impacted sites. Presser and Luoma (2010) established that accurate predictions of body burdens in both freshwater and marine fishes could be made based on dietary intake alone, with a 1:1 relationship occurring between the two; these data appear to coincide with the results of the current study as well as those discussed previously (Presser and Luoma, 2010; Stewart et al., 2010). Thus, knowledge of the proportional concentrations of Se in the maternal diet and corresponding eggs quantified in the current laboratory study for amphibians may potentially be utilized to not only assess the risk of adverse reproductive and population effects, but also to determine the actual Se exposures of females at contaminated sites when foraging behaviours and movement patterns are unclear. For example, Hopkins et al. (2006) determined that female eastern narrow-mouth toads (*Gastrophryne carolinensis*) collected at Savannah River, South Carolina, USA near the site of a coal-burning power plant transferred up to 100 µg Se/g d.m. to their eggs when compared to toads at a reference site, thus indicating a high degree of Se contamination leading to excess dietary consumption in predators and increased likelihood of detrimental reproductive effects even though measured concentrations of Se in the exposure site water were only 3.93 µg Se/L.

2.6.2 Fertilization success, embryo mortality and hatchability

In ovo Se exposure via maternal transfer in *X. laevis* had no biologically significant effects on fertilization success, embryo mortality or hatchability. Although a statistically significant difference was detected for endpoints related to embryo mortality and hatchability in the 28.1 µg Se/g egg d.m. group when compared to the control group, the difference of less than 4% would likely have minimal impact in the context of large clutch sizes. Thus, the resultant absence of substantial effects for these three reproductive endpoints is not uncommon when compared to studies involving fishes (Kennedy et al., 2000; Holm et al., 2005; Muscatello et al., 2006; Hardy et al., 2010). The previously mentioned study by Hopkins et al. (2006) reported that eastern narrow-mouth toads collected near a coal-burning power plant had an 11% reduction in hatching success when compared to toads at the reference site. In the current study, adult females accumulated up to 97.5 µg Se/g egg d.m. with no effect on fertilization success, embryo mortality or hatchability indicating that species variability amongst fishes and amphibians, as well as contaminant mixture effects, may be confounding factors that contribute to negative effects for these endpoints in other studies. Comparatively, *X. laevis* appears to have a similar tolerance to Se toxicity as fishes with respect to fertilization success, embryo mortality, and hatchability.

2.6.3 Selenium-induced anatomical abnormalities in *X. laevis*

An extensive examination of the anatomical features of 5 dpf *X. laevis* tadpoles revealed differences in the frequency of particular types of abnormalities when compared to those reported in fishes. The ocular lens, craniofacial, and gut abnormalities were the most prevalent in the highest dose group, with far fewer incidences of tail fin and spinal abnormalities, and edema,

overall. In contrast to these findings, greater incidences of edema and skeletal curvatures are commonly reported in studies focusing on fishes (Lemly, 1993b; Holm et al., 2005; Muscatello et al., 2006; Janz et al., 2010). In addition, analysis of anatomical alterations pertaining to the ocular lens and gut during early developmental stages has not been performed previously in either fishes or amphibians, offering novel information in relation to Se toxicity.

A special consideration should be made in relation to the malformations detected in both the ocular lens and craniofacial regions, and the unique set of characteristics they displayed in the current study. Craniofacial abnormalities appeared predominantly as microphthalmia of one or both eyes, extension of retinal tissue into the optic stalk, microcephaly, asymmetrical head/mouth structures, and excessively sloped/rounded heads. These abnormal craniofacial features, in combination with those observed in the eye lens, suggest that the toxic effects of embryonic Se exposure may interfere with function of transcription factors and related gene expression critical to proper development prior to absorption of the yolk. A description of the distribution of essential metals (i.e., iron, copper, zinc, and selenium) in the *X. laevis* oocyte revealed that Se is distributed moderately throughout the animal pole, vegetal pole, equatorial cytosol and nucleus (Popescu et al., 2007). With greater levels of Se transferred via the maternal diet to the oocyte, it can be inferred that increased amounts of SeMet could potentially accumulate throughout the cell, including the nucleus, causing damage through oxidative stress. One example of a vulnerable and vital transcription factor/gene region is paired box protein-6 (*Pax6*). It becomes activated in the late gastrula stage in the presumptive anterior neural plate and plays a crucial role in the development of the eye, brain, spinal cord, pancreas and intestinal enteroendocrine cells (Wride, 1996; Altmann et al., 1997; Yan et al., 2006; Nakayama et al., 2015). In addition, studies have demonstrated that alterations in the expression of *Pax6* results in

similar ocular and craniofacial malformations in humans, mice and *Xenopus* sp. (Wride, 1996; Altmann et al., 1997; Yan et al., 2006; Nakayama et al., 2015) as those detected in the 5 dpf *X. laevis* tadpoles of the current study. Further research is clearly required to investigate mechanisms of ocular and craniofacial deformities in oviparous vertebrates exposed to elevated Se.

The eye lens was a distinct anatomical feature that was observed to be the most sensitive teratogenic effect following *in ovo* Se exposure in *X. laevis* tadpoles at 5 dpf when compared to the suite of anatomical features investigated. Aquatic vertebrates, such as fishes and larval amphibians, rely principally on the lens rather than the cornea to refract light onto the retina (Schwab, 2007). The ocular lens of amphibians is a large, virtually spherical, transparent structure with an exceptionally high refractive index when compared to other vertebrates. In order for the lens to both effectively refract light and accurately focus in its entirety, it is positioned far forward in the ocular globe, and bulges through the pupil to dwell close to the cornea (Robinson and Lovicu, 2004). In the current study, alterations to the eye lens predominantly appeared as a severe reduction of its spherical form to the extent that it was extensively recessed behind the opening of the pupil. Other malformations, although less common in comparison, were cataract-like milky opaqueness of the lens, or excessive bulging (ectopy) of the lens from the ocular globe. A recent study using confocal x-ray fluorescence imaging reported that zebrafish larvae exposed *in ovo* to SeMet via maternal transfer preferentially accumulated high concentrations of Se in the lens core and moderate levels in the epithelium (Choudbury et al., 2015). While no structural changes to the ocular lens were reported (Choudbury et al., 2015), it is possible that the exposure concentration (i.e., 34.1 μg Se/g egg d.m.) utilized was insufficient to produce the types of lens abnormalities observed in the current

study. Comparatively, a deformity analysis performed on fishes collected from Belews Lake, North Carolina, USA reported the manifestation of cataracts at tissue concentrations in the range of 80 to 132 μg Se/g d.m., although ocular abnormalities were not the most prevalent teratogenic response in these fish populations (Lemly, 1993b). While the connection between Se exposure and cataractogenesis has been identified previously in fishes (Woock et al., 1987; Lemly, 1993b, 2002; Choudbury et al., 2015), the prominent structural changes to the eye lens observed in 5 dpf *X. laevis* tadpoles in the current study have not previously been reported.

In early stages of development, the eye lens is molded into its unique structure through the processes of cell proliferation, cell migration, cell differentiation and apoptosis (Yan et al., 2006). Through the generation of oxidative stress, SeMet has the capability to interfere with all of these stages of lens development, thus dramatically affecting its final structure and functional competency. For example, the presence of elevated levels of Se in 12-14 day old Sprague-Dawley rats reduced DNA synthesis, prolonged cell migration time, and diminished cell differentiation in germinative lens epithelial cells during S or pre-S phase (Cenedella, 1989). With the greatest concentrations of Se present in the lens core (Choudbury et al., 2015), it is also possible that lens proteins located within fiber cells, known as crystallins, are altered or damaged. Lens crystallins constitute 90% of the soluble protein in lens fiber cells, and are vital to maintaining transparency while achieving a high refractive index for normal optical functions (Mostafapour and Reddy, 1978). In addition, crystallin proteins are known to contain high amounts of S-containing methionine, making it probable that substitution with SeMet would occur under conditions of elevated Se exposure. The potential to accumulate high levels of SeMet makes fiber cells vulnerable to damage through metabolic processes that could alter the organization and structure of crystallin proteins, or diminish the functioning of other vital

cellular components. Structural differences and necrosis of a large number of fiber cells could potentially lead to abnormal shape and inflexibility of the ocular lens following elevated Se exposure. Moreover, the scarcity of cataracts detected in the current study may be due to factors related to amphibian biology, Se concentration or insufficient time for their development. Nonetheless, if the amphibian eye lens is a sensitive target of elevated Se exposure during development, as observed in the current study, this could have significant ecological consequences in native amphibians inhabiting Se-contaminated aquatic ecosystems.

2.6.4 Implications of environmental Se for amphibian populations

Embryonic Se exposure via chronic maternal dietary exposure has been established as a distinctive exposure route that elicits the formation of morphological abnormalities in fishes, which have resulted in population collapse at Se-contaminated sites in the past. The data presented in the current study offers the foundation for characterizing Se toxicity in amphibians through this key route of exposure. Both frequency and severity of developmental malformations increased in 5 dpf *X. laevis* tadpoles with rising Se concentrations in eggs, allowing for an EC_{10} value of 44.9 µg Se/g egg d.m. to be calculated. Teratogenic effects in *X. laevis* occurred at greater egg Se concentrations than those observed in most fishes. Toxicity threshold values are predominantly between 15 to 25 µg Se/g egg/ovary d.m. in fishes, with the highest EC_{10} value of 54 µg Se/g egg d.m. estimated in Dolly Varden trout (*Salvelinus malma*) (Janz et al., 2010; DeForest et al., 2012). Therefore, *X. laevis* are as sensitive to *in ovo* Se exposure via maternal transfer as the most tolerant fish species studied to date. This suggests that the US EPA's proposed egg-based tissue threshold for fish of 15.8 µg Se/g egg d.m. may be protective for amphibians as well (US EPA, 2015). However, a cautionary approach is necessary with the

utilization of these results in that *X. laevis* is a unique anuran model that may not adequately represent the most sensitive of amphibian species, particularly native North American amphibians, nor the wide array of reproductive strategies employed by this taxon.

CHAPTER 3

EFFECTS OF ELEVATED *IN OVO* SELENIUM EXPOSURE ON LATE STAGE DEVELOPMENT OF *XENOPUS LAEVIS* TADPOLES

3.1 Preface

This investigation was performed as one aspect of a large generational bioassay that comprises this thesis. It was undertaken with the purpose to determine the prolonged effects of elevated *in ovo* selenium exposure on amphibian late stage larval development. Adult *Xenopus laevis* females were administered elevated concentrations of dietary selenium in the form of SeMet for 68 days after which they were bred with untreated males. The resultant embryos collected from this process were incubated in eggcups until 5 days post fertilization (dpf) then transferred to aquaria to be raised to metamorphosis under uncontaminated laboratory conditions. Mortality, morphometrics, time to metamorphosis as well as the distribution of developmental stages were recorded during the experimental process. The results from this study indicate elevated *in ovo* selenium exposure exclusively has minimal adverse effects on late stage development of *X. laevis* tadpoles in the absence of teratogenic abnormalities induced prior to 5 dpf; however, indications of its potential to prolong time to metamorphosis of individuals at high concentrations were observed.

This chapter has been submitted to *Bulletin of Environmental Contamination and Toxicology* under joint authorship with Jorgelina R. Muscatello (Stantec Consulting Ltd.) and David M. Janz (University of Saskatchewan).

3.2 Abstract

Selenium (Se) is known to produce teratogenicity in aquatic oviparous vertebrates; however, the subtle residual effects associated with elevated *in ovo* Se exposure on later stages of development have not been sufficiently investigated, especially with respect to amphibians. The objective of this study was to determine the consequences of elevated *in ovo* Se exposure on the survival, growth and maturation rate of late stage larval anuran, as represented by *Xenopus laevis*. Adult *X. laevis* females (n=9-10) were fed diets augmented with L-selenomethionine for 68 days and successively bred with untreated males for the purpose of obtaining embryos. At 5 days post fertilization (dpf), a subsample of tadpoles were reared under uncontaminated conditions until 50% of individuals within a tank had completed metamorphosis, at which point euthanization of the entire tank occurred. Subsequently, froglet morphometrics along with developmental stage were recorded for each tadpole/froglet collected. The measured Se concentrations in embryos were 1.6 (control), 10.8, 28.1 and 81.7 μg Se/g d.m. which corresponded with the maternal dietary concentrations of 0.7, 10.9, 30.4, or 94.2 μg Se/g dry mass (d.m.), respectively. There were no significant differences detected among the treatment groups in relation to mortality or time to metamorphosis during the rearing period. A significant increase in froglet body weight and snout to vent length upon completion of metamorphosis were observed in the 81.7 μg Se/g d.m. *in ovo* exposed group when compared to the 10.8 and 28.1 μg Se/g d.m. groups, but not in relation to the control group. Additionally, a significant increase in the proportion of tadpoles at earlier stages of larval development occurred in the 81.7 μg Se/g d.m. *in ovo* exposed group when compared to the other three treatment groups. Overall, this research suggests that *in ovo* Se exposure has the potential to effect growth, resilience and late stage larval development in the model amphibian, *X. laevis*.

3.3 Introduction

Selenium (Se) is a fundamental micronutrient necessary for optimal growth and development in vertebrates. A suite of 45 Se-containing proteins have been identified in vertebrates to date with functions ranging from redox homeostasis, thyroid hormone activation or deactivation, selenocysteine synthesis, and Se transport; however, the physiological roles of a substantial number of selenoproteins remains largely unknown (Lobanov et al., 2009; Janz, 2012; Mariotti et al., 2012). While adequate dietary intake of Se is required to maintain peak performance of all selenoproteins including antioxidant defense mechanisms (Janz et al., 2010; Stewart et al., 2010), elevated levels of Se have resulted in the generation of reactive oxygen species and subsequently oxidative stress (Palace et al., 2004; Spallholz et al., 2004). The cellular damage associated with this process is considered to be the predominant mechanism by which Se produces adverse effects in vertebrates, although uncertainties do remain and further investigation is vital to elucidate the biochemical behavior of Se under toxic conditions (Janz et al., 2010). Dietary Se levels only 7-30 times greater than nutritional requirements have been reported to produce toxic effects in fish (Janz et al., 2010; Stewart et al., 2010) yet there continues to be insufficient data pertaining to either the nutritional requirements of amphibians or their sensitivity to elevated levels of Se (Janz et al., 2010; Ferrie et al., 2014). Nonetheless, the narrow range of dietary Se concentrations that have shown to either promote or suppress vertebrate fitness particularly in aquatic oviparous species is one of the reasons that establishing environmental threshold levels that can be utilized to protect populations is of utmost importance.

Although Se is a naturally occurring element in the environment, anthropogenic activities including mining and power generation can mobilize and release large quantities into surrounding aquatic systems (Maher et al., 2010). This influx of inorganic Se, in the forms of either selenate or selenite, is readily absorbed from the water by organisms at the base of the food web (i.e. algae, microbes) and subsequently biotransformed into a variety of organoselenium species of which selenomethionine (SeMet) is of keen interest (Fan et al., 2002; Luoma and Presser, 2009). Unlike other forms of Se, the resemblance of SeMet to the amino acid methionine allows it to be incorporated into proteins in a dose-dependent manner that is physiologically unregulated (Janz et al., 2010; Stewart et al., 2010). Thus, SeMet has the potential for bioaccumulation within tissues and transference within the food web to higher trophic level organisms such as birds, fish and amphibians (Orr et al., 2006; Janz et al., 2014). Moreover, adult females belonging to aquatic oviparous vertebrate populations have demonstrated the capacity to integrate excess dietary SeMet into the yolk protein vitellogenin during its synthesis within the liver resulting in its subsequent transport to and accumulation within their developing oocytes (Janz et al., 2010).

Elevated *in ovo* Se concentrations are associated with the production of morphological abnormalities in larval fish and amphibians that coincide with the utilization of yolk proteins during early stages of development (Thomas and Janz, 2014; Massé et al., 2015). Craniofacial, vertebral and fin malformations along with the presence of edema have been predominantly reported; however, abnormalities related to the eyes and their lens structures have been observed as well (Lemly, 1993b; Massé et al., 2015). The egg and ovary based EC_{10} values related to teratogenic abnormalities for most fishes studied to date ranges from 15 to 25 μg Se/g dry mass (d.m.), while this same value for amphibians is based solely on one non-native laboratory

species, *Xenopus laevis*, at 44.9 µg Se/g d.m. (Janz et al., 2010; Massé et al., 2015; Thomas and Janz, 2015). With marginal knowledge related to Se toxicity in amphibians, existing data on native anuran species has been gathered predominantly from field research at deposition sites for coal combustion wastes where elevated levels of Se coincide with a greater incidence of larval morphological abnormalities as well as diminished swim performance, predator avoidance, food acquisition, growth, survival, developmental rates, and metamorphic success (Raimondo et al., 1998; Hopkins et al., 2000; Snodgrass et al., 2004; Metts et al., 2012; Metts et al., 2013). However, Se is merely one contaminant at these sites among many that have been recognized to either interfere with the bioavailability of Se or with the normal physiological functioning and development of aquatic vertebrates (i.e. arsenic, cadmium, mercury) (Rowe, 2014). Considering the impact that Se could have on the survival and recruitment of individual larval anuran into a population, it is critical to expand Se's toxicological profile with regards to amphibians with the goal of establishing a protective regulatory guideline for aquatic organisms that is comprehensive and species inclusive. Thus, the objective of this study was to gain a better understanding of the potential impact of elevated *in ovo* Se exposure on late stage larval anuran development exclusively under uncontaminated rearing conditions by assessing mortality, morphometry and developmental rates of *Xenopus laevis* tadpoles/froglets from 5 days post fertilization (dpf) until completion of metamorphosis.

3.4 Materials and methods

3.4.1 Test species

Adult *Xenopus laevis* females and males were purchased from Xenopus 1 (Dexter, MI, USA) and were maintained at 17±1°C water temperature and 12h light:12h dark photoperiod in

the Aquatic Toxicology Research Facility (ATRF) located at the Toxicology Centre, University of Saskatchewan prior to and during the experimental process. Adult *X. laevis* females (*n*=9-10) were administered food augmented with L-selenomethionine (≥ 98% purity; Sigma Aldrich, Oakville, ON, Canada) at measured concentrations of 0.7 ± 0.01 (control), 10.9 ± 0.20, 30.4 ± 0.43, and 94.2 ± 3.10 μg Se/g d.m. for 68 days. Upon completion of the dietary exposure period, females were bred with untreated males with the assistance of human chorionic gonadotropin (hCG; Sigma Aldrich, Oakville, ON, Canada) injections to the dorsal lymph sac to stimulate amplexus. A subsample of the resultant embryos (*n*=100) from individual females were collected and stored at -80°C for determination of total Se concentrations. A second subsample of embryos selected from each female (*n*=500) were placed in 150 mL embryo cups (50 embryos per cup) contained within a large Min-O-Cool™ tank (84" L X 24" W X 22" D) and immersed in 22 ± 1°C facility water for up to 5 days post fertilization (dpf), at which point yolk absorption was complete. The 5 dpf tadpoles generated from this process were a part of one large experimental bioassay and a subample were utilized to assess the effects of *in ovo* selenium exposure on late stage larval development for this particular experiment. A detailed description of this entire experimental process is outlined in Massé et al. (2015) or Chapter 2 of this thesis.

3.4.2 Experimental design

X. laevis 5 dpf tadpoles, exposed to elevated *in ovo* concentrations of Se via maternal transfer with swimming capability, were transferred from the previously described embryo cups to continuously aerated 9 L aquaria contained within a diluter system to maintain a controlled temperature (22 ± 1°C) and photoperiod (12 h light and 12 h dark). Each aquarium housed 30 tadpoles from an individual female (*n*=9-10) with 3-5 replicate tanks for every female assigned a

random position within the diluter system. Daily static renewal of aquaria water was completed by allowing the water to flow through at a rate of 0.15-0.2 L/min for one hour twice a day prior to feeding in the morning and during waste removal in the afternoon. Mortalities and water quality parameters were monitored and recorded daily for the duration of the rearing period (pH=7-8, total ammonia < 0.5 mg/L).

X. laevis tadpoles were allowed to develop under uncontaminated conditions with no supplementation of Se to facility water or diet. Sera Micron™ (Sera North America, Inc., Montgomeryville, PA, USA) was administered daily at varying amounts during the rearing period to compensate for growth, mortality and sampling. A 1/8th teaspoon (398.4 ± 24.5 mg; n=10) of Sera Micron™ was mixed vigorously with 40 mL of facility water in a 50 mL falcon tube and divided among tanks. The 5 dpf tadpoles were given 10 mL of the Sera Micron™ mixture for the first 15 days of the rearing period, 20 mL of the mixture for days 16 to 30, and 40 mL of the mixture after day 30. Beginning on day 30, the amount of Sera Micron™ mixture administered was decreased to 20 mL if there were 20 or less tadpoles present and 10 mL if there were 10 or less tadpoles present. The duration and termination of this rearing period was dependent on the time for 50% of the tadpoles to complete metamorphosis in an individual tank; however, this time period never exceeded 60 days or 65 dpf.

3.4.3 Assessment of maturation rate

The period of time for 50% of *X. laevis* tadpoles to complete metamorphosis was recorded as days post fertilization (dpf) which included the 5 days of development prior to the transfer of tadpoles into aquaria and the initiation of the rearing period. Beginning on day 30 (35 dpf), daily monitoring and recording of froglet number as well as the corresponding post

fertilization day was performed. This day was selected as the baseline for both monitoring and calculation since the tadpole number within individual aquaria had typically stabilized. As tadpoles completed metamorphosis, they were removed from the tank and euthanized with a lethal dose of buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate; 700 mg/L) since changes to dietary preference of froglets were unable to be accommodated. The percentage of tadpoles to complete metamorphosis was calculated by dividing the number froglets present by the total number of tadpoles present in the aquarium on day 30 (35 dpf). When 50% was attained, the post fertilization day was noted and the remaining tadpole/froglets were euthanized. All calculations included froglets that were collected prior to termination of the rearing period.

3.4.4 Assessment of froglet morphometrics and distribution of developmental stages

The completion of metamorphosis by 50% of tadpoles within an individual aquarium resulted in the euthanization of all remaining tadpoles/froglets with a lethal dose of buffered MS-222. Body mass, snout to vent length and developmental stage were determined and recorded for each tadpole or froglet collected. Larval developmental stages (i.e. NF stage) were determined in accordance with the classifications outlined in the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1994). The stages of *X. laevis* larval development have been divided into four metamorphic stage ranges for the present study: premetamorphosis (NF stages 45-55), prometamorphosis (NF stages 56-59), metamorphic climax (NF stages 60-65) and metamorphic completion (NF stage 66; i.e. froglet). The number of tadpoles belonging to each of the three developmental ranges at the completion of metamorphosis by 50% of tadpoles in a tank were converted into percentages. Each percentage was attained by dividing the number of

tadpoles/froglets belonging to a range of NF developmental stages by the total number of tadpoles/froglets present at day 30 (35 dpf).

3.4.5 Quantification of selenium in embryos and tadpole diet

The total Se concentration of the Sera Micron™ tadpole diet (n=3) and the subsample of embryos (n=100) collected from *X. laevis* females (n=9-10) exposed to elevated dietary concentrations of SeMet were determined using inductively coupled plasma-mass spectrometry (ICP-MS). Samples were processed in a manner consistent with the methods outlined in Chapter 2 of this thesis (Massé et al., 2015). TORT-2 (lobster hepatopancreas, NRC, Ottawa, ON, Canada) was used as the certified reference material for determination of Se recovery. The ICP-MS limit of quantification was ≤ 0.39 ng Se/g for all samples.

3.4.6 Statistical analyses

All data were tested for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene Median tests, respectively. If necessary, data were $\log_{10}$ transformed before performing parametric statistical analysis (SigmaPlot 11.0, Systat Software, San Jose, CA, USA). One-way analysis of variance (ANOVA) followed by a Holm-Sidak's method of multiple pairwise comparisons was used to detect significant differences between treatment groups. If data did not meet parametric assumptions after $\log_{10}$ tranformation, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's method was employed for statistical comparisons. Data that passed parametric assumptions were presented as mean ± S.E.M., and those that did not were presented as the median (25^{th} percentile, 75^{th} percentile). An alpha value of 0.05 was designated for both parametric and non-parametric ANOVA tests.

3.5 Results

3.5.1 Selenium concentrations in embryos and tadpole diet

The concentration of Se naturally present in *n*=3 samples of the Sera Micron™ tadpole diet was 0.96 ± 0.03 µg Se/g d.m.. Previously reported in Massé et al. (2015), the mean concentrations of total Se measured in pooled samples of 100 embryos collected from adult *X. laevis* females exposed to dietary levels of 0.7, 10.9, 30.4, and 94.2 µg Se/g d.m. for 68 days were 1.6 ± 0.07, 10.8 ± 0.68, 28.1 ± 2.91 and 81.7 ± 3.40 µg Se/g d.m., respectively (Table 3.1).

3.5.2 Mortality

The percent mortality of tadpoles for the duration of rearing period showed no significant difference among the treatment groups (p = 0.191; Table 3.1). Interestingly, the highest mortalities were observed in tadpoles belonging to both the 1.6 (control) and 81.7 µg Se/g d.m. groups (Table 3.1). The mean percent mortality of offspring from each adult female ranged from 2.2 to 59.0%, 1.0 to 54.5%, 0 to 62.2% and 7.2 to 70.5% for the 1.6, 10.8, 28.1 and 81.7 µg Se/g d.m. treatment groups, respectively.

3.5.3 Time to metamorphosis

The time for 50% of tadpoles to complete metamorphosis was not significantly different among treatment groups (p = 0.868; Table 3.1). The mean time for metamorphosis to occur among different *in ovo* exposure groups ranged from 44 to 54 dpf in the control group, 44 to 56 dpf in the 10.8 µg Se/g d.m. group, 46 to 57 dpf in the 28.1 µg Se/g d.m. group, and 43 to 64 dpf in the 81.7 µg Se/g d.m. group.

3.5.4 Froglet morphometrics

The greatest body mass and snout to vent length upon completion of metamorphosis (NF stage 66) was observed in *X. laevis* exposed to the highest *in ovo* Se concentration of 81.7 µg Se/g d.m. (Table 3.1). The median froglet body mass from the highest *in ovo* exposed group was 0.130 g greater than froglets belonging to the 10.8 and 28.1 µg Se/g d.m. groups ($p < 0.05$; Table 3.1) with no significant difference in body mass was detected between the control group and the other three treatment groups. The mean body mass ranged from 0.251 to 0.463 g, 0.247 to 0.452 g, 0.244 to 0.397 g and 0.234 to 0.574 g for the 1.6, 10.8, 28.1 and 81.7 µg Se/g d.m. treatment groups, respectively. A significantly greater increase of 1.8 to 2.0 mm was observed in the median snout to vent lengths of froglets belonging to the 81.7 µg Se/g d.m. *in ovo* exposed group when compared to the 10.8 and 28.1 µg Se/g d.m. groups ($p < 0.05$; Table 3.1) in addition to no significant difference in snout to vent length detected between froglets from the control group and the other three exposure groups. The mean snout to vent lengths ranged from 13.5 to 17.0 mm, 13.5 to 16.8 mm, 13.3 to 16.3 mm and 13.3 to 17.9 mm for the 1.6, 10.8, 28.1 and 81.7 µg Se/g d.m. treatment groups, respectively.

Table 3.1: Mortality, time to metamorphosis, and froglet morphometrics in developing *Xenopus laevis* exposed to elevated levels of selenium *in ovo* via maternal transfer.

Embryonic Selenium Concentration (μg Se/g d.m.)	Time to Metamorphosis (dpf)	Mortality (%)	Froglet Body Mass (g)	Froglet Snout to Vent Length (mm)
1.6 ± 0.07	49 ± 1	18.1 (3.9, 51.3)	0.285[ab] (0.260, 0.322)	14.4[ab] (14.0, 15.3)
10.8 ± 0.68	51 ± 1	8.9 (3.1, 43.4)	0.273[a] (0.250, 0.310)	14.1[a] (13.7, 15.1)
28.1 ± 2.91	50 ± 1	7.4 (2.8, 36.0)	0.273[a] (0.270, 0.305)	13.9[a] (13.6, 15.0)
81.7 ± 3.40	51 ± 2	24.3 (21.0, 56.7)	0.405[b*] (0.342, 0.475)	15.9[b*] (15.1, 17.3)

Data are presented as mean ± S.E.M, or median (25th percentile, 75th percentile), of n=9-10 females.

[a,b] Different lower case letters denote a significant difference between treatment groups using Kruskal-Wallis One Way Analysis of Variance on Ranks and Dunn's post hoc test (*, $p < 0.05$)

3.5.5 Distribution of developmental stages

The distribution of developmental stages present at the time 50% of tadpoles completed metamorphosis exhibit a significant increase in the number of tadpoles at earlier stages of development within the group exposed to the highest *in ovo* levels of Se when compared to the other three groups. An increase of 2.3 to 3.3 % in premetamorphic tadpoles (NF stages 45-55) was detected in the 81.7 μg Se/g d.m. *in ovo* exposed tadpoles when compared to the other treatment groups ($p < 0.005$; Table 3.2). Although there was < 5% of tadpoles at NF stages 45-55 across all treatment groups, a pattern illustrating an increase in premetamorphic tapdoles with

increasing *in ovo* exposure was observed. An 11 to 14.4% increase in tadpoles at prometamorphic stages (NF stages 55-59) in the 81.7 μg Se/g d.m. *in ovo* exposed group ($p < 0.001$; Table 3.2) was detected in comparison to the other treatment groups. A significantly greater percentage of tadpoles (7.9 to 12.8%) at metamorphic climax stages (NF stages 60-65) were detected in the 1.6, 10.8 and 28.1 μg Se/g d.m. *in ovo* exposed groups when compared to the highest *in ovo* exposed group of 81.7 μg Se/g d.m.. Although not surprising, no difference was observed in the number of tadpoles that completed metamorphosis (NF stage 66) since 50% was the predetermined time point related to this particular stage of development. However, it does illustrate the 1.6, 10.8 and 28.1 μg Se/g d.m. *in ovo* exposed tadpoles synchronously completed metamorphosis to a greater extent than those belonging to the 81.7 μg Se/g d.m. exposed group. This is demonstrated by the three lowest *in ovo* exposed treatment groups exceeding the 50% endpoint by 10.3 to 12.2% while the highest *in ovo* exposed group exceeded it by only 5.7%. Overall, tadpoles exposed *in ovo* to 81.7 μg Se/g d.m. displayed greater dispersion of developmental stages when compared to the other three treatment groups.

Table 3.2: The distribution of developmental stages of *in ovo* selenium-exposed *Xenopus laevis* upon completion of metamorphosis by 50% of tadpoles.

Embryonic Selenium Concentration (µg Se/g d.m.)	NF Stages 45-55 (%)	NF Stages 56-59 (%)	NF Stages 60-65 (%)	NF Stage 66 (%)
1.6 ± 0.07	0.2 ± 0.2[a]	9.3 ± 1.8[a]	28.3 ± 1.7[a]	62.2 ± 3.1
10.8 ± 0.68	0.5 ± 0.2[a]	5.9 ± 1.8[a]	33.2 ± 1.7[a]	60.3 ± 1.4
28.1 ± 2.91	1.2 ± 0.7[a]	8.8 ± 1.3[a]	28.5 ± 1.0[a]	61.6 ± 1.3
81.7 ± 3.40	3.5 ± 0.7[b**]	20.3 ± 1.3[b***]	20.4 ± 1.7[b***]	55.7 ± 1.8

Data are mean ± S.E.M. of offspring from *n*=9-10 females.
[a,b] Different lowercase letters denote a significant difference between treatment groups using one-way ANOVA and Holm-Sidak post hoc test (**, $p < 0.005$; ***, $p < 0.001$)

3.6 Discussion

To our knowledge, this is the first study investigating the prolonged effects of elevated *in ovo* Se exposure on the survival, growth and maturation of a larval anuran exclusively within an uncontaminated rearing environment. The most pronounced outcome that emerged was the significantly greater percentage of *X. laevis* tadpoles remaining at earlier stages of development in the 81.7 µg Se/g egg d.m. treatment group thus suggesting that high *in ovo* Se concentrations may hinder the progression to metamorphic climax. The observed higher proportion of tadpoles at premetamorphic and prometamorphic stages of development along with a lower proportion at metamorphic climax in the highest *in ovo* Se-exposed group could be attributed to variations in individual embryo concentrations and the corresponding frequency of morphological abnormalities. The quantity of Se deposited from the diet of female birds and reptiles to their developing eggs is associated with differing reproductive strategies specifically pertaining to the

method of egg production. Stinkpot turtles (*Sternotherus odoratus*; synchronous egg producers) exhibited significant lower variability and higher repeatability of embryo Se concentrations when compared to tree swallows (*Tachycineta bicolor*; sequential egg producers) from the same site impacted by a coal ash spill (Van Dyke et al., 2013). Thus, the concentration of Se present through maternal transfer in individual *X. laevis* embryos will likely vary considerably in this rapid asynchronous egg producer, even though the overall mean concentration was equivalent to 81.7 µg Se/g egg d.m. for a subsample of 100 embryos belonging to the highest *in ovo* dosed group in the present study. Therefore, the portion of tadpoles at premetamorphic and prometamorphic stages might have had higher *in ovo* Se concentrations that contributed to their inability to progress to metamorphic climax within the same time period. These results coincide with the prolonged time to metamorphosis observed in a field study performed on larval southern toads (*Bufo terrestris*) collected at a Se-contaminated coal combustion waste site (Metts et al., 2012). A significantly increased incidence of craniofacial, vertebral, ocular and gut abnormalities previously reported in 5 dpf *X. laevis* tadpoles exposed *in ovo* to 81.7 µg Se/g egg d.m. (Massé et al., 2015) could be the cause for reduced growth and development by diminishing the capacity to acquire and utilize food efficiently (Rowe et al., 1996). In contrast, the 81.7 µg Se/g d.m. *in ovo* exposed tadpoles that completed metamorphosis within the normal time frame may have had lower *in ovo* Se concentration and therefore fewer morphological abnormalities impeding their growth. In addition, further research is required to determine if elevated *in ovo* Se exposure could interfere with the production, activation or utilization of sufficient thyroid hormone concentrations necessary to initate metamorphic climax.

The survival of tadpoles throughout the rearing period exhibited no treatment related differences. However, the 10.8 and 28.1 µg Se/g d.m. *in ovo* exposed tadpoles appeared to have

improved survival when compared to the 1.6 and 81.7 µg Se/g d.m. groups despite the absence of statistical significance. Growth and development is a highly metabolically active period in an organism's life, and perhaps a moderate Se reserve may prove beneficial for the optimal functioning of selenoproteins that are particularly involved in thyroid activation/deactivation or maintaining redox homeostasis in larval anurans. In addition, Se has been linked to enhanced immunocompetence in adult common eiders (*Somateria mollissima*) fed a Se-enriched diet (20 mg/kg d.m.); in contrast, eiders fed a 60 mg Se/kg displayed signs of impaired immunocompetence (Franson et al., 2007; Janz et al., 2010). Thus, our data tenuously suggests that moderately elevated *in ovo* Se concentrations may provide increased larval anuran resilience without producing teratogenic malformations.

The significantly greater froglet body mass and snout to vent length in the highest *in ovo* Se group when compared to the other three treatment groups was potentially attributed to the tadpole selection process and differing tank densities rather than effects due to elevated Se. A comparable study involving zebrafish (*Danio rerio*) exposed *in ovo* to Se through maternal transfer, reported no Se-related effect on the total length, body mass or cumulative mortality after 140 dpf (Thomas and Janz, 2014), thus demonstrating the atypical nature of the data in the present study. The selection of 5 dpf tadpoles with swimming capability was performed with the intention of observing the prolonged effects of *in ovo* Se exposure on the survivors during late larval development. However, this process increased the probability for the selection of individual embryo/larvae that were either exposed to lower *in ovo* Se concentrations through deposition variability or those with higher tolerance to the Se exposure. In Chapter 2, the mean frequency of abnormalities in 5 dpf tadpoles from the highest *in ovo* exposed group was reported to be 80.7%, thus demonstrating the limitations when selecting tadpoles for this portion of the

experiment. The higher mortality observed in both the 1.6 and 81.7 µg Se/g egg d.m. groups consequently resulted in a lower tank density. Although the quantity of food dispensed per tank was reduced according to tadpole number, the adequate food rations combined with the alleviation of competition for resources may have resulted in greater growth (Dash and Hota, 1980). Interestingly, the decrease in tadpole density and greater growth of larval *X. laevis* did not reduce the time to metamorphosis in either the 1.6 or 81.7 µg Se/g egg d.m. groups when compared to the other groups. Overall, the mean time for 50% of tadpoles to complete metamorphosis in the present study ranged between 49 to 51 dpf which is comparable to the typical rate of development (58 dpf) stated in the literature when taking into consideration differing husbandry conditions (Nieuwkoop and Faber, 1994; Hilken et al., 1995). While density influences the threshold size at which tadpoles undergo metamorphosis, it can also prevent tadpoles from initiating metamorphic climax if limited resources minimize growth (Dash and Hota, 1980; Semlitsch and Caldwell, 1982). In general, a decrease in density should result in enhanced growth and developmental rate (Hilken et al., 1995). However, an adequate food supply along with the promoting influence of kinship provides an explanation as to how the 10.8 and 28.1 µg Se/g d.m. *in ovo* exposed tadpoles achieved threshold size and completed metamorphosis within a similar time frame despite greater tank density (Blaustein and Waldman, 1992; Girish and Saidapur, 2003).

In conclusion, the present study indicates *in ovo* selenium exposure through maternal transfer has minimal prolonged effects on the later stages of larval *X. laevis* development when reared under uncontaminated conditions. The presence of morphological abnormalities induced within the first 5 dpf through elevated egg concentrations appear to be the defining factor to the survival, growth and maturation of late stage *X. laevis* tadpoles exposed solely through this route

of exposure (Massé et al., 2015). However, further investigation is necessary with regards to the combined effects of *in ovo* and dietary exposure in larval anuran development under controlled laboratory conditions. Gray tree frog (*Hyla chrysoscelis*) tadpoles administered a diet containing 50 μg Se/g d.m. exhibited decreased growth and survival as well as a greater incidence of hind limb deformities (Lockard et al., 2013). Moreover, larval southern toads (*Bufo terrestris*) exposed to elevated levels of Se through *in ovo* maternal transfer and diet at a coal combustion waste site experienced a reduction in survival to metamorphosis by 85% when compared to a reference site (Metts et al., 2012). The combination of adverse effects of Se toxicity corresponding to these two exposure routes could have a significant negative impact on larval anuran development and the fitness of native populations.

CHAPTER 4

TISSUE-SPECIFIC SELENIUM ACCUMULATION AND TOXICITY IN ADULT FEMALE *XENOPUS LAEVIS* CHRONICALLY EXPOSED TO ELEVATED DIETARY SELENOMETHIONINE

4.1 Preface

An evaluation of the potential non-reproductive effects of chronically elevated dietary Se exposure on adult amphibian females is explored and described by the research presented within this chapter. The extent of tissue accumulation upon conclusion of the 68 day exposure period and the generation of embryo-to-tissue concentration relationships are depicted to establish organismal Se distribution in an amphibian model which may aid in the realm of risk assessment. In addition, biometric indices, energetic status and stress response were assessed in order to gain knowledge of the potential sublethal effects of Se exposure on the health of adult *Xenopus laevis* females that could have implications for successful reproduction. The results of this research indicate that adult anuran females, as represented by *Xenopus laevis*, are relatively tolerant to elevated dietary Se consumption despite the teratogenic effects observed in their offspring.

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4.2 Abstract

Selenium (Se) is a developmental toxicant that is also capable of altering the bioenergetic and endocrine status of adult fishes. To date, aquatic ecotoxicological research has predominantly focused on the toxic effects of Se in fishes with minimal information related to amphibians. The objective of this study was to investigate the potential for ecological risk associated with chronically elevated dietary Se consumption in adult female amphibians utilizing the model species *Xenopus laevis*. Adult *X. laevis* females were fed a diet augmented with L-selenomethionine at measured concentrations of 0.7 (control), 10.9, 30.4, or 94.2 μg Se/g dry mass for 68 days, after which they were bred with untreated males. Ovary, egg, liver, muscle and blood samples were collected from female frogs upon completion of the exposure period and subsequent breeding to ascertain Se tissue distribution, muscle and liver triglyceride and glycogen levels, and plasma cortisol concentrations. The concentrations of Se measured in female tissues excluding the liver significantly increased in proportion with dietary intake. No significant differences were observed among treatment groups with respect to biometric indices, energy stores, or stress response of adult female *X. laevis* after Se exposure, which suggests that this amphibian species is capable of accumulating substantial quantities of this element in their tissues with no adverse effects on fitness.

4.3 Introduction

Selenium (Se) is a micronutrient essential for optimal vertebrate health. Selenocysteine, the 21st amino acid, is a major component of selenoproteins, which play a key role in disease prevention (Lobanov et al., 2009). Selenoproteins primarily function as oxidoreductases, which prevent the occurrence of cellular damage, repair damage that does transpire, and maintain redox homeostasis of proteins (Lobanov et al., 2009). The suite of selenoproteins that contribute to the selenoproteome of any given species can vary greatly, but generally follows an evolutionary trend between aquatic and terrestrial organisms and the availability of Se in their corresponding environments (Lobanov et al., 2009; Mariotti et al., 2012). Of the 45 selenoprotein subfamilies detected thus far, representative bony fishes possess 41 while frogs, birds and mammals have only 24, 25 and 28, respectively (Mariotti et al., 2012). A dietary Se requirement for the maintenance of normal physiological selenoprotein activities has been experimentally determined for fish and aquatic birds to range between 0.1 and 0.5 µg Se/g dry mass (d.m.) and 0.3 and 1.1 µg Se/g d.m., respectively (Stewart et al., 2010). For amphibians, data concerning nutritional requirements is unavailable; nonetheless a preliminary recommendation of 0.3 µg Se/g d.m. has been generated based on published data of comparable vertebrate species (Ferrie et al., 2014). Although it is necessary to ensure minimum Se requirements are attained, the range between essentiality and toxicity is exceptionally narrow, particularly in aquatic oviparous species where dietary concentrations greater than 3 or 5 µg Se/g d.m. have been reported to cause adverse effects in fishes and birds, respectively (Janz et al., 2010).

Selenium is a globally distributed metalloid that is naturally present in soils and sediments as well as shale, coal and phosphate deposits within the earth's crust (Maher et al.,

2010). It becomes mobilized and introduced into the aquatic environment primarily through anthropogenic activities such as mining, oil refining, fossil fuel combustion, fertilizer production, and agricultural irrigation practices (Maher et al., 2010). The influxes of soluble anionic forms of Se (i.e., selenate and selenite) released by these industrial practices into surrounding waterbodies are readily absorbed and biotransformed by primary producers and microorganisms into organoselenide variants, of which selenomethionine (SeMet) is the most prevalent (Fan et al., 2002; Orr et al., 2006; Luoma and Presser, 2009; Janz et al., 2014). The structural similarity between the amino acid methionine and SeMet allows for the indiscriminate and dose-dependent substitution of the latter during protein synthesis, which in turn facilitates its bioaccumulation and trophic transfer within aquatic food webs (Fan et al., 2002; Orr et al., 2006; Luoma and Presser, 2009; Stewart et al., 2010; Janz et al., 2010; Janz et al., 2014]. Consequently, elevated dietary Se consumption in species occupying higher trophic levels can result in excessive tissue accumulation (up to 100 µg Se/g d.m.) and adverse reproductive effects while aqueous Se concentrations remain relatively low (< 10 µg Se/L) (Ohlendorf et al., 1990; Lemly, 1997a; Hopkins et al., 2006; Janz et al., 2010).

Selenium exhibits an interestingly dynamic relationship between adult oviparous females and their developing progeny. The major route of Se exposure in adult oviparous vertebrates is through dietary sources, while embryonic exposure occurs most notably through *in ovo* exposure via maternal transfer (Janz et al., 2010). Consequently, the greatest adverse impact of elevated maternal dietary consumption of Se on aquatic oviparous vertebrates is the production of teratogenic abnormalities in their progeny (Janz et al., 2010). Adult females transfer excess Se from their diet to their oocytes during vitellogenesis. The synthesis of the yolk precursor protein, vitellogenin, takes place in the liver after which it is transported and incorporated into

developing ovarian follicles. Vitellogenin, and its derivatives lipovitellin and phosvitin, are ordinarily comprised of varying numbers of methionine residues; however, a greater proportion of SeMet is substituted for methionine with increased maternal dietary intake (Janz et al., 2010). The catabolism of these SeMet rich yolk proteins for energy utilization by the developing embryo initiates a series of biochemical reactions in which an excessive generation of reactive oxygen species causes damage to DNA, proteins and lipids during early stages of development (Palace et al., 2004; Janz et al., 2010). Embryonic mortalities and teratogenic larval abnormalities are hypothesized to be the result of this process in fishes, birds and amphibians, and ultimately the primary cause for failed recruitment of individuals into populations (Ohlendorf et al., 1990; Lemly, 1997a; Palace et al., 2004; Hopkins et al., 2006; Muscatello et al., 2006; Janz et al., 2010; Massé et al., 2015). The majority of Se research has been focused on characterizing toxic effects on fishes and birds, with few studies conducted in amphibians. Field studies involving amphibians inhabiting areas surrounding coal burning power plants have shown that adult females are capable of accumulating excessive levels of Se in their tissues in a manner that coincides with the magnitude of teratogenic abnormalities present in their progeny (Hopkins et al., 2006). However, the presence of other highly toxic contaminants (e.g., arsenic, cadmium, mercury) may be greater contributors to the toxic effects observed at these sites than those generated by Se. Thus, an investigation to exclusively characterize the toxicological profile of Se in amphibians will provide a baseline for reference and assist in determining if Se is the primary contaminant responsible for the adverse effects observed at these sites.

While adult oviparous species are relatively tolerant of the toxic effects of Se in comparison to early developmental stages of their progeny, there is minimal information relating how chronic sublethal exposure could negatively impact their capacity to engage in energetically

expensive activities associated with reproduction, food acquisition, predator avoidance, hibernation/estivation and migration, particularly for amphibians. The accumulation of SeMet in metabolically active proteineous tissues such as the liver, muscle, and ovaries could potentially create an oxidative intracellular environment that results in damage to vital components and ultimately a decline in fitness. Previous studies with adult and juvenile fishes have indicated that elevated dietary SeMet exposure causes alterations in the utilization of energy stores (i.e., triglycerides and glycogen) and the production of a physiological stress response (i.e., cortisol production) (Thomas and Janz, 2011; Wiseman et al., 2011; Thomas et al., 2013; McPhee and Janz, 2014). In addition, these biochemical responses corresponded to increased oxygen consumption and diminished swimming efficiency of SeMet exposed fishes (Thomas and Janz, 2011; Thomas et al., 2013; McPhee and Janz, 2014). Therefore, a comprehensive approach is imperative when identifying reasons for oviparous vertebrate population declines in Se-impacted aquatic ecosystems, especially since sublethal effects often provide a more sensitive indicator of toxicity that could ultimately produce a similar adverse outcome over a prolonged period.

A tissue-based criterion for Se has been proposed by the United States Environmental Protection Agency (US EPA) for the protection of aquatic organisms based on toxicological data related to fishes, the presumed most sensitive taxa (US EPA, 2015). Recently, a laboratory study assessing the frequency of teratogenic abnormalities in *Xenopus laevis* tapdoles exposed *in ovo* to Se via maternal transfer presented evidence that early life stages of this amphibian species are more tolerant to Se toxicity than most fishes (Massé et al., 2015). However, it remains largely unknown if the draft US EPA criterion will be effective in the protection of amphibians inhabiting sites contaminated by Se with the absence of sufficient data describing the toxic effects of Se across distinct life stages within this highly diverse and unique taxon. Therefore, the

objective of this study was to investigate patterns of tissue accumulation, energetic status, and physiological stress response in adult female *X. laevis* exposed chronically to elevated levels of dietary Se. The goal was to assist in predicting associated teratogenic risk during early stages of development from female tissue Se concentrations, as well as evaluating potential effects on the fitness of adult females that could impact their survival and reproductive success.

4.4 Materials and methods

4.4.1 Test species and laboratory conditions

Sexually mature adult *Xenopus laevis* breeding pairs were purchased from Xenopus 1 (Dexter, MI, USA) and housed in the Aquatic Toxicology Research Facility (ATRF) located at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK. Laboratory conditions consisted of 17 ± 1°C water temperature and 12 h light:12 h dark photoperiod. Nasco™ juvenile frog brittle (Newmarket, ON, Canada) was fed *ad libitum* daily to frogs. Males and females were housed separately in large Min-O-Cool™ aquaria (84" L X 24" W X 22" D) with a 12" water depth and flow-through conditions (0.75–0.9 L/min). A two month acclimation time to these laboratory conditions preceded the commencement of the experiment.

4.4.2 Diet preparation and experimental design

The dietary concentrations of Se administered to female *X. laevis* in this study were selected based on environmental relevance and were comparable to those reported in invertebrates, fishes, amphibians and birds collected at Se contaminated field sites (Ohlendorf et al., 1990; Lemly, 1997a; Hopkins et al., 2006; Orr et al., 2006). Seleno-L-methionine (≥ 98% purity; Sigma Aldrich, Oakville, ON, Canada) was dissolved in deionized water and added to ground Nasco™ juvenile frog brittle at nominal concentrations of 10, 30, and 90 µg Se/g d.m. as

described previously in Chapter 2 of this thesis (Massé et al., 2015). The control diet was comprised of equivalent quantities of deionized water and ground brittle without the addition of seleno-L-methionine. Food was pelleted and stored at -20^{o}C in airtight containers. Representative samples (n=6) of each diet were taken prior to and during the feeding trial for total Se analysis.

Each *X. laevis* female (n=40) was bred immediately prior to the onset of the dietary exposure in order to stimulate the release of stage VI oocytes (post-vitellogenic and preovulatory) from the ovary (Rasar and Hammes, 2006). This initial breeding was performed to minimize differences among female oogenesis cycles as well as maximize the transference of SeMet from the maternal diet to stage III, IV and V (actively vitellogenic) oocytes upon initiation of the exposure period (Rasar and Hammes, 2006). Breeding procedures performed pre and post exposure were described previously in Chapter 2 (Massé et al., 2015). Briefly, an initial priming dose of human chorionic gonadotropin (25 IU hCG; Sigma Aldrich, Oakville, ON, Canada) and a second higher dose (500 IU hCG for females, 250 IU hCG for males) 24 hours later were injected sub-dermally into the dorsal lymph sacs of *X. laevis* to stimulate amplexus (Sive et al., 2000). Immediately after the second hCG injection, each female was placed in a 20 L covered aquarium with a randomly selected untreated male to spawn overnight in a darkened area of the exposure room.

Following pre-exposure breeding, eight *X. laevis* females were weighed, divided evenly into four partitioned sections of a Min-O-Cool™ aquaria and maintained under the laboratory conditions stated previously. Pairs of females were assigned a section of the tank with those receiving the control diet occupying the section nearest to the in-flow and those receiving the

highest SeMet augmented diet occupying the furthest section. This same design was employed for a total of five tanks, which provided n=10 females per treatment group. *X. laevis* females were fed 2 g daily of either the control or seleno-L-methionine augmented diets for 68 days and excess food was siphoned from the tank 4 hours after administration. Water quality was monitored on a daily basis throughout the exposure period (pH=7-8, total ammonia < 0.25 mg/L, dissolved O_2 > 80%).

Upon completion of the dietary exposure, females underwent the breeding procedures described above and eggs were collected the morning after spawning. The jelly coating surrounding the eggs was removed by gently swirling for 3 min with a 2% L-cysteine solution (Sigma Aldrich, Oakville, ON, Canada), and promptly rinsed afterwards with Modified Barth's Saline solution (Sive et al., 2000). A random subsample of eggs (n=100) was collected from each female and stored at -80°C for determination of Se concentration. Females were euthanized for collection of tissue samples 24 h after oviposition by immersion in a buffered MS-222 (ethyl 3-aminobenzoate methanesulfonate; 5 g/L) solution to induce deep sedation prior to decapitation (Green, 2010). Snout-to-vent length and body weight was measured for each female prior to euthanasia. Liver, ovaries, adductor muscles, abdominal fat bodies and spleen were collected, weighed and stored at -80°C. Blood samples were collected from anesthetized females via cardiocentesis with a 22 gauge needle attached to a 5 mL heparinized syringe (Green, 2010). These experimental procedures presented in this chapter were a portion of a large bioassay that was performed and approved by the University of Saskatchewan's Animal Research Ethics Board (protocol no. 20120070), and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

4.4.3 Assessment of female biometric indices

The change in body weight over the exposure period and thus changes to overall body condition due to treatment was obtained by subtracting the initial weight at the onset of the dietary exposure from the final weight measured upon completion of the exposure period. Liver, ovaries, abdominal fat bodies and spleen were weighed at the time of collection for determination of organo-somatic indices [i.e., Liver Somatic Index (LSI), Ovarian Somatic Index (OSI), Fat Body Somatic Index (FBSI) and Splenic Somatic Index (SSI)] which were calculated by dividing the organ mass (liver, ovaries, abdominal fat bodies or spleen) by the final body mass (e.g., LSI = (liver mass / body mass)*100).

4.4.4 Quantification of selenium in female diet and tissues

Liver, muscle, and ovary tissues from each female in addition to experimental diets (n=6 per diet) were lyophilized using a freeze dryer (Dura-Dry^{TM} MP, FTS systems, Stone Ridge, NY, USA) and subsequently homogenized using a mortar and pestle. The wet and dry tissue and egg masses were recorded prior to and after freeze-drying to determine percentage moisture content. A portion of the homogenized dried tissue and diet samples measuring 100 mg were cold digested in Teflon vials using 5 mL of ultrapure nitric acid and 1.5 mL hydrogen peroxide. Fully digested samples were then concentrated on a hot plate (< 75 °C), reconstituted in 5 mL of 2% ultrapure nitric acid, and stored at 4°C until total Se concentrations could be determined using inductively coupled plasma mass spectrometry (ICP-MS). Randomly selected eggs from the clutch of each female underwent the same process except the mass was equivalent to the 100 eggs collected and freeze dried. A certified reference material (TORT-2, lobster hepatopancreas,

NRC, Ottawa, ON, Canada) was used to determine the recovery of Se. The ICP-MS level of detection limit was ≤ 0.87 ng Se/g for all samples.

4.4.5 Quantification of triglyceride and glycogen concentrations

Female *X. laevis* liver and muscle samples were thawed on ice, weighed out into 200 mg random subsamples, homogenized in 0.2 M sodium citrate buffer (pH = 5; Fisher Chemical, Fair Lawn, NJ, USA) using a Tissue Tearor™ (Biospec Products, Inc., Bartlesville, OK, USA), and the final tissue homogenate was stored at -80^{o}C until analysis.

Both triglyceride and glycogen concentrations for liver and muscle samples were determined through commercially available standard solutions and reagents purchased from Sigma-Aldrich (Oakville, ON, Canada). The procedural methods for the triglyceride and glycogen assays (McGowan et al., 1983; Gómez-Lechón et al, 1996) underwent further validation in our lab for tissue and whole body samples (Thomas and Janz, 2011;Wiseman et al., 2011; Thomas et al., 2013; McPhee and Janz, 2014). Standard curves were created for the triglyceride and glycogen assays using glycerol and purified Type IX bovine liver glycogen, respectively. Liver and muscle homogenate samples were run in triplicate and mean results with less than 10% coefficient of variation (%CV; standard deviation/mean) were used for data analysis.

4.4.6 Quantification of blood plasma cortisol concentrations

In preparation for the cortisol assay, *X. laevis* female whole blood samples were centrifuged at 3000*g* for 20 minutes at 4^{o}C to obtain plasma, and stored at -20^{o}C. A 100 µL volume of plasma was diluted in 900 µL of phosphate-buffered saline (PBS) provided by the kit manufacturer described below. Cortisol was extracted using diethyl ether and subsequently

placed under a constant stream of nitrogen gas at 50°C to evaporate the ether (MultiVap™118 Nitrogen Evaporator/OA-Sys™ heating system; Organomation Associates, Inc., Berlin, MA, USA). Samples were then reconstituted in PBS for storage at -20°C until cortisol analysis was performed. An enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research, Oxford, MI, USA) in addition to a SpectaMAX 190 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) were employed to quantify plasma cortisol concentrations. Each sample was run in triplicate with mean results below 15%CV used for data analysis.

4.4.7 Statistical analyses

All data were tested for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene Median tests. If necessary, data were $\log_{10}$ transformed before performing parametric statistical analysis. One-way analysis of variance (ANOVA) followed by a Holm-Sidak test was used to detect significant differences among treatment groups. Data that passed parametric assumptions were presented as mean ± standard error of the mean (S.E.M.). Raw data values were reported for all variables including those that required $\log_{10}$ transformation for statistical analysis. Best fit relationships between egg and female tissue (ovary, muscle or liver) Se concentrations were evaluated using regression analysis. All statistical tests were performed using SigmaPlot version 11.0 (Systat Software, San Jose, CA, USA) with a 95% ($\alpha = 0.05$) level of confidence.

4.5 Results

4.5.1 Biometric indices of *X. laevis* females

The mean body weights of females prior to and upon completion of the exposure period showed no significant differences among the four treatment groups; however, the change in body

weight over the exposure period demonstrated a trend in weight loss with increasing dietary Se exposure. The mean initial and final female body weights for the four dietary treatment groups (0.7, 10.9, 30.4 and 94.2 µg Se/g d.m.) were 83.9 ± 2.5, 80.8 ± 1.7, 82.7 ± 3.6 and 82.2 ± 3.1 g wet mass (w.m.), and 82.0 ± 2.9, 76.7 ± 1.7, 77.2 ± 3.4 and 75.7 ± 3.0 g w.m., respectively. The range of initial and final body weights for individual frogs across all treatment groups were 61.0 to 95.0 g w.m. and 57.0 to 94.7 g w.m., respectively. The mean change in body weight (final-initial) were -1.9 ± 1.1, -4.0 ± 1.1, -5.5 ± 1.4, and -6.5 ± 1.4 g for the 0.7, 10.9, 30.4 and 94.2 µg Se/g d.m. groups, respectively (p = 0.088; data not shown).

The LSI, OSI, FBSI and SSI in *X. laevis* females exhibited no significant differences in relation to elevated levels of dietary Se over a 68 day dietary exposure (Table 4.1). The organo-somatic indices ranged across all treatment groups from 2.85 to 9.86 for LSI, 4.11 to 17.71 for OSI, 0.62 to 8.82 for FBSI and 0.042 to 0.145 for SSI. Although no statistically significant changes were detected for these four organo-somatic indices, the OSI in particular indicated a pattern in which the females from the SeMet-augmented treatment groups had lower mean values than those observed in the females from the control group (p = 0.161; Table 4.1).

Table 4.1: Organo-somatic indices of adult female *Xenopus laevis* upon completion of a 68 day dietary exposure to elevated levels of selenium (Se).

Dietary Selenium Concentration[a]	Liver Somatic Index[b]	Ovarian Somatic Index[b]	Fat Body Somatic Index[b]	Splenic Somatic Index[b]
0.7 ± 0.01	4.39 ± 0.29	10.43 ± 1.13	2.34 ± 0.36	0.09 ± 0.01
10.9 ± 0.20	3.91 ± 0.20	8.56 ± 0.87	3.35 ± 0.85	0.10 ± 0.01
30.4 ± 0.43	4.14 ± 0.21	9.13 ± 0.54	2.14 ± 0.42	0.09 ± 0.01
94.2 ± 3.10	4.93 ± 0.60	7.82 ± 0.65	2.40 ± 0.36	0.08 ± 0.01

[a] μg Se/g dry mass, n=6 food samples.
[b] (organ weight/body weight)*100, n=9-10 females.
Data are presented as mean ± S.E.M.

4.5.2 Selenium concentration in female diet and tissues

The measured Se concentrations in diets administered to females over the exposure period (0.7 ± 0.01, 10.9 ± 0.20, 30.4 ± 0.43 and 94.2 ± 3.10 μg Se/g d.m.) corresponded with the nominal concentrations (control, 10, 30, 90 μg Se/g d.m.) set out in the experimental design (Table 4.2). The concentrations of Se measured in individual female tissues ranged across treatment groups from 0.59 to 226.49 μg Se/g d.m. in liver, 1.55 to 114.27 μg Se/g d.m. in ovary, 0.95 to 29.94 μg Se/g d.m. in muscle and 1.21 to 97.55 μg Se/g d.m. in eggs (Fig. 4.1). The mean moisture content of *X. laevis* tissues across all treatment groups ranged from 69.3 to 70.3% for liver, 68.4 to 69.9 % for ovary, 76.4 to 76.8% for muscle and 91.6 to 92.6% for eggs.

The quantity of Se present in the ovary, muscle and eggs were significantly different among treatment groups ($p < 0.001$) with increasing tissue concentrations rising in proportion

with dietary concentrations (Table 4.2). The tissue bioconcentration factors (i.e., [tissue Se] / [diet Se]; TBFs) for females assigned the 0.7, 10.9, 30.4 and 94.2 μg Se/g d.m. dietary treatment groups were 2.3, 1.0, 0.9, and 0.9 for eggs, and 2.7, 1.1, 1.0, and 1.0 for ovaries, respectively. The accumulation of Se in the eggs and ovaries of females were comparable, with females from the control group exhibiting TBFs approximately twice as high as females assigned the SeMet augmented diets (Table 4.2). The TBFs for female muscle were 1.2, 0.3, 0.2, and 0.2 for the 0.7, 10.9, 30.4 and 94.2 μg Se/g d.m. treatment groups, which demonstrates a very low rate of Se incorporation into this particular tissue (Table 4.2). Both ovary and muscle Se concentrations present in each *X. laevis* female exhibited a strong positive linear relationship with the concentrations measured in eggs (ovary: $r^2 = 0.97$, $p < 0.001$; muscle: $r^2 = 0.91$, $p < 0.001$; Figs. 4.1A, B).

The concentration of Se in the liver of *X. laevis* females did not adhere to the direct and consistent accumulation related to dietary levels that was observed in the other three tissues analyzed. A significant increase in the concentration of Se from the livers of females fed SeMet augmented diets was observed when compared to females fed the control diet ($p < 0.001$); moreover, a statistical difference was detected among all treatment groups ($p < 0.001$) except between the 10.9 and 30.4 μg Se/g d.m. groups (Table 4.2). A greater proportion of Se appeared to be naturally retained within the liver of *X. laevis* females fed the control (Se normal) diet, with a TBF equal to 5.7; however, a decline in this proportion occurred with increasing dietary concentrations of Se up to 30.4 μg Se/g d.m. (TBFs of 1.9 and 0.6 for the 10.4 μg Se/g d.m. and 30.4 μg Se/g d.m. groups, respectively), after which a TBF of 1.1 was observed in the highest

dietary Se dose group (Table 4.2). A strong positive quadratic relationship was observed between Se concentrations present in the liver of females and their eggs ($r^2 = 0.81$, $p < 0.001$; Fig. 4.1C).

Table 4.2: Selenium (Se) concentrations measured in adult female *Xenopus laevis* tissues after a 68 day exposure to elevated levels of dietary Se.

Dietary Selenium Concentration[a]		**Tissue Selenium Concentration[b]**			
Nominal	**Measured**	**Liver**	**Muscle**	**Ovary**	**Egg[c]**
Control	0.7 ± 0.01	3.96 ± 0.54A	0.85 ± 0.06A	1.88 ± 0.10A	1.6 ± 0.07A
10	10.9 ± 0.20	21.11 ± 3.02B	3.02 ± 0.17B	12.09 ± 0.65B	10.8 ± 0.68B
30	30.4 ± 0.43	17.51 ± 3.00B	7.20 ± 0.55C	30.51 ± 2.37C	28.1 ± 2.91C
90	94.2 ± 3.10	105.88 ±16.53C	19.66 ± 1.89D	90.93 ± 4.80D	81.7 ± 3.40D

[a] μg Se/g dry mass, *n*=6 food samples.

[b] μg Se/g dry mass, *n*=9-10 females.

[c] subsample of 100 eggs collected from each female, *n*=9-10.

Different capital letters designate significant differences ($p < 0.001$) among treatment groups for a single variable.

Data are presented as mean ± S.E.M.

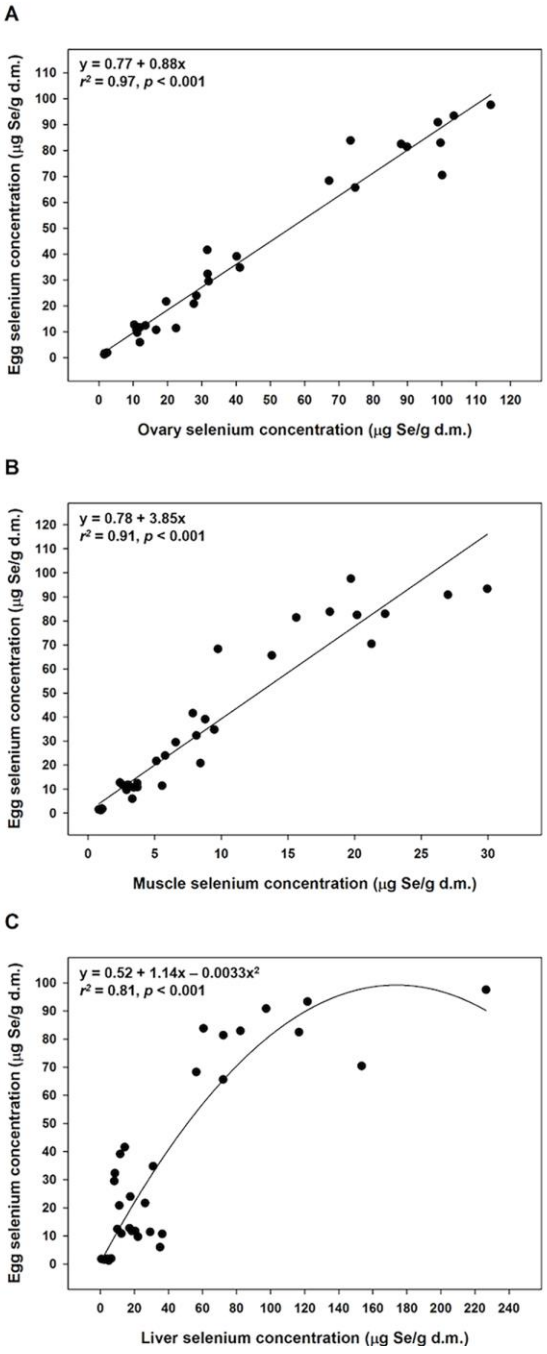

Figure 4.1: Relationships between selenium (Se) concentrations (µg/g dry mass) in (A) ovary, (B) muscle or (C) liver of adult *Xenopus laevis* females (n=9-10) exposed to one of four dietary concentrations of Se (0.7, 10.9, 30.4 or 94.2 µg Se/g dry mass) for 68 days, and the corresponding Se concentrations measured in subsamples of 100 eggs collected from each female. Each point represents the concentrations measured in the tissue and eggs for an individual female.

4.5.3 Triglyceride, glycogen and cortisol concentrations

The triglyceride and glycogen concentrations of liver and muscle samples collected from *X. laevis* females upon completion of the 68 day exposure period and subsequent breeding demonstrated no significant differences among the groups in relation to dietary Se treatment (Table 4.3). The concentrations of triglycerides for individual females across all treatment groups ranged from 0.74 to 2.63 mg/g w.m. in liver and 0.55 to 1.57 mg/g w.m. in muscle. The concentrations of glycogen for individual females across all treatment groups ranged from 29.97 to 136.73 mg/g w.m. in liver and 2.97 to 23.07 mg/g w.m. in muscle. Although no statistically significant differences were detected among the dietary treatment groups in relation to liver glycogen concentrations, the data indicated the mean levels measured in females belonging to the SeMet augmented groups were lower than those observed in females from the control group (p = 0.139; Table 4.3). The mean glycogen concentrations measured in female muscle samples ranged from 10.36 to 14.60 mg/g w.m., which were not significantly different among treatment groups (p = 0.297; Table 4.3).

The plasma cortisol concentrations revealed no significant alterations to the physiological stress response of recently bred *X. laevis* females due to a 68 day exposure to elevated levels of dietary Se (Table 4.3). The range of plasma cortisol concentrations for individual females across all treatment groups ranged from 0.63 to 2.26 ng/mL.

Table 4.3: Concentrations of triglyceride and glycogen in liver and muscle in addition to plasma cortisol levels from adult female *Xenopus laevis* fed elevated levels of dietary selenium (Se) for 68 days.

Dietary Se Concentration[a]	Liver Triglyceride[b]	Muscle Triglyceride[b]	Liver Glycogen[b]	Muscle Glycogen[b]	Plasma Cortisol[c]
0.7 ± 0.01	1.41 ± 0.08	0.95 ± 0.11	86.14 ± 9.39	13.73 ± 2.00	1.30 ± 0.12
10.9 ± 0.20	1.32 ± 0.17	0.96 ± 0.11	63.77 ± 3.73	10.86 ± 1.97	1.14 ± 0.09
30.4 ± 0.43	1.61 ± 0.20	0.95 ± 0.08	67.24 ± 9.76	10.36 ± 1.13	1.06 ± 0.08
94.2 ± 3.10	1.47 ± 0.07	1.16 ± 0.09	61.27 ± 7.81	14.60 ± 2.09	1.19 ± 0.15

[a] µg Se/g dry mass, *n*=6 food samples.
[b] mg/g wet mass, *n*=9-10 females.
[c] ng/mL, *n*=9-10 females
Data are presented as mean ± S.E.M.

4.6 Discussion

To our knowledge, this is the first study investigating patterns of Se accumulation and distribution within the tissues of an amphibian chronically exposed to elevated levels of dietary SeMet, and associated effects on physiological processes related to female fitness. Muscle, liver, ovary or egg samples are typically the best indicators of toxic effects at Se-impacted field sites rather than traditional media such as water or sediment (Janz et al., 2010). *In ovo* exposure via maternal transfer and subsequent embryonic development are the most critical exposure route and toxicological endpoint for Se in aquatic oviparous vertebrates (Janz et al., 2010). Therefore, the ability to accurately predict adverse developmental effects from Se concentrations measured in eggs, or indirectly through concentrations in female tissues, allows for alternate sampling

methods for monitoring studies. In the present study, strong relationships between Se concentrations in female *X. laevis* tissues (i.e., ovary, muscle or liver) and their egg clutches were ascertained and are comparable to observations reported for fishes collected from Se-contaminated sites. Egg-ovary Se relationships have previously been reported to be strong (r^2 > 0.75) and consistent across multiple fish species (deBruyn et al., 2008) with diverse reproductive strategies, and for *X. laevis* in the present study, which indicates that early life stage Se exposure can be accurately predicted from ovarian tissues of adult female amphibians despite having an asynchronous pattern of oogenesis or having multiple spawning periods per year as is the case for *X. laevis*. Although the egg-muscle Se relationship in fishes has been reported to be strong (r^2 > 0.7) as observed in the present study, it is also variable among fish species (deBruyn et al., 2008), a pattern which may hold true for amphibians with further investigation into Se accumulation by native North American amphibian species. Eastern narrow-mouth toads (*Gastrophyrne carolinensis*) and southern toads (*Bufo terrestris*) collected from the same Se-contaminated site surrounding a coal-fired power plant showed r^2 values of 0.94 and 0.64, respectively, between female carcasses and egg concentrations, indicating interspecies variation even within a particular site (Hopkins et al., 2006; Metts et al., 2013). Female liver Se concentrations provided the least reliable estimation of egg concentrations of the three tissues examined in the present study, similar to multiple fish studies in which such relationships were highly variable (deBruyn et al., 2008), which is attributed to the active role of liver in the biotransformation, regulation, and elimination of Se. Thus, *X. laevis* was observed to have similar Se tissue accumulation and distribution patterns as those reported in fishes, which establishes a foundation for amphibian research in this regard.

The extent of anuran Se tissue accumulation and the resultant effects on the survival and development of their progeny appears to be highly dependent on species ecology and physiology. The mean whole body Se concentrations of adult female eastern narrow-mouth toads and southern toads were 42 and 17 μg Se/g d.m., respectively, when collected near the same coal-fired power plant, indicating that differences in foraging strategies were the most probable explanation for disparities in Se accumulation (Hopkins et al., 1998, 2006). Furthermore, similar Se concentrations in female tissues and eggs do not necessarily result in comparable toxic effects for early life stages among different species. A concurrent study to the present one (Massé et al., 2015) examined early life stage toxicities of *X. laevis* tadpoles exposed to elevated levels of Se *in ovo* via maternal transfer, and reported no significant increases in the frequency of teratogenic abnormalities at egg Se concentrations of 10.8 and 28.1 μg/g d.m. when compared to the control group (1.6 μg/g d.m.). However, the highest (81.7 μg Se /g egg d.m.) exposure group had a 62.3 % greater occurrence of teratogenic abnormalities (Massé et al., 2015). In comparison, a previous field study investigating maternal transfer of Se in *G. carolinensis* reported egg concentrations of 43.96 ± 37.62 μg/g d.m. that coincided with an overall 55-58% greater incidence of tadpole abnormalities at the Se-contaminated site in comparison to the reference site (Hopkins et al., 2006). Moreover, the mean Se concentration of 5.28 μg/g d.m for *B. terrestris* eggs collected from a coal fly ash contaminated area corresponded to reduced hatching success, offspring viability and female reproductive success, but no change in the frequency of abnormalities when compared to embryos/larvae from the reference site, suggesting that contaminants other than Se were impacting amphibians to a greater extent (Metts et al., 2013). Within this limited data, it appears that early life stage toxicities occur at higher egg Se concentrations in amphibians than what is reported for most fishes to date (i.e. 15-25 μg Se/g egg

d.m.), which may be due to factors such as an evolutionarily smaller amphibian selenoproteome in comparison to fishes as well as increased resilience related to the polyploidy genome of *X. laevis* (Comai, 2005; Janz et al., 2010; Mariotti et al., 2012). In addition, a high degree of variability in female tissue Se accumulation and frequency of teratogenic abnormalities was demonstrated by these studies in amphibians, indicating the vital importance of gathering both species-specific and site-specific data when determining toxicity thresholds for Se with respect to amphibians.

The accumulation of Se within female tissues increased with dietary levels at a rate that reflected the metabolic activity for each tissue. The TBFs for each *X. laevis* tissue illustrated that a smaller proportion of the total Se dietary dose was retained in tissues of SeMet treatment groups when compared to the control group, suggesting greater retention at low dietary Se levels together with either efficient elimination or limited incorporation of Se at elevated dietary intake (Hodson and Hilton, 1983). The concentrations of Se in female ovarian tissue and eggs approximated their dietary level and appeared to reach a steady state within the 68 day exposure period; however, a similar pattern did not appear in either liver or muscle tissue. The concentration of Se in female muscle increased with dietary dose yet remained at approximately a quarter the concentration consistently throughout the SeMet treatment groups, thus indicating a slower incorporation of Se into muscle possibly due to longer tissue turnover rates. Muscle tissue half-lives have been determined to range from 116-173 days in adult bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), and yellow perch (*Perca flavescens*), with fish mass being an effective predictor of carbon turnover rates (Weidel et al., 2011). For example, juvenile fathead minnows (*Pimephales promelas*) have been reported to achieve a steady state equilibration between Se body burden and diet after exposure for one week, while

juvenile bluegill tissue Se levels reflected dietary intake though a steady state was not attained until approximately 100 days (Stewart et al., 2010). Thus, muscle may provide a more accurate estimate of long term exposure (months to years) while egg/ovary tissue would indicate short term exposure patterns such as during seasonal spawning (weeks to months) in adults. The liver TBFs showed a diminishing proportion of dietary Se levels being retained for females from the 10.9 and 30.4 µg Se/g d.m. groups, followed by an increase in the 94.2 µg Se/g d.m. group. This pattern suggests a progressively efficient elimination of Se from liver in females fed the 0.7, 10.9 and 30.4 µg Se/g d.m.; however, the highest dietary dose group possibly exhibits a reduced ability in the elimination of Se when compared to the other three groups and appeared to achieve a steady state with the corresponding dietary dose. Nonetheless, no definite conclusions as to accumulation or elimination rates can be determined since our results only reflect one time point (Tahjian et al., 2006). Juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to dietary concentrations of either 3.74 µg Se/g d.m. for 12 weeks or 13.1 µg Se/g d.m. for 24 weeks displayed similar liver accumulation (44.2 and 47.84 µg Se/g d.m., respectively) as the present study, illustrating that a threshold for liver accumulation exists much like we observed in the 10.9 and 30.4 µg Se/g d.m. treatment groups (Hilton et al., 1982). Although not specific to liver accumulation, fathead minnows fed a daily dose of 53.94 µg Se/g d.m. displayed whole body burdens that plateaued between days 30 to 60 of the exposure period, after which a sharp rise occurred (Bertram and Brooks, 1986). Thus, it is possible that this apparent steady state represents a threshold for liver in which excessive Se accumulation surpasses the capacity to regulate Se due to cellular damage and tissue necrosis. Histological examination of redear sunfish (*Lepomis microlophus*) exposed to 20 µg Se/g d.m. in their diet showed central necrosis of the liver along with reduced rough endoplasmic reticulum and glycogen within hepatocytes

(Sorenson et al., 1983). Therefore, oxidative damage, depleted energy and diminished protein synthesis within hepatocytes could be interfering with an active metabolic process required for Se biotransformation or elimination, and subsequently the reason for a greater accumulation in the liver tissue of females fed the 94.2 μg Se/g d.m. diet in the present study.

Elevated dietary and tissue Se concentrations from adult and juvenile fishes collected from both Se-contaminated sites and laboratory studies have been associated with alterations in energy homeostasis and the physiological stress response (Bennett and Janz, 2007; Thomas and Janz, 2011; Wiseman et al., 2011; Goertzen et al., 2012; Thomas et al., 2013; McPhee and Janz, 2014). In the present study, triglyceride, glycogen and cortisol levels displayed no substantial changes that corresponded with SeMet treatment. Overall, these three variables exhibited similar concentrations to literature values reported for amphibians, taking into consideration post breeding conditions, diverse life history behaviors and alternate analytical methods (Bryne and White, 1975; Woof and Janssens, 1978; Merkle and Hanke, 1988; Merkle, 1989; Wright et al., 2003; Gurushankara et al., 2007). The marginal decrease observed in liver glycogen concentrations of the SeMet treated females could be due to slightly higher metabolic costs related to exposure despite still being within normal range of literature values. Typically under nonbreeding conditions, *X. laevis* females have markedly high levels of glycogen content in the liver (15% of liver w.m.) (Spornitz, 1975; Atar-Zwillenberg and Spornitz, 2002). However, rapid depletion of liver glycogen concentrations does occur under breeding conditions where *X. laevis* liver glycogen content dramatically decreases to approximately 5% of the liver w.m. (Atar-Zwillenberg and Spornitz, 2002). The average percentage of glycogen present in the whole liver of *X. laevis* females in the present study ranged from 6.1-8.4%, which is similar to values reported previously in *X. laevis* after administration of hCG or after vitellogenesis has transpired

(Spornitz, 1975; Atar-Zwillenberg and Spornitz, 2002). Although excess dietary Se is linked to greater concentrations of both triglyceride and glycogen in several fish species (Bennett and Janz, 2007; Thomas and Janz, 2011; Wiseman et al., 2011; Goertzen et al., 2012), decreases in glycogen in combination with no change to triglyceride or cortisol concentrations of resting juvenile fathead minnows fed 9.9 and 26.5 μg Se/g d.m. have been reported (McPhee and Janz, 2014), thus demonstrating that age, dietary Se concentration and species sensitivity play vital roles in how energy stores are regulated in relation to exposure. The absence of adverse physiological effects on energy storage and physiological stress in the present study may be due to these same factors in addition to a relatively small sample size and favourable experimental conditions. Increased metabolic demands and depleted energy reserves as a result of elevated dietary Se exposure could be easily counterbalanced due to minimal exertion required to obtain food and refuge by *X. laevis* in the present study. Consequently, potential differences for these three variables in response to elevated Se exposure may only become apparent under conditions akin to stressors encountered in their natural environment.

X. laevis females administered the SeMet augmented diet showed no major signs of ill health related to the exposure. All organo-somatic indices displayed no significant differences across treatment groups and corresponded to literature values of control *X. laevis* females (Merkle, 1989). A trend in weight loss cannot be adequately explained when no significant changes were observed in either the rudimentary (organo-somatic indices) and more precise (triglyceride, glycogen, cortisol) measurements of organismal health and fitness; however, it could be due to an additive effect of slightly reduced OSI and liver glycogen content in SeMet treated females compared to the control females. Although not statistically significant, there appeared to be a dose-dependent decrease in OSI. With slight decreases in liver glycogen levels,

females exposed to elevated SeMet may have had diminished capacity for synthesis and transport of vitellogenin to the developing oocytes, resulting in decreased vitellogenic loading of stage III, IV and V oocytes leading to an overall reduction in ovarian tissue weight. While not actively monitored, no remarkable changes were observed in food intake among the treatment groups. If SeMet treated females developed an aversion to the food, a reduction in tissue Se concentrations, energy stores and organo-somatic indices would likely be pronounced. Both the tissue moisture content and energy reserves (LSI, FBSI, triglyceride, glycogen) indicated no dose-dependent changes associated with malnourishment (Merkle and Hanke, 1988).

The results of the present study in combination with prior related research suggests the tissue thresholds for Se in fish put forth by the US EPA in their draft criterion will be protective for amphibians. The EC_{10} for egg based tissue thresholds related to teratogenic abnormalities in *X. laevis* was estimated at 44.9 μg Se/g d.m. (Massé et al., 2015). Using the regression equations generated in the present study, the corresponding EC_{10} for female ovarian and muscle tissue thresholds would be 50.1 and 11.5 μg Se/g d.m., respectively. The US EPA proposes egg-ovary and muscle tissue thresholds for Se to be 15.8 and 11.3 μg Se/g d.m., respectively, for freshwater fishes (US EPA, 2015); hence, this criterion should be protective for early life stage amphibians as well. In addition, *X. laevis* adult females exposed to elevated dietary Se levels exhibited minimal to no toxic effects related to body condition indices, energetic status, and physiological stress response even at concentrations well above (94.2 μg Se/g d.m.) those producing comparable sublethal toxic effects in fishes (< 30 μg Se/g d.m.) (Janz et al., 2010; Thomas and Janz, 2011; Wiseman et al., 2011; Thomas et al., 2013; McPhee and Janz, 2014). To date, investigations into the toxic effects of Se on amphibians indicate that adult female amphibians

and their progeny are relatively more tolerant to elevated dietary and *in ovo* Se levels when compared to most fishes. However, further research is required to elucidate if similar results are generated in native North American species with very diverse reproductive and foraging strategies, as well as differing ecological niches and physiology, considering *X. laevis* is known to be a relatively resilient anuran within this taxon.

CHAPTER 5

GENERAL DISCUSSION

5.1 Introduction

The Canadian mining industry contributed $54 billion to the Gross Domestic Product in 2013 making it a major economic driver in this country (Marshall, 2015); however, being one of the largest mining nations in the world does have consequences. The Elk River Valley of southeastern British Columbia is a noteworthy site of continuous Se contamination due to mining operations. Teck Coal Limited has five open-pit coal mines situated in the valley that produce 70% of Canada's total annual coal exports (BC MEM, 2015). In 2013, these mines produced 25.3 million tonnes of coal (BC MEM, 2015). The high volume of waste rock produced from these mines is deposited in valley fills, which leach and drain into the Elk River watershed. Effluent discharges have been reported to exceed 300 µg Se/L with surrounding ponds and marshes ranging from 50 to 80 µg Se/L and the main Elk River ranging from 5.8 to 9.6 µg Se/L (Young et al., 2010a). The high degree of Se loading in lotic, lentic and marsh ecosystems of this watershed has resulted in bioaccumulation throughout the food web with early life stage toxicities reported in fishes, birds and frogs. Columbia spotted frog (*Rana luteiventris*) embryos collected from an impacted site in the Elk River Valley had Se concentrations ranging from 10 to 38 µg Se/g d.m with a greater incidence of spinal deformities observed in the area (Young et al., 2010a). To date, the risk attributable solely to Se contamination particularly in regards to amphibians within the Elk River Valley has remained indiscernible due to numerous confounding factors (e.g., water chemistry, hydrology) and difficulties with sample collection (Young et al., 2010a). Within a

broader scope of environmental Se contamination, amphibian populations may play a key role in the transfer of elevated levels of dietary Se to more sensitive species in addition to being subjected to adverse effects themselves.

5.2 Amphibians as victims and vectors of Se toxicity

Amphibians play a fundamental role in both aquatic and terrestrial ecosystems. A year long survey of an isolated 10 ha freshwater wetland on the Savannah River Site reported the presence of 24 amphibian species (i.e. 17 anuran, 7 salamanders) that produced a high number of metamorphic amphibians (> 360,000 individuals) equating to > 1400 kg of biomass; thus, demonstrating the tremendous capacity of this class of vertebrate to guide the energy and nutrient flow within a food web (Whitfield Gibbons et al., 2006). A number of field studies have demonstrated the contributions of tadpoles, frogs and salamanders to food web regulation and ecosystem stability by their ability to profoundly influence invertebrate populations, primary production, nutrient cycling and leaf litter decomposition in addition to providing an influx of energy and nutrients to nearby aquatic and terrestrial habitats with their seasonal migrations and emergences (Beard et al., 2002; Davic and Welsh, 2004; Ranvestel et al., 2004; Regester et al., 2006). Within the contents of Chapters 2 and 4 of this thesis, my results demonstrate that female *X. laevis* tissues and eggs can accumulate high concentrations of Se (i.e. up to 226 µg Se/g d.m.) from dietary sources, which coincide with an increase (i.e. up to 92.5%) in the occurrence of morphological abnormalities in their progeny. Although dietary exposure of late stage larval *X. laevis* to elevated levels of Se was not within the scope of this thesis, previous research has documented that bullfrog larvae (*Rana catesbeiana*) collected from a swamp containing coal combustion wastes accumulated higher levels of Se than snails (*Helisoma trivolvis*), clams

(*Corbicula fluminea*), odonate larvae (*Tramea* sp. and *Erythemis* sp.), eastern mosquitofish (*Gambusia holbrooki*), juvenile spotted sunfish (*Lepomis punctatus*) and juvenile largemouth bass (*Micropterus salmoides*) from the same site (Unrine et al., 2007). Consequently, this data indicates that amphibians could be significant vectors of Se transfer between aquatic and terrestrial habitats, and a major Se exposure pathway for birds, fish and mammals (Unrine et al., 2007). Moreover, the adverse effects of elevated Se on the development of larval amphibians could contribute to population declines and ultimately ecosystem instability surrounding contaminated sites.

5.3 Effects of elevated *in ovo* Se exposure on the early stages of larval development in *X. laevis*

Within chapter 2 of this thesis, my data indicates that amphibians, as represented by the model amphibian *X. laevis*, are a more tolerant taxon to elevated *in ovo* selenium concentrations than most fishes and birds studied to date. EC_{10} values relating to larval mortality and deformities in fish have predominantly ranged from 17 to 24 μg Se/g egg d.m. in fishes, while EC_{10} values for impaired hatchability and teratogenic effects in birds has typically ranged from 5-37 μg Se/g egg d.m. (Janz et al., 2010). However, sensitivity within vertebrate classes can vary considerably with tolerant species such as the Dolly Varden trout (*Salvelinus malma*) and the American avocet (*Recurvirostra americana*) having calculated EC_{10} values of 54 and 74 μg Se/g egg d.m., respectively, related to teratogenic effects (Janz et al., 2010). The analogous EC_{10} value for *X. laevis* was estimated at 44.9 μg Se/g egg d.m. in my research, indeed making it more tolerant to Se toxicity than most fish and bird species studied to date with regards to this particular sensitive toxicological endpoint, but without further comparable studies involving

other anuran species it is difficult to determine if *X. laevis* is a tolerant, average or sensitive species within this taxon.

Alterations to the structure of the ocular lens of 5 dpf *X. laevis* tadpoles exposed to elevated *in ovo* Se concentrations was a distinct teratogenic abnormality detected in my extensive deformity analysis of early life stage larval anuran development in Chapter 2. Although the relationship between Se toxicity and the eye lens has been documented as cataractogenesis in fishes and mammals previously (Woock et al., 1987; Lemly, 1993b, 2002; Manikandan et al., 2009; Choudbury et al., 2015) it was novel to observe alterations to the contour of the eye lens as early as yolk resorption in larval *X. laevis*. As mentioned in Chapter 2, oxidative damage to essential components of lens epithelial cells or fiber cells through the excessive accumulation and subsequent metabolism of SeMet could generate the malformations observed in my research by interfering with numerous biochemical and molecular processes required for proper development. However, it is important to remark upon the role abnormal musculoskeletal structure and neurotoxicity could contribute to lens shape and position within the ocular cavity. Abnormal lens and craniofacial structure were the two most sensitive indicators of *in ovo* selenium toxicity in 5 dpf tadpoles with estimated EC_{10} values of 43.4 and 48.6 μg Se/g egg d.m., respectively, thus demonstrating that these particular abnormalities were often observed simultaneously within individual tadpoles. Consequently, alterations to the proportions and angles of craniofacial bone structure and the origins of associated ocular musculature could interfere with the biomechanical dynamics required for proper anatomical positioning and functional visual accommodation of the lens. Additionally, the association between Se toxicity and motor neuron degeneration offers an alternative explanation to the atypical lens structure observed in this thesis research (Vinceti et al., 2010; Maraldi et al., 2011;

Estevez et al., 2012). The contraction of the protractor lentis muscle moves the lens forward and away from the retina along the optic axis during visual accommodation (Douglas et al., 1986); however, paralysis of this muscle through neuromuscular toxicity would result in the lens being pulled toward the retina giving the appearance of being flattened and recessed. Nonetheless, further investigation is required to elucidate the mechanisms involved between elevated *in ovo* Se concentrations and abnormal eye lens development in oviparous vertebrates.

5.4 Effects of elevated *in ovo* Se exposure on the late stages of larval development in *X. laevis*

In Chapter 3, elevated *in ovo* Se concentration had minimal adverse effect on the survival or maturation of late stage larval *X. laevis* aside from the disadvantages connected to the generation of deformities within the first five days after fertilization. As mentioned previously, the results obtained related to frequency of mortality, time to metamorphosis and growth could be attributed to differing tadpole density among treatment groups, the promoting influence of kinship and varying embryo Se concentrations within a clutch. In a previous laboratory study, larval Cope's gray tree frogs (*Hyla chrysolscelis*) fed a diet of 50.1 μg Se/ g d.m. exhibited decreased growth and survival through metamorphic climax in addition to the induction of hind limb deformities (Lockard et al., 2013). While the dietary Se concentrations utilized in this study were extremely high, it does highlight the possibility that combined *in ovo* and dietary exposure of larval anuran could exacerbate Se's influence on growth and development during these early life stages. Moreover, the amphibian is a unique model organism for investigating the impact excessive Se exposure could have on thyroid hormonal status in embryonic development and metamorphosis as well as immunocompetence. Data concerning the influence of Se toxicity on

thyroid physiology is negligible in the literature even though the thyroid gland typically has the highest selenium content per gram of tissue among organs and its efficient protection and functioning is extremely dependent on adequate Se consumption (Köhrle, 1999). Thus, the thyroid gland could be a particularly vulnerable organ to oxidative damage produced through excessive SeMet consumption, tissue accumulation and ultimately biotransformation into reactive oxygen species. While thyroid hormone status is vital to proper development and successful metamorphosis in larval anurans, increased disease susceptibility due to innate immune system suppression and feeding cessation during metamorphic climax could impact survival as well (Murphy et al., 2000). Consequently, it is unknown the ranges of *in ovo* and dietary Se concentrations that could potentially hinder or promote the health and successful completion of metamorphosis among larval anuran.

5.5 Effects of chronically elevated dietary Se exposure on adult *X. laevis* females

The focus of research regarding Se toxicity in birds and fishes is predominantly focused on either the effects of maternal transfer on developing offspring or the effects of dietary Se exposure on juvenile fitness leaving minimal data associated with the impact elevated dietary Se levels could have on non-reproductive endpoints of adult fitness for comparison (Janz et al., 2010). Adult zebrafish fed a 27.5 μg/g d.m. Se diet exhibited both increased mortality as well as enhanced growth and energy status when compared to the control group (Thomas et al., 2013) none of which were observed in my research. Instead, the results presented in Chapter 4 of this thesis showed that female *X. laevis* administered food augmented with L-selenomethione at concentrations up to 94.2 μg Se/g d.m. displayed no signs of mortality or reduced fitness due to chronic exposure other than a marginal reduction in weight and liver glycogen levels.

Comparably, a one year study investigating the metabolic performance of male southern toads (*Bufo terrestris*) collected from a Se-rich coal ash basin site and continually exposed to ash-contaminated sediment and food within microcosms reported similar results to the ones observed in this thesis (Ward et al., 2006). The male toads exhibited less weight gain and had a significantly reduced respiratory quotient after exercise with no adverse effects related to standard or exercise metabolic rate, plasma glucose levels, and hepatic or muscle percentage indices overall (Ward et al., 2006). A reduced respiratory quotient with prolonged exercise suggests a greater reliance on protein metabolism for energy instead of carbohydrates (i.e. glycogen) in the ash-exposed toads, which is possibly related to diminished energy reserves from weight loss (Ward et al., 2006). Although female *X. laevis* health remained relatively unchanged over the 68 day dietary Se exposure period, my data indicates that diminishing biotic performance and negative physiological consequences in adult anurans are likely over time, particularly in relation to energy expensive behaviours such as breeding, hibernation/estivation and migration.

5.6 The contribution of Se toxicity to declining amphibian populations

The results of my thesis research in its entirety suggest that amphibians are more robust within Se-contaminated environments than other oviparous vertebrate species; however, as a group this taxon is experiencing unprecedented population declines demonstrating their extreme vulnerability to stressors such as habitat destruction, climate change (i.e. increased UV radiation) and pathogens in addition to chemical contamination (Buck et al., 2012; Li et al., 2013; Yu et al., 2015). As demonstrated in my thesis, anurans have the capacity for effective physiological and behavioural compensation when confronted with elevated dietary and *in ovo* Se exposure

exclusively, but understandably the substantial strain produced by multiple environmental stressors could exceed their capability to manage and thrive. In fact, one of the distinctive features of amphibians, their permeable skin, could potentially be the location where Se toxicity, UV radiation, and *Batrachochytrium dendrobatidis* unite to produce a fatal outcome. For decades, excessive dietary Se consumption in human case studies has demonstrated its connection with highly keratinized areas of the body with chronic exposure resulting in skin lesions, alopecia and brittle nails possibly due to regional Se-induced oxidative damage (Nuttall, 2006). The aquatic fungal pathogen *Batrachochytrium dendrobatidis* attacks keratin contained within the outer layers of post-metamorphic amphibian skin or mouthparts of tadpoles causing chytridiomycosis, a disease devastating global amphibian populations (Buck et al., 2012; Li et al., 2013). Moreover, the increase in UV radiation reaching the troposphere results in irradiation of the skin that impairs natural antioxidant defenses (e.g., gluthathione peroxidase) resulting in the generation of high levels of reactive oxygen species in vulnerable cell types like melanocytes, keratinocytes and fibroblasts (McKenzie, 2000; Burke et al., 2003; Farmer et al., 2003; Denat et al., 2014). Thus, the oxidative damage produced through elevated UV and Se exposure to key cell types may compromise the skin's ability to act as a protective barrier against pathogens as well as perform other functions such as osmoregulation, thermoregulation, hydration and cutaneous gas exchange (Clarke, 1997). Although dietary and *in ovo* Se toxicity is a potential contributor to declining amphibian populations at contaminated sites, there remains vast uncertainties as to Se's behaviour within an amphibian biological model and the definitive impact it has on this distinct and diverse taxon.

5.7 Recommendations for future research

While the sum of my thesis research provides a foundation for a largely under-studied area of Se-related toxicology, further research is necessary to gain a comprehensive understanding of the threat amphibian populations are confronted with at Se contaminated sites. A few areas that require further investigation are listed below.

- In the present study, I provided a baseline for dietary and *in ovo* Se toxicity in adult and larval anurans, respectively, using the model species, *Xenopus laevis*. Additional laboratory-based studies exploring these two routes of Se exposure and the impact on both maternal fitness and larval development in native North American species with differing reproductive strategies and life histories are essential.
- In Chapter 2, an EC_{10} value pertaining to early life stage teratogenic abnormalities in *X. laevis* was estimated. To adequately protect anuran species from *in ovo* Se toxicity, further research to develop similar EC_{10} values for both anuran and urodele native North American species is required.
- In Chapter 2, evidence that *in ovo* Se toxicity induced ocular lens abnormalities in 5 dpf *X. laevis* tadpoles was presented. Future research investigating the biochemical and molecular mechanisms of Se toxicity within the eye is necessary.
- In Chapter 3, elevated *in ovo* Se exposure had minimal effect on late stage larval survival, growth, and development. A comprehensive understanding of different exposure routes and their separate or combined effects (i.e. *in ovo*, dietary, *in ovo* and dietary) on late larval development should be investigated in multiple species of this taxon.
- In Chapter 3, the potential for elevated *in ovo* Se exposure to promote survival and growth in late stage larval *X. laevis* was speculated. An understanding of the possible

influence of elevated *in ovo* and/or dietary Se exposure on thyroid and energetic status in larval anurans and its relationship with metamorphosis could provide a unique perspective in Se toxicology research.

- In Chapter 4, adult females presented with no alterations to biometric indices, energetic status, or stress response despite extensive accumulation of Se in their tissues from elevated dietary consumption. However, a trend in weight loss and reduced liver glycogen stores in SeMet exposed females indicate a potential for the reduced ability of adults to cope effectively with natural stressors. Thus, further laboratory studies investigating the effect of Se toxicity on the capacity of both adult female and male anurans to perform energy expensive behaviours (i.e. hopping, mating calls, estivation/hibernation) should be undertaken.
- Within my thesis research, I administered elevated dietary concentrations of Se to adult female *X. laevis* by supplementing a commercially produced pelleted food source with SeMet. A comparison of Se's toxicological potency in relation to the varying nutritional composition of food sources (i.e. protein, carbohydrate, and lipid ratios) utilized in combination with either SeMet supplementation or inherently high Se concentrations could provide an explanation for differing results between similar dietary studies.

REFERENCES

Ahn, S., Koketsu, M., Ishihara, H., Lee, S., Ha, S., Lee, K., Kang, T., Kim, S., 2006. Regulation of melanin synthesis by selenium-containing carbohydrates. Chem. Pharm. Bull. 54, 281-286.

Altman, C.R., Chow, R.L., Lang, R.A., Hemmati-Brivanlou, A., 1997. Lens induction by Pax-6 in *Xenopus laevis*. Dev. Biol. 185, 119-123.

Amaya, E., Offield, M.F., Grainger, R.M., 1998. Frog genetics: *Xenopus tropicalis* jumps into the future. Trends Genet. 14, 253-255.

Atar-Zwillenberg, D.R., Atar, M., Spornitz, U.M., 2002. Hormonal regulation of the glycogen/glucose balance in *Xenopus laevis*. Microsc. Microanal. 8 (Suppl.2), 886-887.

Bantle, J.A., Dumont, J., Finch, R., Linder, G., 1991. Atlas of abnormalities: a guide for the performance of FETAX. Oklahoma State University Publications, Stillwater, OK.

BC MEM (British Columbia Ministry of Energy and Mines), 2015. British Columbia coal industry overview 2014. Mines and Mineral Resources Division, British Columbia Geological Survey Information Circular 2015-03. www.em.gov.bc.ca/geology.

BC MoE (British Columbia Ministry of Environment), 2014. Ambient water quality guidelines for selenium technical report update. Water Protection and Sustainability Branch, Environmental Sustainability and Strategic Policy Division, Victoria, BC, pp.1-270.

Beard, K.H., Vogt, K.A., Kulmatiski, A., 2002. Top-down effects of a terrestrial frog on forest nutrient dynamics. Oecologia 133, 583-593.

Bennett, P.M., Janz, D.M., 2007. Bioenergetics and growth of young-of the-year northern pike (*Esox lucius*) and burbot (*Lota lota*) exposed to metal mining effluent. Ecotoxicol. Environ. Saf. 68, 1-12.

Bergeron, C.M., Bodinof, C.M., Unrine, J.M., Hopkins, W.A., 2010. Bioaccumulation and maternal transfer of mercury and selenium amphibians. Environ. Toxicol. Chem. 29, 989-997.

Bertram, P.E., Brooks, A.S., 1986. Kinetics of accumulation of selenium from food and water by fathead minnows. Water Res. 20, 877-884.

Birge, W.J., Westerman, A.G., Spromberg, J.A., 2000. Comparative toxicology and risk assessment of amphibians. In: Sparling, D.W., Linder, G., Bishop, C.A., (Eds.), Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola, FL. pp. 727-791.

Blaustein, A.R., Waldman, B., 1992. Kin recognition in anuran amphibians. Anim. Behav. 44, 207-221.

Brachet, J., 1979. Oogenesis and maturation in amphibian oocytes. Endeavour 3, 144-149.

Bridges, C.M., Dwyer, F.J., Hardesy, D.K., Whites, D.W., 2002. Comparative contaminant toxicity: Are amphibian larvae more sensitive than fish? Bull. Environ. Contam. Toxicol. 60, 562-569.

Brown, D.D., Cai, L., 2007. Amphibian metamorphosis. Dev. Biol. 306, 20-33.

Bryne, J.J., White, R.J., 1975. Cyclic changes in liver and muscle glycogen tissue lipid and blood glucose in a naturally occurring population of *Rana catesbeiana*. Comp. Biochem. Physiol. A 50, 709-715.

Buck, J.C., Scheessele, E.A., Relyea, R.A., Blaustein, A.R., 2012. The effects of multiple stressors on wetland communities: pesticides, pathogens and competing amphibians. Freshwater Biol. 57, 61-73.

Burger, J., Snodgrass, J., 2000. Oral deformities in several species of frogs from the Savannah River Site, USA. Environ. Toxicol. Chem. 19, 2519-2524.

Burke, K.E., Clive, J., Combs, G.F., Nakamura, R.M., 2003. Effects of topical L-selenomethionine with topical and oral vitamin E on pigmentation and skin cancer induced by ultraviolet irradiation in Skh:2 hairless mice. J. Am. Acad. Dermatol. 49, 458-472.

Cannatella, D.C., de Sa, R.O., 1993. *Xenopus laevis* as a model organism. Syst. Biol. 42, 476-507.

CCME (Canadian Council of Ministers of Environment), 2007. Canadian water quality guidelines for the protection of aquatic life: Summary table. In: Canadian environmental quality guidelines, 1999, Canadian Council of Ministers of Environment, Task Force on Water Quality Guidelines, Winnipeg, MB, pp.1-9.

Cenedella, R.J., 1989. Cell cycle specific effects of selenium on the lens epithelium studied *in vivo* by the direct chemical approach. Curr. Eye Res. 8, 429-433.

Choudhury, S., Thomas, J.K., Sylvain, N.J., Ponomarenko, O., Gordon, R.A., Heald, S.M., Janz, D.M., Krone, P.H., Coulthard, I., Geoge, G.N., Pickering, I.J., 2015. Selenium preferentially accumulates in the eye lens following embryonic exposure: a confocal x-ray fluorescence imaging study. Environ. Sci. Technol. 49, 2255-2261.

Clarke, B.T., 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol. Rev. 72, 365-379.

Cockrem, J.F., 2013. Individual variation in glucocorticoid stress responses in animals. Gen. Comp. Endocr. 181, 45-48.

Comai, L., 2005. The advantages and disadvantages of being polyploid. Nat. Rev. Genet. 6, 836-846.

Dantzer, B., Fletcher, Q.E., Boonstra, R., Sheriff, M.J., 2014. Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? Conserv. Physiol. 2, 1-18.

Dash, M.C., Hota, A.K., 1980. Density effects on the survival, growth rate, and metamorphosis of *Rana tigrina* tadpoles. Ecology 61, 1025-1028.

Davic, R.D., Welsh, H.H., 2004. On the ecological role of salamanders*. Annu. Rev. Ecol. Evol. Syst. 35, 405-434.

deBruyn, A.M., Hodaly, A., Chapman, P.M., 2008. Tissue selection criteria: selection of tissue types for the development of a meaningful selenium tissue threshold in fish. In: Selenium tissue thresholds: tissue selection criteria, threshold development endpoints, and potential to predict population or community effects in the field. Submitted to North America Metals Council – Selenium Working Group, Washington, DC, USA, pp. 1-32.

DeForest, D.K., Gilron, G., Armstrong, S.A., Robertson, E.L., 2011. Species sensitivity distribution evaluation for selenium in fish eggs: considerations for development of a Canadian tissue-based guideline. Integr. Environ. Assess. Manage. 8, 6-12.

Denat, L., Kadekaro, A.L., Marrot, L., Leachman, S.A., Abdel-Malek, Z.A., 2014. Melanocytes as instigators and victims of oxidative stress. J. Invest. Dermatol. 134, 1512-1518.

Denver, R.J., 2009a. Stress hormones mediate environment-genotype interactions during amphibian development. Gen. Comp. Endocr. 164, 20-31.

Denver, R.J., 2009b. Structural and functional evolution of vertebrate neuroendocrine stress systems. Ann. NY Acad. Sci. 1163, 1-16.

Dinsmore II, S.C., Swanson, D.L., 2008. Temporal patterns of tissue glycogen, glucose, and glycogen phosphorylase activity prior to hibernation in freeze-tolerant chorus frogs, *Pseudacris triseriata*. Can. J. Zool. 86, 1095-1100.

Douglas, R.H., Collet, T.S., Wagner, H.J., 1986. Accommodation in anuran Amphibia and its role in depth vision. J. Comp. Physiol. A 158, 133-143.

Driedger, K., Weber, L.P. Rickwood, C.J., Dubé, M.G., Janz, D.M., 2010. Growth and energy storage in juvenile fathead minnows exposed to metal mine waste water in simulated winter and summer conditions. Ecotoxicol. Environ. Saf. 73, 727-734.

Dumont, J.N., 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. J. Morphol. 136, 153-179.

Elinson, R.P., del Pino, E.M., 2012. Developmental diversity of amphibians. WIREs Dev. Biol. 1, 345-369.

Estevez, A.O., Mueller, C.L., Morgan, K.L., Szewczyk, N.J., Teece, L., Miranda-Vizuete, A., Estevez, M., 2012. Selenium induces cholinergic motor neuron degeneration in *Caenorhabditis elegans*. NeuroToxicology 33, 1021-1032.

Falso, P.G., Noble, C.A., Diaz, J.M., Hayes, T.B., 2015. The effect of long-term corticosterone treatment on blood cell differentials and function in laboratory and wild-caught amphibian models. Gen. Comp. Endocr. 212, 73-83.

Fan, T.W.M., Teh, S.J., Hinton, D.E., Higashi, R.M., 2002. Selenium biotransformations into proteinaceous forms by foodweb organisms of selenium-laden drainage waters in California. Aquat. Toxicol. 57, 65-84.

Farmer, P.J., Gidanian, S., Shahandeh, B., Di Bilio, A.J., Tohidian, N., Meyskens, F.L., 2003. Melanin as a target for melanoma chemotherapy: pro-oxidant effect of oxygen and metals on melanoma viability. Pigm. Cell Res. 16, 273-279.

Ferrie, G.M., Alford, V.C., Atkinson, J., Baitchman, E., Barber, D., Blaner, W.S., Crawshaw, G., Daneault, A., Dierenfeld, E., Finke, M., Fleming, G., Gagliardo, R., Hoffman, E.A., Karasov, W., Klasing, K., Koutsos, E., Lamkton, J., Lavin, S.R., Lentini, A., Livingston, S.; Lock, B., Mason, T., McComb, A., Morris, C., Pessier, A.P., Olea-Popelka, F., Probst, T., Rodriguez, C., Schad, K., Semmen, K., Sincage, J., Stamper, M.A., Steinmetz, J., Sullivan, K., Terrell, S., Wertan, N., Wheaton, C.J., Wilson, B., Valdes, E.V., 2014. Nutrition and health in amphibian husbandry. Zoo Biol. 33, 485-501.

Fitzpatrick, L.C., 1979. Life-history patterns of storage and utilization of lipids for energy in amphibians. Am. Zool. 16, 725-732.

Formation Environmental. HabiTech Inc., 2012. Technical support document: proposed site-specific selenium criterion, Sage and Crow Creeks, Idaho. Prepared for J.R. Simplot Company, pp.1-256.

Franson, J.C., Wells-Berlin, A., Perry, M.C., Shearn-Bochsler, V., Finley, D.L., Flint, P.L., Hollmén, T., 2007. Effects of dietary selenium on tissue concentrations, pathology, oxidative stress, and immune function in common eiders (*Somateria mollissima*). J. Toxicol. Environ. Health A 70, 861-874.

Gilbert, S.F., 2000. Developmental biology (6th Ed.). Sinauer Associates, Sunderland, MA.

Girish, S., Saidapur, S.K., 2003. Density-dependent growth and metamorphosis in the larval bronze frog *Rana temporalis* is influenced by genetic relatedness of the cohort. J. Biosci. 28, 489-496.

Gladyshev, V.N., 2012. Selenoproteins and selenoproteomes. In: Hatfield, D.L., Berry, M.J., Gladyshev, V.N., (Eds.), Selenium: its molecular biology and role in human health. Springer Science and Business Media, New York, NY, pp. 109-123.

Goertzen, M.M., Driessnack, M.K., Janz, D.M., Weber, L.P., 2011. Swimming performance and energy homeostasis in juvenile laboratory raised fathead minnow (*Pimephales promelas*) exposed to uranium mill effluent. Comp. Biochem. Physiol. C 154, 420-426

Gómez-Lechón, M.J., Ponsoda, X., Castel, J.V., 1996. A microassay for measuring glycogen in 96-well-cultured cells. Anal. Biochem. 236, 296-301.

Green, S.L., 2002. Factors affecting oogenesis in the South African clawed frog (*Xenopus laevis*). Comparative Med. 52, 307-312.

Green, S.L., 2010. The laboratory *Xenopus* sp.. CRC Press, Boca Raton, FL, USA.

Gurdon, J.B., 1996. Introductory comments: *Xenopus* as a laboratory animal. In: Tinsley, R.C., Kobel, H.R., (Eds.), The biology of *Xenopus*. Oxford University Press Inc., New York, NY, pp. 3-6.

Gurushankara, H.P., Meenakumari, D., Krishnamurthy, S.V., Vasudev, V., 2007. Impact of malathion stress on lipid metabolism in *Limnonectus limnocharis*. Pestic. Biochem. Physiol. 88, 50-56.

Hamilton, S.J., 2004. Review of selenium toxicity in the aquatic food chain. Sci. Total Environ. 326, 1-31.

Hardy, R.W., Oram, L.L., Möller, G., 2010. Effects of dietary selenomethionine on cutthroat trout (*Oncorhynchus clarki bouvieri*) growth and reproductive performance over a life cycle. Arch. Environ. Contam. Toxicol. 58, 237-245.

Henry, P.F.P., 2000. Aspects of amphibian anatomy and physiology. In: Sparling, D.W., Linder, G., Bishop, C.A., (Eds.), Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola, FL. pp. 71-110.

Herman, C.A., 1992. Endocrinology. In: Feder, M.E., Burggren, W.W., (Eds.), Environmental physiology of the amphibians. University of Chicago Press, Chicago, IL, pp.40-54.

Hilken, G., Dimigen, J., Iglauer, F., 1995. Growth of *Xenopus laevis* under different laboratory rearing conditions. Lab. Anim. 29, 152-162.

Hilton, J.W., Hodson, P.V., Slinger, S.J., 1982. Absorption, distribution, half-life and possible routes of elimination of dietary selenium in juvenile rainbow trout (*Salmo gairdneri).* Comp. Biochem. Physiol. C 71, 49-55.

Hodson, P.V., Hilton, J.W., 1983. The nutritional requirements and toxicity to fish of dietary and waterborne selenium. Ecol. Bull. 35, 335-340.

Hoffman, D.J., Heinz, G.H., 1988. Embryonic and teratogenic effects of selenium in the diet of mallards. J. Toxicol. Environ. Health 24, 477-490.

Holm, J., Palace, V., Siwik, P., Sterling, G., Evans, R., Baron, C., Werner, J., Wautier, K., 2005. Developmental effects of bioaccumulated selenium in eggs and larvae of two salmonid species. Environ. Toxicol. Chem. 24, 2373-2381.

Hopkins, W.A., Congdon, J.D., Ray, J.K., 2000. Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. Environ. Toxicol. Chem. 19, 862−868.

Hopkins, W.A., DuRant, S.E., Staub, B.P., Rowe, C.L., Jackson, B.P., 2006. Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. Environ. Health Perspect. 114, 661−666.

Hopkins, W.A., Mendonça, M.T., Congdon, J.D., 1999. Responsiveness of the hypothalamo-pituitary-internal axis in an amphibian (*Bufo terrestris*) exposed to coal combustion wastes. Comp. Biochem. Physiol. C 122, 191-196.

Hopkins, W.A., Mendonça, M.T., Rowe, C.L., Congdon, J.D., 1998. Elevated trace element concentrations in southern toads, *Bufo terrestris*, exposed to coal combustion waste. Arch. Environ. Contam. Toxicol. 35, 325-329.

Janz, D.M., 2012. Selenium. In: Wood, C.W., Farrell, A.P., Brauner, C.J., (Eds.), Fish Physiology Vol 31A, Homeostasis and toxicology of essential metals. Elsevier, San Diego, CA, pp. 327-374.

Janz, D.M., Deforest, D.K., Brooks, M.L., Chapman, P.M., Gilron, G., Hoff, D., Hopkins, W.A., McIntyre, D.O., Mebane, C.A., Palace, V.P., Skorupa, J.P., Wayland, M., 2010. Selenium toxicity to aquatic organisms. In: Chapman, P.M., Adams, W.J., Brooks, M.L, Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., (Eds.), Ecological assessment of selenium in the aquatic environment. CRC Press, Boca Raton, FL, pp. 141-231.

Janz, D.M., Liber, K., Pickering, I.J., Wiramanaden, C.I., Weech, S.A., Gallego-Gallegos, M., Driessnack, M.K., Franz, E.D., Goertzen, M.M., Phibbs, J., Tse, J.J., Himbeault, K.T., Robertson, E.L., Burnett-Seidel, C., England, E., Gent, A., 2014. Integrative assessment

of selenium speciation, biogeochemistry, and distribution in a northern coldwater ecosystem. Integr. Environ. Assess. Manag. 10, 543-554.

Kelly, J.M., Janz, D.M., 2008. Altered energetics and parasitism in juvenile northern pike (*Esox lucius*) inhabiting metal-mining contaminated lakes. Ecotoxicol. Environ. Saf. 70, 357-369.

Kennedy, C.J., McDonald, L.E., Loveridge, R., Strosher, M.M., 2000. The effect of bioaccumulated selenium on mortalities and deformities in the eggs, larvae, and fry of a wild population of cutthroat trout (*Oncorhynchus clarki lewisi*). Arch. Environ. Contam. Toxicol. 39, 46-52.

Kerby, J.L., Richards-Hrdlicka, K.L., Storfer, A., Skelly, D.K., 2010. An examination of amphibian sensitivity to environmental contaminants: Are amphibians poor canaries? Ecol. Lett. 13, 60-67.

Kime, D.E., Nash, J.P., Scott, A.P., 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. Aquaculture 177, 345-352.

Köhrle, J., 1999. The trace element selenium and the thyroid gland. Biochimie 81, 527-533.

Lemly, A.D., 1985. Toxicology of selenium in a freshwater reservoir: implications for environmental hazard evaluations and safety. Ecotox. Environ. Safe. 34, 223-227.

Lemly, A.D., 1993a. Metabolic stress during winter increases the toxicity of selenium in fish. Aquat. Toxicol. 27, 133-158.

Lemly, A.D., 1993b. Teratogenic effects of selenium in natural populations of freshwater fish. Ecotox. Environ. Safe. 26, 181-204.

Lemly, A.D., 1997a. A teratogenic deformity index for evaluating impacts of selenium on fish populations. Ecotox. Environ. Safe. 37, 259-266.

Lemly, A.D., 1997b. Ecosystem recovery following selenium contamination in a freshwater reservoir. Ecotox. Environ. Safe. 36, 275-281.

Lemly, A.D., 2002. Symptoms and implications of selenium toxicity in fish: the Belews Lake case example. Aquat. Toxicol. 57, 39-49.

Lemly, A.D., 2004. Aquatic selenium pollution is a global environmental safety issue. Ecotox. Environ. Safe. 59, 44-56.

Li, Y., Cohen, J.M., Rohr, J.R., 2013. Review and synthesis of the effects of climate change on amphibians. Integr. Zool. 8, 145-161.

Lin, Y.H., Shiau, S.Y., 2005. Dietary selenium requirements of juvenile grouper, *Epinephelus malabaricus*. Aquaculture 250, 356-363.

Linder, G., Palmer, B.D., Little, E.E., Rowe, C.L., Henry, P.F.P., 2010. Physiological ecology of amphibians and reptiles: natural history and life history attributes framing chemical exposure in the field. In: Sparling, D.W., Linder, G., Bishop, C.A., Krest, S.K., (Eds.), Ecotoxicology of amphibians and reptiles (2nd Ed.). CRC Press, Pensacola, FL, pp. 105-166.

Lobanov, A.V., Hatfield, D.L., Gladyshev, V.N., 2009. Eukaryotic selenoproteins and selenoproteomes. Biochim. Biophys. Acta 1790, 1424-1428.

Lockard, L., Rowe, C.L., Heyes, A., 2013. Dietary selenomethionine exposure induces physical malformations and decreases growth and survival to metamorphosis in an amphibian (*Hyla chrysoscelis*). Arch. Environ. Contam. Toxicol. 64, 504-513.

Luoma, S.M., Presser, T.S., 2009. Emerging opportunities in management of selenium contamination. Environ. Sci. Technol. 43, 8483-8487.

Maher, W., Roach, A., Doblin, M., Fan, T., Foster, S., Garrett, R., Möller, G., Oram, L., Wallschläger, D., 2010. Environmental sources, speciation, and partitioning of selenium. In: Chapman, P.M., Adams, W.J., Brooks, M., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., (Eds.), Ecological assessment of selenium in the aquatic environment. CRC Press, Boca Raton, FL, pp. 47-92.

Manikandan, R., Thiagarajan, R., Beulaja, S., Chindhu, S., Mariammal, K., Suhandiran, G., Arumugam, M., 2009. Anti-cataractogenic effect of curcumin and aminoguanidine against selenium-induced oxidative stress in the eye lens of Wistar rat pups: an *in vitro* study using isolated lens. Chem-Biol. Interact. 181, 202-209.

Maraldi, T., Riccio, M., Zambonin, L., Vinceti, M., De Pol, A., Hakim, G., 2011. Low levels of selenium compounds are selectively toxic for a human neuron cell line through ROS/RNS increase and apoptotic process activation. NeuroToxicology 32, 180-187.

Mariotti, M., Ridge, P.G., Zhang, Y., Lobanov, A.V., Pringle, T.H., Guigo, R., Hatfield, D.L., Gladyshev, V.N., 2012. Composition and evolution of the vertebrate and mammalian selenoproteomes. *PLoS ONE* 7, e33066. doi: 10.1371/journal.pone.0033066.

Marshall, B., 2015. Facts and figures of the Canadian mining industry 2014. The Mining Association of Canada. http://mining.ca/documents/facts-and-figures-2014.

Massé, A.J., Muscatello, J.R., Janz, D.M., 2015. Dose-dependent early life stage toxicities in *Xenopus laevis* exposed *in ovo* to selenium. Environ. Sci. Technol. 49, 13658-13666.

McDiarmid, R.W., Mitchell, J.C., 2000. Diversity and distribution of amphibians and reptiles. In: Sparling, D.W., Linder, G., Bishop, C.A., (Eds.), Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola, FL. pp. 15-69.

McGowan, M.W., Artiss, J.D., Strandbergh, D.R., Zak, B., 1983. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clin. Chem. 29, 538-542.

McKenzie, R.C., 2000. Selenium, ultraviolet radiation and the skin. Clin. Exp. Dermatol. 25, 631-636.

McPhee, D.L., Janz, D.M., 2014. Dietary selenomethionine exposure alters swimming performance, metabolic capacity and energy homeostasis in juvenile fathead minnow. Aquat. Toxicol. 155, 91-100.

Merkle, S., 1989. Sexual differences as adaptation to the different gender roles in the frog *Xenopus laevis* Daudin. J. Comp. Physiol. B 159, 473-480.

Merkle, S., Hanke, W., 1988. Long-term starvation in *Xenopus laevis* Daudin – II. Effects on several organs. Comp. Biochem. Physiol. A 90, 491-495.

Metts, B.S., Buhlmann, K.A., Scott, D.E., Tuberville, T.D., Hopkins, W.A., 2012. Interactive effects of maternal and environmental exposure to coal combustion wastes decrease survival of larval southern toads (*Bufo terrestris*). Environ. Pollut. 164, 211-218.

Metts, B.S., Buhlmann, K.A., Tuberville, T.D., Scott, D.E., Hopkins, W.A., 2013. Maternal transfer of contaminants and reduced reproductive success of southern toads (*Bufo [Anaxyrus] terrestris*) exposed to coal combustion waste. Environ. Sci. Technol. 47, 2846-2853.

Miller, L.L., Wang, F., Palace, V.P., Hontela, A., 2007. Effects of acute and subchronic exposures to waterborne selenite on the physiological stress response and oxidative stress indicators in juvenile rainbow trout. Aquat. Toxicol. 83, 263-271.

Misra, S., Niyogi, S., 2009. Selenite causes cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes by inducing oxidative stress. Toxicol. in Vitro 23, 1249-1258.

MoEE (Ministry of Environment and Energy), 1994. Water management policies, guidelines, provincial water quality objectives of the Ministry of Environment and Energy. Ontario Ministry of Environment and Energy, Toronto, ON, pp.1-67.

Mónaco, M.E., Villecco, E.I., Sánchez, S.S., 2007. Implication of gap junction coupling in amphibian vitellogenin uptake. Zygote 15, 149-157.

Moore, F.L., Miller, L.J., 1984. Stress-induced inhibition of sexual behavior: corticosterone inhibits courtship behaviors of male amphibian (*Taricha granulosa*). Horm. Behav. 18, 400-410.

Mostafapour, M.K., Reddy, V.N., 1978. Studies on lens proteins. I. Subunit structure of beta crystallins of rabbit lens cortex. Invest. Ophthalmol. Visual Sci. 17, 660-665.

Murphy, J.E., Phillips, C.A., Beasley, V.R., 2000. Aspects of amphibian ecology. In: Sparling, D.W., Linder, G., Bishop, C.A., (Eds.), Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola, FL. pp.141-178.

Muscatello, J.R., Belknap, A.M., Janz, D.M., 2008. Accumulation of selenium in aquatic systems downstream of a uranium mining operation in northern Saskatchewan, Canada. Environ. Pollut. 156, 387-393.

Muscatello, J.R., Bennett, P.M., Himbeault, K.T., Belknap, A.M., Janz, D.M., 2006. Larval deformities associated with selenium accumulation in northern pike (*Esox lucius*) exposed to metal mining effluent. Environ. Sci. Technol. 40, 6506-6512.

Nakayama, T., Fisher, M., Nakajima, K., Odeleye, A.O., Zimmerman, K.B., Fish, M.B., Yaoita, Y., Chojnowski, J.L., Lauderdale, J.D., Netland, P.A., Grainger, R.M., 2015. *Xenopus pax6* mutants affect eye development and other organ systems, and have phenotypic similarities to human aniridia patients. Dev. Biol. 408, 328-344.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Garland Publishing Inc., New York, NY.

Norris, D.O., 2007. Vertebrate endocrinology (4th Ed.). Elsevier Academic Press, Burlington, MA.

NRC (National Research Council), 1993. Nutrient requirements of fish. National Academies Press, Washington, DC.

Nuttall, K.L., 2006. Evaluating selenium poisoning. Ann. Clin. Lab. Sci. 36, 409-420.

Ohlendorf, H.M., Hothem, R.L., Bunck, C.M., Marois, K.C., 1990. Bioaccumulation of selenium in birds at Kesterson Reservoir, California. Arch. Environ. Contam. Toxicol. 19, 495-507.

Ohlendorf, H.M., Kilness, A.W., Simmons, J.L., Stroud, R.K., Hoffman, D.J., Moore, J.F., 1988. Selenium toxicosis in wild aquatic birds. J. Toxicol. Environ. Health 24, 67-92.

Orr, P.L., Guiguer, K.R., Russel, C.K., 2006. Food chain transfer of selenium in lentic and lotic habitats of a western Canadian watershed. Ecotox. Environ. Safe. 63, 175-88.

Palace, V.P., Spallholz, J.E., Holm, J., Wautier, K., Evans, R.E., Baron, C.L., 2004. Metabolism of selenomethionine by rainbow trout (*Oncorhynchus mykiss*) embryos can generate oxidative stress. Ecotox. Environ. Safe. 58, 17-21.

Papp, L.V., Lu, J., Holmgren, A., Khanna, K., 2007. From selenium to selenoproteins: synthesis, identity, and their role in human health. Antioxid. Redox Sign. 9, 775-808.

Phibbs, J., Wiramanaden, C.I.E., Hauck, D., Pickering, I.J., Liber, K., Janz, D.M., 2011. Selenium uptake and speciation in wild and caged fish downstream of a metal mining and milling discharge. Ecotox. Environ. Safe. 74, 1139–1150.

Pinder, A.W., Storey, K.B., Ultsch, G.R., 1992. Estivation and hibernation. In: Feder, M.E., Burggren, W.W., (Eds.), Environmental physiology of the amphibians. The University of Chicago Press, Chicago, IL, pp. 250-274.

Popescu, B.F.G., Belak, Z.R., Ignatyev, K., Ovsenek, N., Nichol, H., 2007. Asymmetric distribution of metals in the *Xenopus laevis* oocyte: a synchrotron x-ray fluorescence microprobe study. Biochem. Cell Biol. 85, 537-542.

Pough, F.H., Magnusson, W.E., Ryan, M.J., Wells, K.D., Taigen, T.L., 1992. Behavioral energetics. In: Feder, M.E., Burggren, W.W., (Eds.), Environmental physiology of the amphibians. The University of Chicago Press, Chicago, IL, pp. 395-436.

Presser, T.S., Luoma, S.N., 2010. A methodology for ecosystem-scale modeling of selenium. Integr. Environ. Assess. Manage. 6, 685-710.

Presser, T.S., Ohlendorf, H.M., 1987. Biogeochemical cycling of selenium in the San Joaquin Valley, California, USA. Environ. Manage. 18, 437-454.

Puls, R., 1994. Mineral levels in animal health: diagnostic data (2nd ed.). Sherpa International, Clearbrook, BC.

Punshon, T., Jackson, B.P., Lanzirotti, A., Hopkins, W.A., Bertsch, P.M., Burger, J., 2005. Application of synchrotron x-ray microbeam spectroscopy to the determination of metal distribution and speciation in biological tissues. Spectrosc. Lett. 38, 343-363.

Raimondo, S.M., Rowe, C.L., 1998. Exposure to coal ash impacts swimming performance and predator avoidance in larval bullfrogs (*Rana catesbeiana*). J. Herpetol. 32, 289-292.

Ranvestel, A.W., Lips, K.R., Pringle, C.M., Whiles, M.R., Bixby, R.J., 2004. Neotropical tadpoles influence stream benthos: evidence for the ecological consequences of decline in amphibian populations. Freshwater Biol. 49, 274-285.

Rasar, M.A., Hammes, S.R., 2006. The physiology of the *Xenopus laevis* ovary. In: Liu, X.L., (Ed.), *Xenopus* protocols: cell biology and signal transduction. Humana Press, Totowa, NJ, pp. 17-30.

Reddy, C.C., Massaro, E.J., 1983. Biochemistry of selenium: an overview. Fundam. Appl. Toxicol. 3, 431-436.

Regester, K.J., Lips, K.R., Whiles, M.R., 2006. Energy flow and subsidies associated with the complex life cycle of ambystomatid salamanders in ponds and adjacent forest in southern Illinois. Oecologia 147, 303-314.

Reilly, C., 2006. Selenium in food and health (2nd ed.). Springer Science and Business Media, New York, NY.

Robinson, M.L., Lovicu, F.J., 2004. The lens: historical and comparative perspectives. In: Lovicu, F.J., Robinson, M.L., (Eds.), Development of the ocular lens. Cambridge University Press, New York, NY, pp. 3-26.

Roe, J.H., Hopkins, W.A., Durant, S.E., Unrine, J.M., 2006. Effects of competition and coal combustion wastes on recruitment and life history characteristics of salamanders in temporary wetlands. Aquat. Toxicol. 79, 176-184.

Rowe, C.L., 2014. Bioaccumulation and effects of metals and trace elements from aquatic disposal of coal combustion residues: recent advances and recommendations for further study. Sci. Total Environ. 485-486, 490-496.

Rowe, C.L., Heyes, A., Hilton, J., 2011. Differential patterns of accumulation and depuration of dietary selenium and vanadium during metamorphosis in the Gray Treefrog (*Hyla versicolor*). Arch. Environ. Contam. Toxicol. 60, 336-342.

Rowe, C.L., Heyes, A., Hopkins, W., 2009. Effects of dietary vanadium on growth and lipid storage in a larval anuran: results from studies employing ad libitum and rationed feeding. Aquat. Toxicol. 91, 179-186.

Rowe, C.L., Hopkins, W.A., Coffman, V.R., 2001. Failed recruitment of southern toads (Bufo terrestris) in a trace element-contaminated breeding habitat: direct and indirect effects that may lead to local population sink. Arch. Environ. Contam. Toxicol. 40, 339-405.

Rowe, C.L., Kinney, O.M., Congdon, J.D., 1998a. Oral deformities in tadpoles of the bullfrog (*Rana catesbeiana*) caused by conditions in a polluted habitat. Copeia 1, 244-246.

Rowe, C.L., Kinney, O.M., Fiori, A., Congdon, J.D., 1996. Oral deformities in tadpoles (*Rana catesbeiana*) associated with coal ash deposition: effects on grazing ability and growth. Freshwater Biol. 36, 723-730.

Rowe, C.L., Kinney, O.M., Nagle, R.D., Congdon, J.D., 1998b. Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. Physiol. Zool. 71, 27-35.

Schrauzer, G.N., 2000. Selenomethionine: a review of its nutritional significance, metabolism and toxicity. J. Nutri. 130, 1653-1656.

Schwab, I.R., 2007. Eye in the Sky. Br. J. Ophthalmol. 91, 994.

Semlitsch, R.D., Caldwell, J.P., 1982. Effects of density on growth, metamorphosis, and survivorship in tadpoles of *Scaphiopus holbrooki*. Ecology 63, 905-911.

Sheridan, M.A., Kao, Y., 1998. Regulation of metamorphosis-associated changes in the lipid metabolism of selected vertebrates. Am. Zool. 38, 350-368.

Shi, Y.B., 2000. Amphibian metamorphosis: from morphology to molecular biology. John Wiley and Sons, Inc., New York, NY.

Simmons, D., Wallschläger, D., 2005. A critical review of the biogeochemistry and ecotoxicology of selenium in lotic and lentic environments. Environ. Toxicol. Chem. 23, 1331-1343.

Singha, U., Pandey, N., Boro, F., Giri, S., Giri, A., Biswas, S., 2014. Sodium arsenite induced changes in survival, growth, metamorphosis and genotoxicity in the Indian cricket frog (*Rana limnocharis*). Chemosphere 112, 333-339.

Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early development of *Xenopus laevis*: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring, NY.

Snodgrass, J.W., Hopkins, W.A., Broughton, J., Gwinn, D., Baionno, J. A., Burger, J., 2004. Species-specific responses of developing anurans to coal combustion wastes. Aquat. Toxicol. 66, 171-182.

Snodgrass, J.W., Hopkins, W.A., Jackson, B.P., Baionno, J.A., Broughton, J., 2005. Influence of larval period on responses of overwintering green frog (*Rana clamitans*) larvae exposed to contaminated sediments. Environ. Toxicol. Chem. 24, 1508-1514.

Snodgrass, J.W., Hopkins, W.A., Roe, J.H., 2003. Relationships among developmental stage, metamorphic timing, and concentrations of elements in bullfrogs (*Rana catesbeiana*). Environ. Toxicol. Chem. 22, 1597-1604.

Sorenson, E.M.B., Bell, J.S., Harlan, C.W., 1983. Histopathological changes in selenium-exposed fish. Am. J. Foren. Med. Path. 4, 111-123.

Spallholz, J.E., 1994. On the nature of selenium toxicity and carcinostatic activity. Free Radical Bio. Med. 17, 45-64.

Spallholz, J.E., Palace, V.P., Reid, T.W., 2004. Methioninase and selenomethionine but not Se-methylselenocysteine generate methylselenol and superoxide in an in vitro chemiluminescent assay: implications for the nutritional carcinostatic activity of selenoamino acids. Biochem. Pharmacol. 67, 547-554.

Spallholz, J.E., Shriver, B.J., Reid, T.W., 2001. Dimethyldiselenide and methylseleninic acid generate superoxide in an in vitro chemiluminescence assay in the presence of glutathione: implications for the anticarcinogenic activity of L-selenomethionine and L-Se-methylselenocysteine. Nutr. Cancer 40, 34-41.

Spornitz, U.M., 1975. Studies on the liver of *Xenopus laevis* – I. The ultrastructure of the parenchymal cell. Anat. Embryol. 146, 245-264.

Stadtman, T.C., 1996. Selenocysteine. Annu. Rev. Biochem. 65, 83-100.

Stebbins, R.C., Cohen, N.W., 1995. A natural history of amphibians. Princeton University Press, Princeton, NJ.

Stewart, R., Grosell, M., Buchwalter, D., Fisher, N., Luoma, S., Mathews, T., Orr, P., Wang, W., 2010. Bioaccumulation and trophic transfer of selenium. In: Chapman, P.M., Adams, W.J., Brooks, M., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., (Eds.), Ecological assessment of selenium in the aquatic environment. CRC Press, Boca Raton, FL, pp. 93-139.

Suzuki, K.T., Ogra, Y., 2002. Metabolic pathway for selenium in the body: speciation by HPLC-ICP MS with enriched Se. Food Addit. Contam. 19, 974-983.

Tahjian, D.H., Hung, S.S.O., 2006. Selenium absorption, distribution, and excretion in white sturgeon orally dosed with L-selenomethionine. Environ. Toxicol. Chem. 25, 2618-2622.

Thomas, J.K., Janz, D.M., 2011. Dietary selenomethionine exposure in adult zebrafish alters swimming performance, energetics and the physiological stress response. Aquat. Toxicol. 102, 79-86.

Thomas, J.K., Janz, D.M., 2014. In ovo exposure to selenomethionine via maternal transfer increases developmental toxicities and impairs swim performance in F1 generation zebrafish (*Danio rerio*). Aquat. Toxicol. 152, 20-29.

Thomas, J.K., Janz, D.M., 2015. Developmental and persistent toxicities of maternally deposited selenomethionine in zebrafish (*Danio rerio*). Environ. Sci. Technol. 49, 10182-10189.

Thomas, J.K., Wiseman, S., Giesy, J.P., Janz, D.M., 2013. Effects of chronic dietary selenomethionine exposure on repeat swimming performance, aerobic metabolism and methionine catabolism in adult zebrafish (*Danio rerio*). Aquat. Toxicol. 130, 112-122.

Thompson, J., Bannigan, J., 2008. Cadmium: Toxic effects on the reproductive system and the embryo. Reprod. Toxicol. 25, 304-315.

Tinsley, R.C., Loumont, C., Kobel, H.R., 1996. Geographical distribution and ecology. In: Tinsley, R.C., Kobel, H.R., (Eds.), The biology of *Xenopus*. Oxford University Press Inc., New York, NY, pp. 35-59.

Unrine, J.M., Hopkins, W.A., Romanek, C.S., Jackson, B.P., 2007. Bioaccumulation of trace elements in omnivorous amphibian larvae: implications for amphibian health and contaminant transport. Environ. Pollut. 149, 182-192.

US EPA (United States Environmental Protection Agency), 2004. Draft aquatic life water quality criteria for selenium-2004. EPA-822-D-04-001, November 2004. Office of Water, Washington, DC, pp.1-334.

US EPA (United States Environmental Protection Agency), 2015. Draft aquatic life ambient water quality criterion for selenium (Freshwater) 2015. EPA 822-P-15-001 Fact Sheet. Office of Water, Washington, DC, pp. 1-3.

Van Dyke, J.U., Beck, M.L., Jackson, B.P., Hopkins, W.A., 2013. Interspecific differences in egg production affect egg trace element concentrations after a coal fly ash spill. Environ. Sci. Technol. 47, 13763-13771.

Vinceti, M., Bonvicini, F., Bergomi, M., Malagoli, C., 2010. Possible involvement of overexposure to environmental selenium in the etiology of amyotrophic lateral sclerosis: a short review. Ann. Ist. Super. Sanitá 46, 279-283.

Vitt, L.J., Caldwell, J.P., 2009. Herpetology (3rd Ed.). Academic Press, Burlington, MA.

Wake, D., Vredenburg, V., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc. Natl Acad. Sci. USA 105, 11466-11473.

Ward, C.K., Appel, A.G., Mendonça, M.T., 2006. Metabolic measures of male southern toads (*Bufo terrestris*) exposed to coal combustion waste. Comp. Biochem. Phys. A 143, 353-360.

Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition. Aquaculture 151, 185-207.

Weidel, B.C., Carpenter, S.R., Kitchell, J.F., Vander Zanden, M.J., 2011. Rates and components of carbon turnover in fish muscle: insights from bioenergetics models and a whole-lake ^{13}C addition. Can. J. Fish Aquat. Sci. 68, 387-399.

Whitfield Gibbons, J., Winne, C.T., Scott, D.E., Willson, J.D., Glaudas, X., Andrews, K.M., Todd, B.D., Fedewa, L.A., Wilkenson, L., Tsaliagos, R.N., Harper, S.J., Greene, J.L., Tuberville, T.D., Metts, B.S., Dorcas, M.E., Nestor, J.P., Young, C.A., Akre, T., Reed, R.N., Buhlmann, K.A., Norman, J., Croshaw, D.A., Hagen, C., Rothermel, B.B., 2006. Remarkable amphibian biomass and abundance in an isolated wetland: implications for wetland conservation. Conserv. Biol. 20, 1457-1465.

Wiseman, S., Thomas, J.K., McPhee, L., Hursky, O., Raine, J.C., Pietrock, M., Giesy, J.P., Hecker, M., Janz, D.M., 2011. Attenuation of the cortisol response to stress in female rainbow trout chronically exposed to dietary selenomethione. Aquat. Toxicol. 105, 643-651.

Woof, C., Janssens, P.A., 1978. Effects of fasting and cortisol administration on carbohydrate metabolism in *Xenopus laevis* Daudin. Gen. Comp. Endocr. 36, 346-359.

Wook, S.E., Garrett, W.R., Partin, W.E., Bryson, W.T., 1987. Decreased survival and teratogenesis during laboratory selenium exposures to Bluegill, *Lepomis macrochirus*.

Bull. Environ. Contam. Toxicol. 39, 998-1005.

Wride, M.A., 1996. Cellular and molecular features of lens differentiation: a review of recent advances. Differentiation 61, 77-93.

Wright, M.L., Guertin, C.J., Duffy, J.L., Szatkowski, M.C., Visconti, R.F., Alves, C.D., 2003. Developmental and diel profiles of plasma corticosteroids in the bullfrog, *Rana catesbeiana*. Comp. Biochem. Physiol. A 135, 585-595.

Yan, Q., Liu, J., Li, D.W., 2006. Apoptosis in lens development and pathology. Differentiation 74, 195-211.

Young, T.F., Finley, K., Adams, W.J., Besser, J., Hopkins, W.A., Jolley, D., McNaughton, E., Presser, T.S., Shaw, D.P., Unrine, J., 2010a. Appendix A: selected case studies of ecosystem contamination by Se. In: Chapman, P.M., Adams, W.J., Brooks, M.L., Delso, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., (Eds.), Ecological assessment of selenium in the aquatic environment. CRC Press, Boca Raton, FL, pp. 257-292.

Young, T.F., Finley, K., Adams, W.J., Besser, J., Hopkins, W.A., Jolley, D., McNaughton, E., Presser, T.S., Shaw, D.P., Unrine, J., 2010b. What you need to know about selenium. In: Chapman, P.M., Adams, W.J., Brooks, M.L., Delso, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., (Eds.), Ecological assessment of selenium in the aquatic environment. CRC Press, Boca Raton, FL, pp. 7-45.

Yu, S., Wages, M., Willming, M., Cobb, G.P., Maul, J.D., 2015. Joint effects of pesticides and ultraviolet-B radiation on amphibian larvae. Environ. Pollut. 207, 248-255.

APPENDIX[a]

[a] The supplementary data included in this appendix was published in the Supporting Information of Environmental Science & Technology 49:13658-13666, under the joint authorship with Jorgelina R. Muscatello (Stantec Consulting Ltd.) and David M. Janz (University of Saskatchewan). The figure or table number is presented as Cx.Sy format, where 'Cx' indicates chapter number; 'Sy' indicates figure or table number.

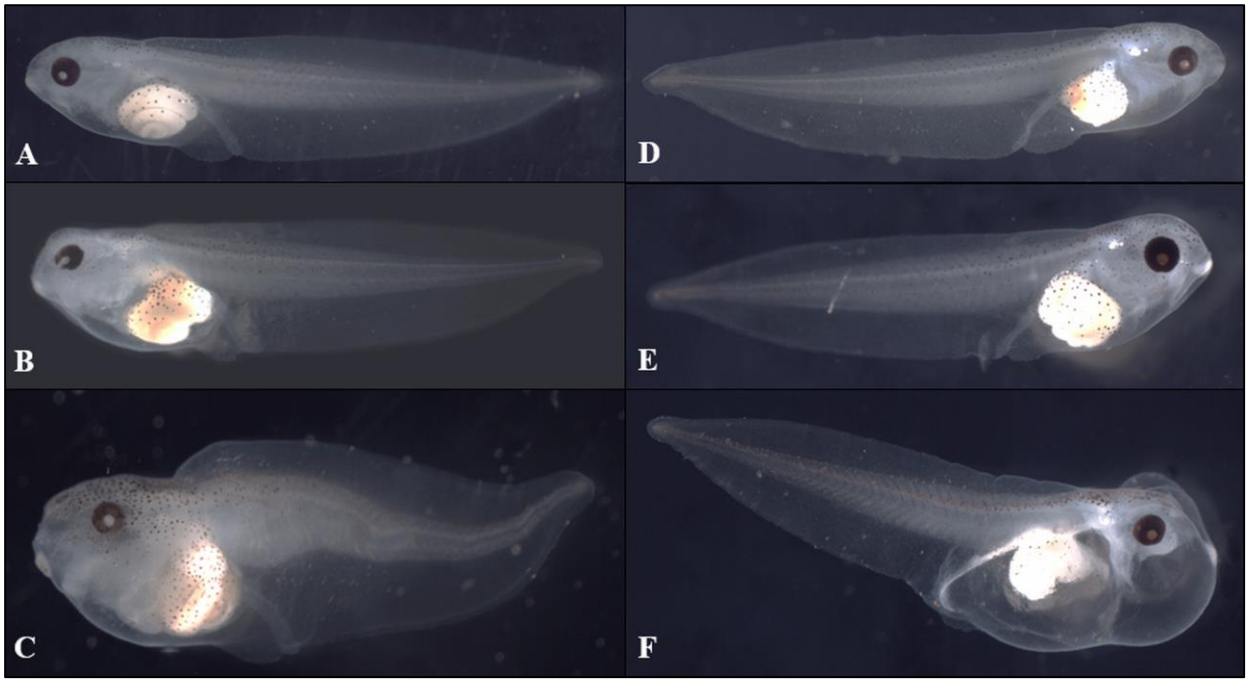

Figure C2.S1: Representative left and right side view images of morphologically normal (A and D) or abnormal (B, C, E, and F) *Xenopus laevis* tadpoles raised to 5 day post-fertilization after *in ovo* exposure to adequate or elevated levels of selenium. Abnormal craniofacial structures in the form of sloping or rounding of the forehead and extended lower mouth are presented in B and E. Abnormal coiling or uncoiling of the gut is shown in B, C, E, and F. The eye of the tadpole in B presents with an incomplete closure of the choroid fissure. Multiple severe abnormalities are observed in C. Image F presents with severe ocular, pericardial, and abdominal edema as well as mild dorsoventral curvature of the spine (lordosis) and microcephaly. Images were taken under 15x magnification using an Olympus model S261 dissecting microscope and Q capture software.

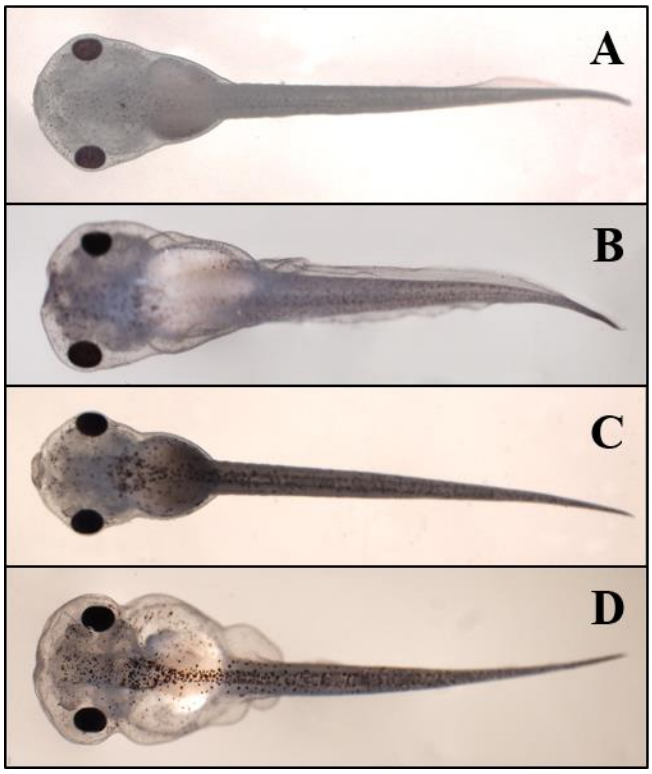

Figure C2.S2: Dorsal view images of morphologically normal (A) or abnormal (B, C, and D) *Xenopus laevis* tadpoles raised to 5 day post-fertilization after *in ovo* exposure to adequate or elevated levels of selenium. A disproportionate head to gut size is observed in B, C, and D when compared to image A (normal). Abnormal craniofacial structures are presented in B, C, and D, with B displaying asymmetrical head and eye structures, and C and D displaying microcephaly. Abnormal coiling or uncoiling of the gut is shown in B and D. Ocular, pericardial, and abdominal edema is presented in B and D. Images were taken under 15x magnification using an Olympus model S261 dissecting microscope and Q capture software.

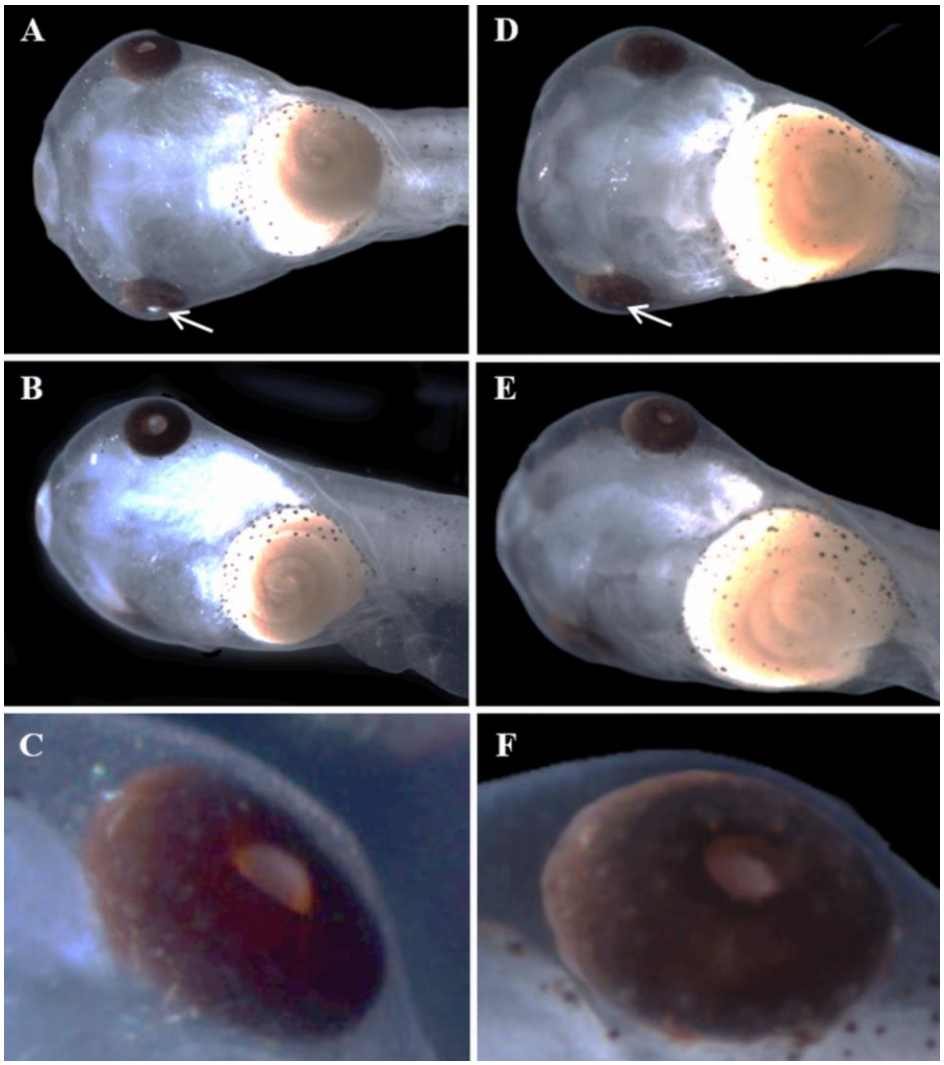

Figure C2.S3: Characteristic ocular lens abnormality prominently observed in *Xenopus laevis* tadpoles raised to 5 day post-fertilization after *in ovo* exposure to elevated levels of selenium. Normal lens structure presented in A, B, and C displays a translucent convex shape protruding from the center of the pigmented region of the eye. Abnormal lens structure in D, E, and F are depicting the loss of curvature of the lens, and its failure to protrude outward. Arrows identify the ocular lens region of the eye. Images A, B, D, and E were taken under 20x magnification while images C and F were taken under 40x magnification using an Olympus model S261 dissecting microscope and Q capture software.

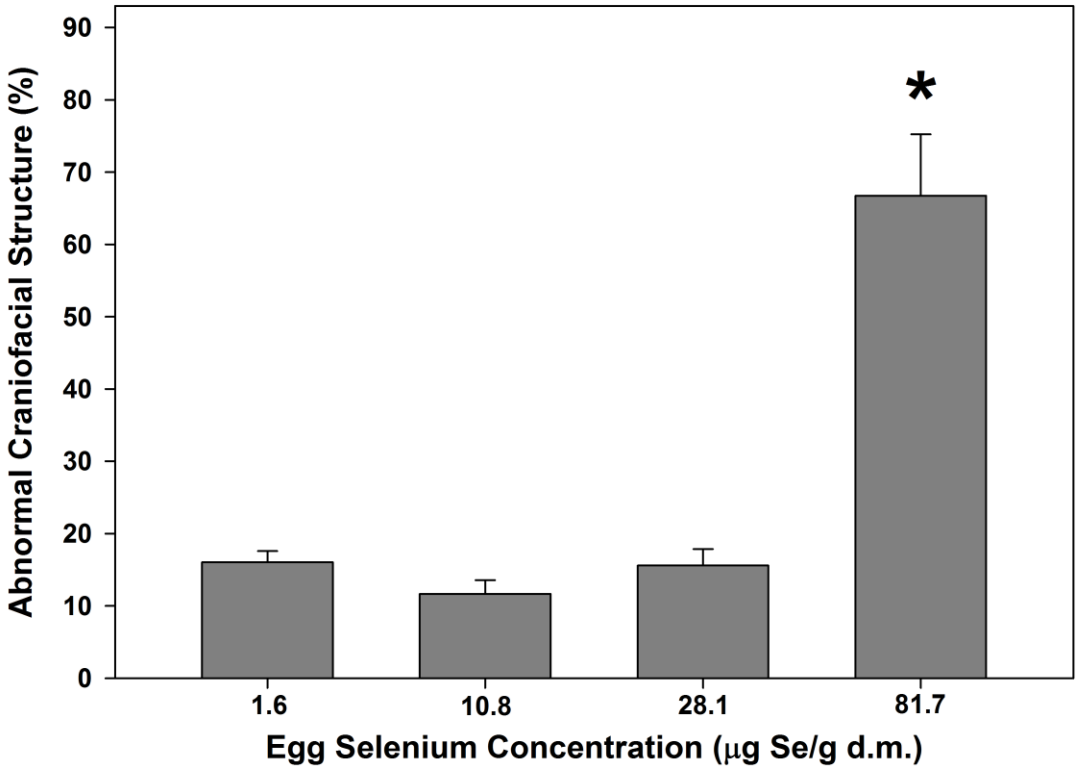

Figure C2.S4: Frequency of abnormal craniofacial structures detected in *Xenopus laevis* tadpoles (subsamples of 200 per female) exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer and subsequently raised to five days post-fertilization. *, Significant difference from control group using one way ANOVA followed by Holm-Sidak test (p<0.001; n=9-10 females).

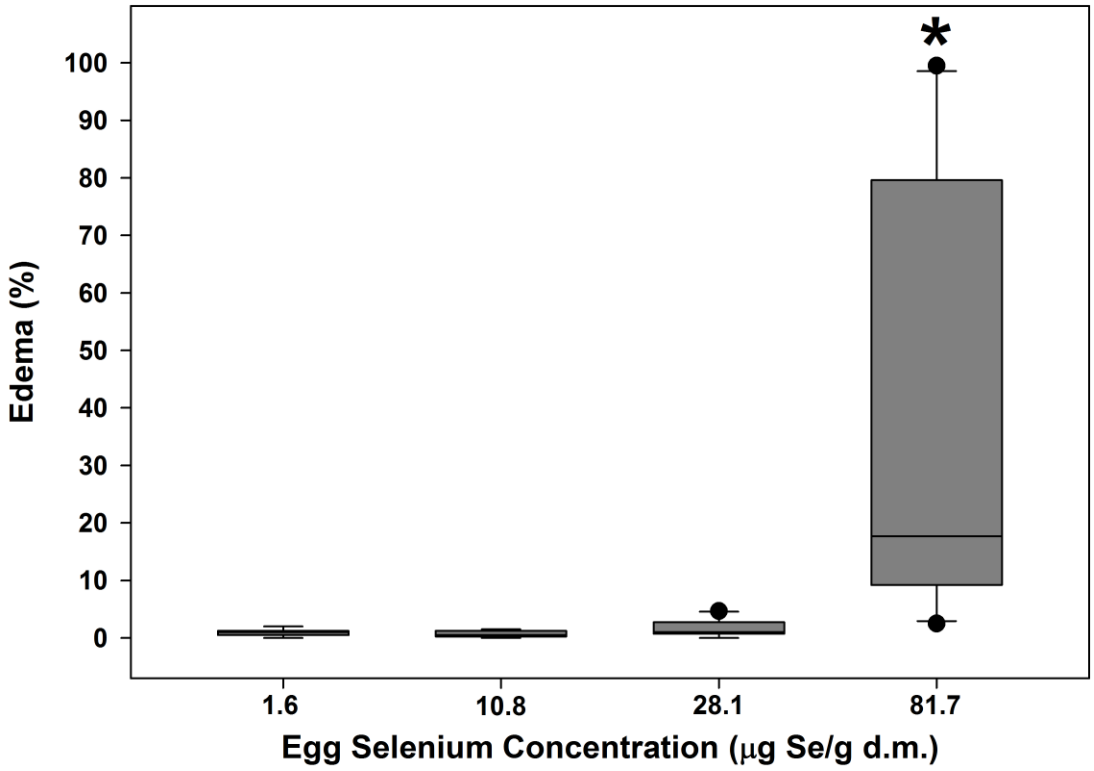

Figure C2.S5: Frequency of edema detected in *Xenopus laevis* tadpoles (subsamples of 200 per female) exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer and subsequently raised to five days post-fertilization. *, Significant difference from control group using Kruskal-Wallis one way ANOVA on Ranks followed by Dunn's test (p<0.001; n=9-10 females)**.**

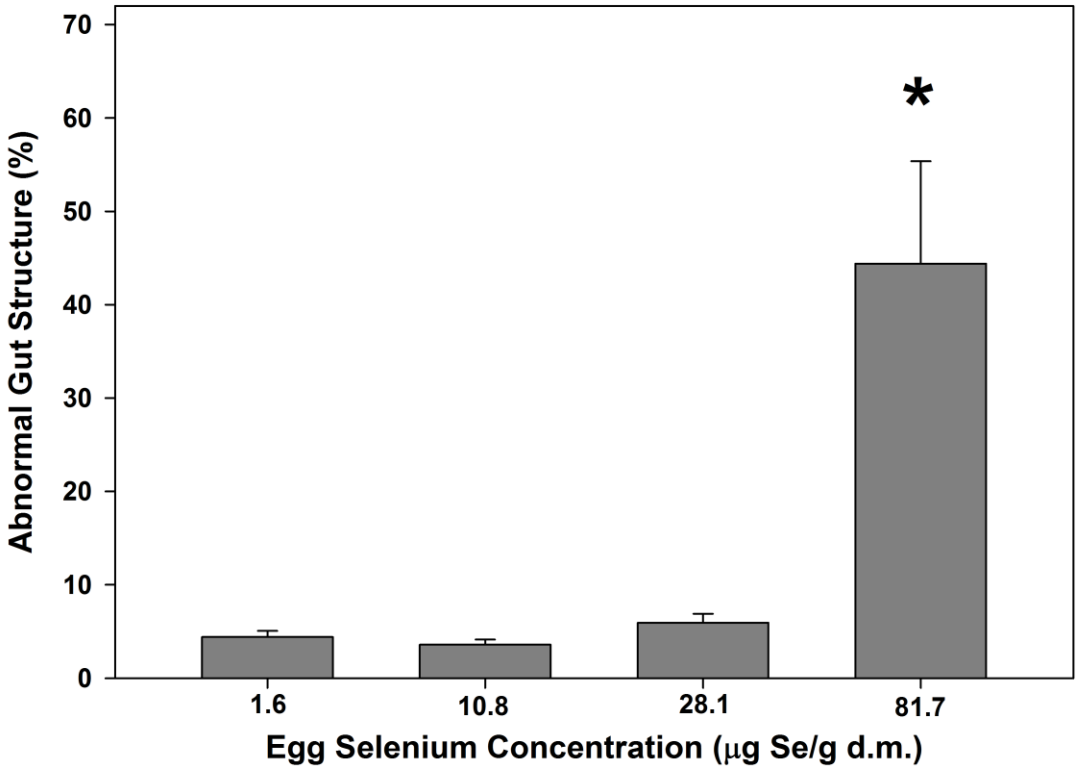

Figure C2.S6: Frequency of abnormal gut structure detected in *Xenopus laevis* tadpoles (subsamples of 200 per female) exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer and subsequently raised to five days post-fertilization. *, Significant difference from control group using one way ANOVA followed by Holm-Sidak test (p<0.001; n=9-10 females).

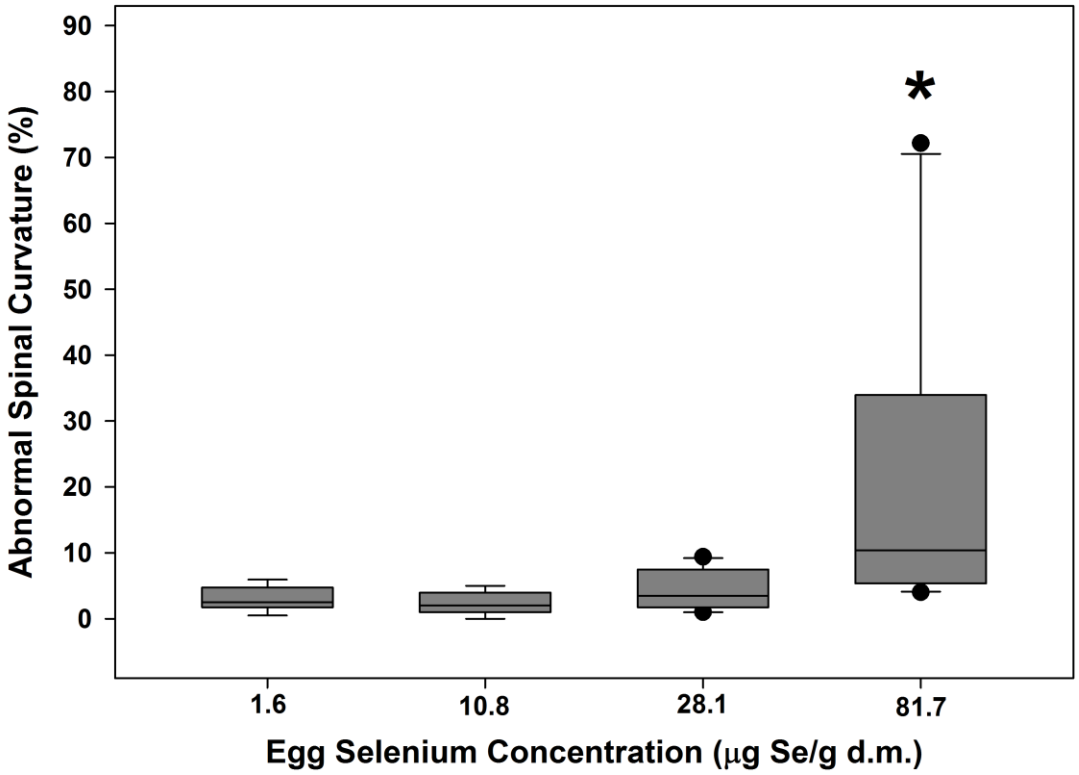

Figure C2.S7: Frequency of abnormal spinal curvatures detected in *Xenopus laevis* tadpoles (subsamples of 200 per female) exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer and subsequently raised to five days post-fertilization. *, Significant difference from control group using Kruskal-Wallis one way ANOVA on Ranks followed by Dunn's test (p<0.001; n=9-10 females)**.**

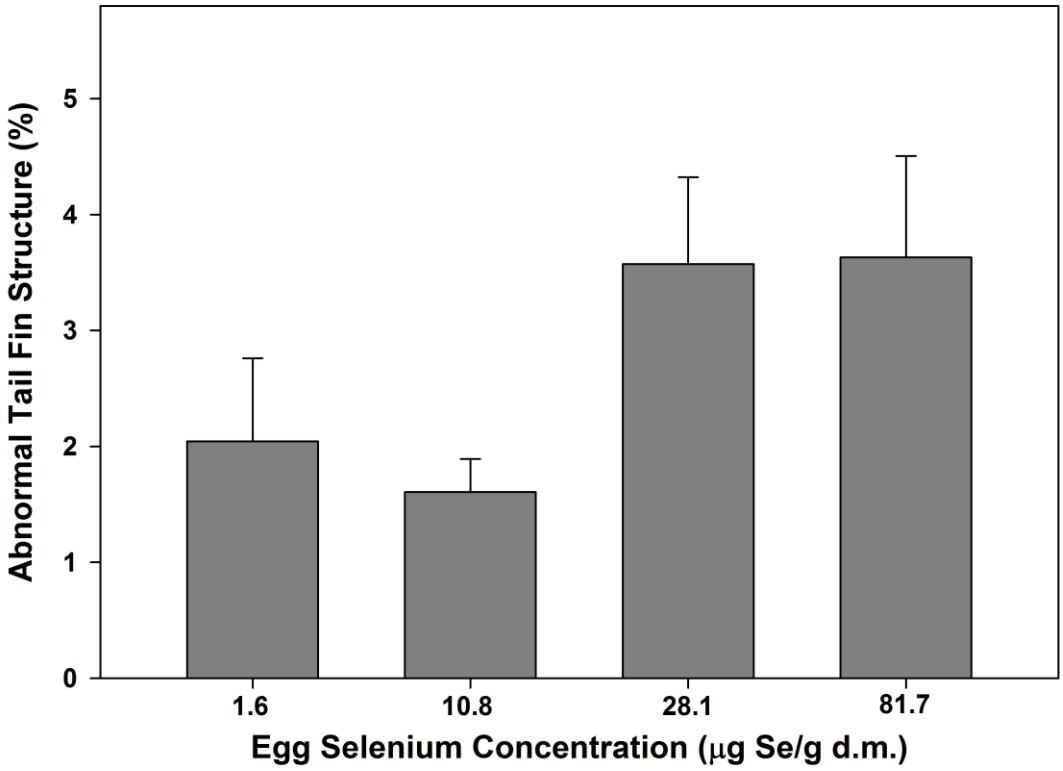

Figure C2.S8: Frequency of abnormal tail fin structure detected in *Xenopus laevis* tadpoles (subsamples of 200 per female; *n*=9-10 females) exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer and subsequently raised to five days post-fertilization.

Table C2.S1: Estimated EC_{10} values based on the presence of any of the listed abnormalities or only specific individual abnormalities in 5 days post-fertilization *Xenopus laevis* tadpoles (200 per female; *n*=9-10 females) exposed *in ovo* to increasing concentrations of selenium after a 68-day maternal dietary exposure to 0.7, 10.9, 30.4, or 94.2 µg Se/g food dry mass (d.m.).

Morphological Classification	EC_{10} Values (µg Se/g egg d.m.)	95% Confidence Interval (µg Se/g egg d.m.)
Total Abnormalities [a]	**44.9**	**41.5 – 48.2**
Abnormal Lens Structure [a]	**43.4**	**41.4 – 45.4**
Abnormal Craniofacial Structure [a]	**48.6**	**44.8 – 52.5**
Edema [b]	**58.8**	**56.7 – 60.8**
Abnormal Gut Formation [a]	**62.8**	**60.0 – 65.7**
Abnormal Spinal Curvature [b]	**64.6**	**62.4 – 66.8**
Abnormal Tail Fin Structure [b]	**208.1**	**130.3 – 285.9**

[a], EC_{10} value estimated using USEPA's Toxicity Relationship Analysis Program
[b], EC_{10} value estimated using ToxStat™ software

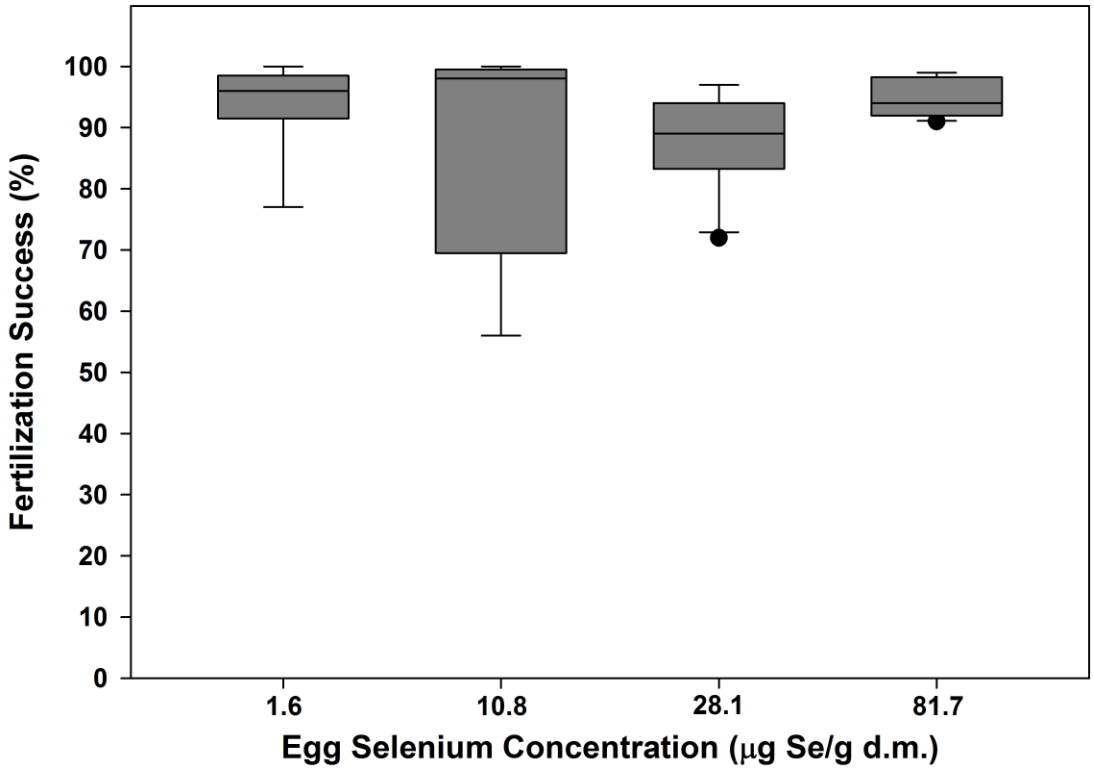

Figure C2.S9: Fertilization success observed in subsamples of *Xenopus laevis* embryos (100 per female; *n*=9-10 females) exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) through *in ovo* maternal transfer (Table 2.1).

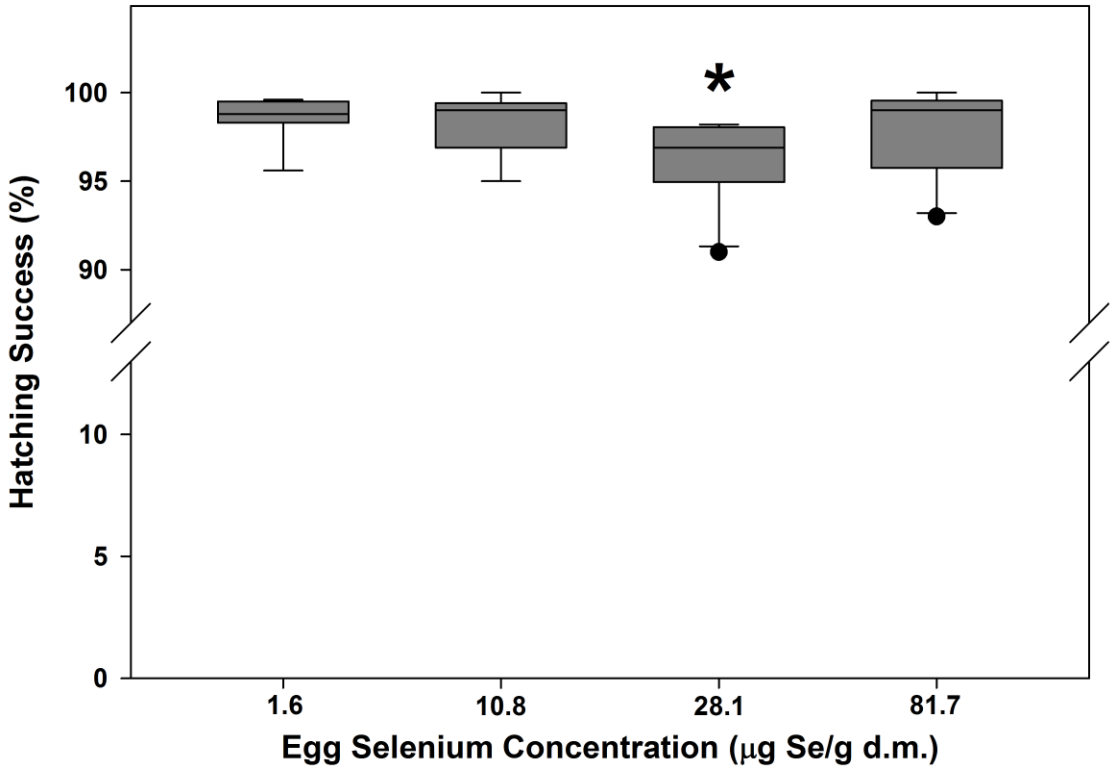

Figure C2.S10: Hatching success observed in subsamples of *Xenopus laevis* embryos (500 per female) exposed to increasing concentrations of selenium (μg Se/g egg dry mass [d.m.]) via *in ovo* maternal transfer (Table 2.1). *, Significant difference from control group using Kruskal-Wallis one way ANOVA on Ranks followed by Dunn's test (p<0.05; n=9-10 females)**.**

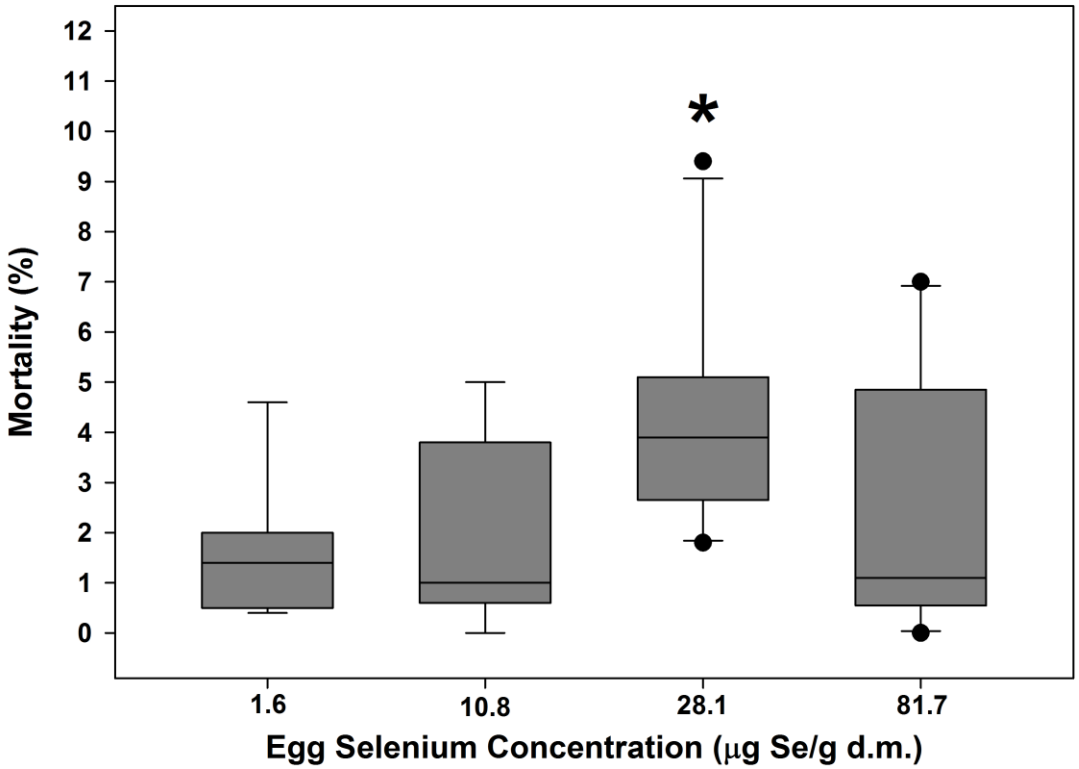

Figure C2.S11: Cumulative mortality observed in the first five days post-fertilization in subsamples of *Xenopus laevis* embryos/tadpoles (500 per female) exposed to increasing concentrations of selenium (μg Se/g egg dry mass[d.m.]) via *in ovo* maternal transfer (Table 2.1). *, Significant difference from control group using Kruskal-Wallis one way ANOVA on Ranks followed by Dunn's test (p<0.05; n=9-10 females)**.**