REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF SELENOMETHIONINE IN THE FATHEAD MINNOW *PIMEPHALES PROMELAS*

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ABSTRACT

Selenium (Se) is an essential trace element that undergoes maternal transfer to offspring where it has a high degree of teratogenic potential in egg-laying vertebrates because of the narrow range between nutritional benefit and toxicity. Studying this phenomenon of Se maternal transfer and subsequent toxicities in offspring is difficult in many fish species for logistical and biological reasons. For instance, Se contaminated sites are often located in remote locations which can make sampling efforts problematic, and certain fish species of concern commonly have long reproductive cycles and/or complex life histories which can be difficult to monitor. Thus, there is a need for improved methods to assess the toxicity of Se across diverse species of embryo-larval fish which could potentially aid site-specific risk assessment of Se contamination. Microinjection methodology is a potential surrogate for simulating the maternal transfer of Se and could be utilized to study this phenomena in non-model species which are unable to be spawned in the laboratory, or are difficult to sample from an Se contaminated site when spawning. Therefore, the overall objective of my research was to compare two potential *in ovo* Se exposure routes, maternal transfer and microinjection, in the fathead minnow *Pimephales promelas* to determine if early life stage toxicities are comparable between these different exposure routes in a freshwater fish. My thesis research fulfilled this objective by characterizing the effects of dietary Se exposure in *P. promelas* on fecundity and the maternal transfer of Se to embryos, the subsequent toxicities in embryo-larval offspring, and then used this information to inform a microinjection study with *P. promelas* embryos to allow for a comparison between the two different *in ovo* exposure routes.

First, a 28-day short-term reproductive assay with *P. promelas* was performed to determine the dynamics of dietary selenomethionine (SeMet) exposure on maternal transfer and its effects on the F_1 generation. Sexually mature *P. promelas* breeding groups (2 females:3 males) were fed a diet of either control (unspiked) or SeMet-spiked food (Low: 3.88 µg Se/g food dry mass [dm]; Medium: 8.75 µg Se/g food dm; High: 29.6 µg Se/g food dm) and allowed to breed. Fecundity did not decrease in female fish exposed to elevated levels of dietary SeMet and the low treatment (3.88 µg Se/g food dm) produced on average the most embryos per female, suggesting a possible supra-nutritional benefit of SeMet on reproduction. Dietary exposure with SeMet-spiked food rapidly induced the maternal transfer of excess Se and embryo

concentrations increased daily until reaching steady-state after approximately 14 days of exposure. *In ovo* exposure to elevated Se did not affect hatchability of embryos or survival until swim-up in early life stage *P. promelas*. However, a dose-dependent increase in the frequency of larval fish with any type of morphological abnormality (e.g. edema, skeletal, finfold, craniofacial) present at swim-up was observed at embryo Se concentrations of 28.4 µg Se/g embryo dm.

In the ancillary embryo microinjection study, embryos were injected with three doses of SeMet (Low: 9.73 µg Se/g embryo dm; Medium: 13.5 µg Se/g embryo dm; High: 18.9 µg Se/g embryo dm) to simulate maternal transfer and provide a point of comparison for the hatchability, survival and deformity endpoints measured in the preceding maternal transfer study. There were no effects of SeMet microinjection on hatchability up to a concentration of 18.9 µg Se/g embryo dm, however this same embryo Se concentration decreased survival until swim-up. Furthermore, this embryo Se concentration caused a greater increase in the frequency of deformed fathead minnow at swim-up in comparison to the highest embryo Se concentration in the maternal transfer study (28.4 µg Se/g embryo dm), suggesting a more toxic response when the dosage is primarily free SeMet rather than maternally transferred Se which is mainly SeMet incorporated into proteins. With this said, the frequency and type of deformities at embryo Se concentrations in the range of 9.73 – 13.5 µg Se/g embryo dm were similar between the two different exposure routes. The deformities observed in *P. promelas* as a response to SeMet exposure through both maternal transfer and microinjection followed a dose-dependent trend, and the most common deformities observed were spinal and finfold abnormalities, which were approximately two-fold more common than edema or craniofacial defects. Overall, this thesis research highlights the utility of embryo microinjection as a proxy for studying the maternal transfer of Se and provides an additional line of evidence for potentially extending this methodology to less commonly studied freshwater fish species of concern.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg	microgram
µg/g	micrograms per gram
µg/L	micrograms per litre
ºC	degrees centigrade
AE	assimilation efficiency
ANOVA	analysis of variance
ATRF	Aquatic Toxicology Research Facility
cm	centimeter
dm	dry mass
df	degrees of freedom
dfp	days post fertilization
DNA	deoxyribonucleic acid
ECCC	Environment and Climate Change Canada
EF	enrichment factor
g	gram
GLM	generalized linear model
GPx	glutathione peroxidase
kg	kilogram
k_e	efflux rate constant
k_g	growth rate
ICP-MS	inductively coupled plasma mass spectrometry
IR	ingestion rate
LOAEC	lowest observed adverse effect concentration
LRT	Likelihood ratio test
m	meter
mg	milligram
MS-222	tricaine methylsulfonate
OECD	Organization of Economic Co-Operation and Development
PAH	polycyclic aromatic hydrocarbon
SD	standard deviation

Se	selenium
Se(0)	solid elemental selenium
Se(-II)	selenide
Se(+IV)	selenite
Se(+VI)	selenate
SeCys	selenocysteine
SEM	standard error of the mean
SeMet	selenomethionine
SSD	species sensitivity distribution
TORT	lobster hepatopancreas
TTF	trophic transfer factor
USA	United States of America
USEPA	United States Environmental Protection Agency

NOTE TO READERS

This thesis was prepared in manuscript style, and will therefore have reiterations of information across certain sections of the chapters. To generate a more powerful manuscript for potential publication, the data generated that was initially planned to contribute to data Chapters 2 and 3 were combined into a single chapter, Chapter 2, and written in the style of a publishable manuscript. Chapter 1 is a general introduction providing a background on selenium and rational for the research that was conducted. Chapter 2 serves as a comprehensive data chapter which describes the research conducted in this thesis and which is currently under review for publication by *Aquatic Toxicology*. Chapter 3 is an overall synthesis and summary of the thesis. The appendices include further information to support the maternal transfer and microinjection studies. All citations in this thesis were combined as a complete list in a section at the end to avoid redundancies in citation lists among chapters.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Selenium ecotoxicity

Selenium (Se) is a naturally occurring trace element that is found in the atmosphere, soils and water-bodies around the world (Johnson et al., 2010; Lemly, 2004; Simmons and Wallschläger, 2009). Vertebrate organisms such as humans and fish do not produce Se but this element is required for proper cellular function based on the role it plays in DNA synthesis, protein repair and defense against oxidative stress (Avery and Hoffman, 2018; Haratake et al., 2015; Janz et al., 2010; Young et al., 2010). Despite the essential role of Se as a trace mineral for organisms, it displays a narrow range between essentiality and toxicity, and accumulation slightly above normal levels can be detrimental to the health of vertebrates (Brandt et al., 2017; Lemly, 2002). The central concern regarding the ecotoxicity of Se is its potential to persist and bioaccumulate in aquatic environments, and its potential deleterious population-level effects on egg-laying vertebrates that offload excess accumulated Se to their offspring via maternal transfer (Brandt et al., 2017; Janz et al., 2010; Lemly, 2004; Luoma and Presser, 2009). Understanding the ecotoxicity of Se in North American ecosystems has become a priority due to the ever-expanding practice of natural resource exploitation and extraction within the country, which mobilizes and increases concentrations of Se in aquatic habitats (Chapman, 2007; Janz et al., 2014).

One of the earliest Se ecotoxicology studies occurred during the 1970s at Belews Lake, North Carolina, USA. Here, uncontaminated waters received wastewater discharge from a coal-burning power plant with Se concentrations between 100 - 200 µg Se/L, which contributed to an overall increase of water Se concentrations and bioaccumulation in the aquatic food web (Lemly, 1985; Lemly, 1997; Young et al., 2010). Benthos concentrations ranged from 4.8 - 15.2 µg Se/g tissue dry mass (dm) and resident fish species reported muscle Se concentrations greater than 40 µg Se/g tissue dm (Lemly, 1985; Lemly 1997; Lemly, 2002). Over 20 fish species were extirpated in Belews Lake with Se contamination as the primary cause (Lemly, 1985; Young et al., 2010). Interestingly, fathead minnow (*Pimephales promelas*) was a species that maintained a reproductive population in Belews Lake during and after the Se contaminated period (Lemly, 1985). Since the incident at Belews Lake, awareness regarding the presence and persistent nature

of Se in aquatic systems has led to Se contamination being identified as an issue of global concern (Chapman, 2007; Janz et al., 2014; Lemly, 2004).

1.1.1 Sources of selenium

Selenium is found naturally within the earth's crusts in average concentrations of 0.05 - 0.09 µg Se/g sediment dm (Johnson et al., 2010). Phosphatic rocks and organic-rich deposits such as coal have greater concentrations of Se and often range from 1 - 20 µg Se/g sediment dm (Johnston et al., 2010). Concentrations of Se mobilized naturally (approximately 4500 tonnes per year), often through weathering of rock, heavy rainfall or volcanic eruptions, are approximately 20-fold lower in comparison to concentrations released through anthropogenic sources (76000 - 88000 tonnes per year) (Johnson et al., 2010; Maher et al., 2010). The predominant sources responsible for introducing Se into aquatic environments are waste by-products from several economically significant industrial processes that occur across the North American landscape. These include metal mining, uranium milling, smelting of pyritic ores, oil and gas refining, combustion of coal and fossil fuels, coal extraction and leachate from waste rock, as well as agricultural irrigation (Brandt et al., 2017, Janz et al., 2014; Lemly, 2004; Muscatello et al., 2008).

1.1.2 Selenium speciation and environmental fate

The diverse speciation of Se allows for its global distribution and influences its fate within various abiotic compartments including the atmosphere, earth's crusts, soils, minerals and aquatic habitats (Johnson et al., 2010; Simmons and Wallschläger, 2004). There are over fifty known species of Se which undergo complex cycling of speciation via geochemical transformation, biochemical modifications and trophic transfer (LeBlanc and Wallschläger, 2016, Luoma and Presser, 2009). It has been established that organic species (e.g. selenomethionine [SeMet] or selenocysteine [SeCys]) are more bioavailable than inorganic species (Janz et al., 2014; Young et al., 2010). The radioisotope, Selenium-79 (^{79}Se), is listed by the Department of Energy as a concern for environmental sites, such as those located in Northern Saskatchewan, which store or process uranium (Thompson et al., 2005).

Within aquatic environments, Se speciation is dependent on the characteristics of the system of interest, and which include hydrology, pH, temperature, water chemistry, and microbial activity (Bezile et al., 2000; Maher et al., 2010). Inorganic Se species generally exist in

one of four oxidation states: elemental Se, Se(0); selenide, Se(-II); selenite, Se(+IV); and selenate, Se(+VI) (Luoma and Presser, 2009). The speciation of Se in aquatic systems is important because it can influence persistence and bioconcentration at the base of the food web in primary producers such as algae and microbes (Conley et al., 2013; Fan et al., 2002; Friesen et al., 2017).

Selenium is cycled in a unique manner within aquatic systems where it is either adsorbed to particles, detritus or sediment, taken up by microorganisms, or is dissolved in solution (Presser and Luoma, 2009; Simmons and Wallschläger, 2004). Under anaerobic conditions, the insoluble species Se(0) and Se(-II) are formed and can be found adsorbed to water column particles and sediment (Luoma and Presser, 2009). Oxidized systems favor the soluble oxyanions Se(+IV) and Se(+VI) (Janz et al., 2014; Luoma and Presser, 2009). The oxyanions of these two species are assimilated and biotransformed by primary producers such as algae into organoselenium compounds (e.g. SeMet, SeCys) (Gómez-Jacinto et al., 2015; Young et al., 2010). If these organisms are not consumed and eventually die, small concentrations of organoselenium and selenite are released, with no species being converted back to selenate (Luoma and Presser, 2009). This process can create systems that lack selenate formation and are dominated by selenite and organoselenium species (Luoma and Presser, 2009; Simmons and Wallschläger, 2004).

1.1.3 Bioconcentration

The bioconcentration of inorganic Se, which is then metabolized and biotransformed into SeMet at the base of the food web, is a crucial step in beginning the cascade of trophic transfer of organic Se from primary producers into the upper trophic levels (Friesen et al., 2017; Kuchapski and Rasmussen, 2015). A consortium of microorganisms (e.g. bacteria, algae, periphyton, and fungi) compose biofilm communities that uptake and assimilate inorganic forms of Se into organic Se, and the rate at which this occurs can be quantified using an enrichment factor (EF), which is simply the ratio of tissue Se concentration and water Se concentration (DeForest et al., 2015; Friesen et al., 2017).

The biofilm community composition influences uptake of Se, which is further affected by water chemistry, flow rate, temperature, lighting and the presence of sulfate (Friesen et al., 2017; Simmons and Wallschläger, 2004). Therefore, EFs can vary by multiple orders of magnitude among different biofilm communities and this is an area of Se research that requires further

investigation (Conley et al., 2013; Friesen et al., 2017). Aquatic systems with predominately selenate as the soluble species have reported EFs between 100 and 500; whereas systems that contain mainly selenite have reported EFs ranging from 1000 to 10,000 (Luoma and Presser, 2009). There are multiple lines of evidence that support selenite being more readily bioconcentrated by primary producers in comparison to selenate, but both species can be taken up efficiently and metabolized into organoselenium species depending on the consortia of primary producers that are present (Besser et al., 1993; Friesen et al., 2017; Janz et al., 2014; Kupchaski and Rasmussen, 2015; Simmons and Wallschläger, 2004).

1.1.4 Bioaccumulation and trophic transfer

Selenium bioaccumulation and trophic transfer into aquatic food webs occurs as secondary and primary consumers feed on primary producers that have bioconcentrated SeMet (Hopkins et al., 2005; Luoma and Presser, 2009). Selenomethionine has been established as the major organoselenium species present in Se contaminated aquatic food webs, composing 60 - 80% of total Se (Janz et al., 2014; Maher et al., 2010; Orr et al., 2006). A trophic transfer factor (TTF) can be derived to explain the relationship between Se concentrations in an organism and its dietary items (Luoma and Presser, 2009).

When Se concentrations at the base of the food web are determined, TTFs can be utilized to predict concentrations in species that reside in upper trophic levels, which is important for characterizing ecological risks of Se. Studies on fish have determined a median TTF of ~1 for uptake of Se from the diet to fish, meaning they accumulate the same tissue Se concentration as their prey, whereas TTFs are more variable for invertebrate species, ranging from 0.6 in amphipods and up to 23 in clams (Luoma and Presser, 2009). Species with greater TTFs have more propensity to bioaccumulate Se and therefore are a more hazardous source of excess Se to upper trophic level predators.

1.2 Selenium in fish

1.2.3 Essentiality and toxicity

Dietary Se requirements have been characterized in a suite of fish species, including rainbow trout (*Oncorhynchus mykiss*) (0.15 - 0.38 µg Se/g diet dm), Nile tilapia (*Oreochromis niolticus*) (1.06 - 2.06 µg Se/g diet dm), channel catfish (*Ictalurus punctatus*) (0.25 µg Se/g diet dm) and gibel carp (*Carassius auratus*) (0.73 - 1.19 µg Se/g diet dm) (Gatlin and Wilson, 1984; Hilton et al., 1980; Lee et al., 2016; Zhu et al., 2017). However, these studies used inorganic Se

(sodium selenite) to supplement Se in the diet even though it is recognized that organic Se is a more bioavailable form for supplementing fish diets. In rainbow trout, an organic selenized yeast containing SeMet was used to supplement the diet at concentrations of 3.5 - 4.3 µg Se/g diet dm and benefits such as improved growth performance, antioxidant capacity, and nutrient utilization were reported (Hunt et al., 2011). Furthermore, consistent Se supplementation just slightly below 5 µg Se/g diet dm has improved plant-based aquaculture diets and the health status of cultured atlantic salmon (*Salmo salar*), which concurs with the physiological benefits observed in rainbow trout when fed a similar dietary Se concentration. However, Se consumption >5µg Se/g diet dm in fish has been reported to increase the potential for physiological toxicities and an overall reduction of fitness (Berntssen et al., 2018; Hunt et al., 2011; Lemly, 1999).

With regards to toxicity, SeMet is more relevant than inorganic Se species based on three reasons: (i) the inefficiency of fish to uptake inorganic species from the water column; (ii) SeMet being the most common dietary Se species due to its significant bioconcentration at the base of the food chain; and (iii) the biochemical processes that incorporate dietary SeMet into proteins (Hamilton, 2004, Janz et al., 2010; Janz et al., 2014; LeBlanc and Wallschläger, 2016).

1.2.1 Mechanisms of toxicity and biochemistry

Accumulation of excess Se above dietary requirements has been identified as a source of potential toxicity in vertebrate organisms (Hamilton, 2004). The main hypotheses regarding the mechanistic basis of Se toxicity are focused on altered protein function and oxidative stress. Altered protein function might occur because of SeMet being non-specifically inserted into a protein being synthesized rather than the intended amino acid, methionine (Brown and Arthur, 2001). Methionyl-tRNA acylase, the enzyme involved in the incorporation of methionine into proteins, does not discriminate between methionine and SeMet because of similarities between Se and sulfur, which leads to the chemical structures of methionine and SeMet being nearly identical (Mangiapane et al., 2014; Schrauzer, 2000; Young et al., 2010). Therefore, incorporation of SeMet into tissues with high rates of protein synthesis can occur in a dose-dependent manner (Janz et al., 2010). Studies investigating protein function after assumed SeMet insertion have reported both normal and impaired function, making it difficult to understand the relationship or effects of SeMet incorporation into proteins that would normally contain methionine (Brown and Arthur, 2001; Palace et al., 2004; Reddy and Massaro, 1983; Stadtman, 1974).

Selenium-induced oxidative stress is an accepted hypothesis that focuses on GPx enzymes, which under normal conditions act in conjunction with reduced glutathione as an intracellular antioxidant (Palace et al., 2004; Reddy and Massaro, 1983). In conditions where excess Se is present in tissues, glutathione peroxidase enzymes (GPx) are utilized for Se metabolism and can become depleted (Misra and Niyogi, 2009). If this occurs, there is insufficient GPx to work in tandem with glutathione as antioxidant, and an overall increase in reactive oxygen species has been reported in this scenario (Misra and Niyogi, 2009). *In vitro* studies investigating oxidative stress mechanisms have used an intermediate metabolite of SeMet, methylselenol, which reacts with glutathione to produce reactive oxygen species such as hydrogen peroxide and superoxide anions (Spallholz et al., 2004). It has been demonstrated that SeMet will generate superoxide in the presence of methioninase enzyme, and this is a proposed mechanism of action that could be responsible for Se induced oxidative stress in early life stage rainbow trout (Holm et al., 2005; Palace et al., 2004, Spallholz et al., 2004).

1.2.2 Selenoproteome

Fish have one of the largest selenoproteomes, containing over 41 selenoprotein subfamilies, in comparison to birds and mammals that have 25 and 28 subfamilies, respectively (Lobanov et al., 2009; Mariotti et al., 2012). Selenium is mainly integrated into selenoproteins for utilization in biochemical functions as SeCys, the 21st amino acid (Avery and Hoffman, 2018; Mariotti et al., 2012) The full suite of selenoprotein functions is undetermined but research has highlighted their involvement as deiodinases in thyroid regulation and growth, and as part of the GPx family of enzymes. (Avery and Hoffman, 2018; Brown and Arthur, 2001; Haratake et al., 2015). For instance, the phospholipid hydroperoxide GPx contains Se and is the only antioxidant enzyme that reduces phospholipid hydroperoxides generated in biological membranes which plays a protective role in biological activity (Haratake et al., 2015; Weitzel et al., 1990). It has been demonstrated that selenoproteins are more effective catalysts than their L-cysteine homologs, which is likely why metabolic pathways evolved to become selenium-dependent (Haratake et al., 2015). Furthermore, selenoproteins do not always function as an enzyme such as in the case of selenoprotein P, which is involved in the transport of Se from plasma into tissues, and selenoprotein H, which has been reported to regulate gene expression of glutathione synthesis (Avery and Hoffman, 2018, Panee et al., 2007).

1.2.3 Maternal transfer and early life stage toxicities

Toxicity in the early life stages is possible because of maternal transfer and the similarity in chemical structure between SeMet and methionine (Janz et al., 2010). The teratogenic effects of Se exposure are often visible with the naked eye in embryo-larval or early life stage fish, and fish are more susceptible to Se toxicity during the early life stages of development. (Berntssen et al., 2018; Lemly et al., 1993; Lemly, 1996). Maternal transfer occurs because Se is incorporated into embryos during vitellogenesis, a process where yolk proteins and nutrients are deposited into immature oocytes, which eventually develop into eggs to be fertilized (Kroll and Doroshov, 1991; Lubzens et al., 2017). Selenium has been shown to incorporate into certain components of yolk proteins, such as phosvitin, lipovitellin, immunoglobulin and vitellogenin (Kroll and Doroshov, 1991). From fertilization until complete adsorption of the yolk-sac, embryos rely on the yolk to provide the required nutrients for development (Finn and Fhyn, 2010; Lubzens et al., 2017).

Initially, the yolk is absorbed at a slow but steadily increasing rate that accelerates right before hatch, and even more so post-hatch (Holm et al., 2005). Therefore, Se is predominantly mobilized and metabolized post-hatch, when platelet components of the yolk proteins are being consumed, and which occurs at a lesser rate during pre-hatch development (Holm et al., 2005). Fish embryos with elevated Se concentrations have been reported to display an increase in the proportion of developmental abnormalities, including spinal curvatures such as lordosis (concave curve of the lumbar region), kyphosis (convex curve of the thoracic region), and scoliosis (S-shaped lateral curve), pericardial and yolk sac edemas, craniofacial deformities (missing or malformed jaw), and fin malformations (Covington et al., 2018; Holm et al., 2005; Muscatello et al., 2006; Lemly, 1997; Thomas and Janz, 2014).

Embryo Se concentrations as low as 6.0 µg Se/g embryo dm significantly reduce survival in 6-day post-fertilization (dpf) zebrafish; however, zebrafish are the most sensitive species of fish to Se exposure during early life stage development (Thomas and Janz, 2014). During an assessment of northern pike (*Esox Lucius*) from a site downstream of a uranium milling operation at McClean Lake, Saskatchewan, Canada, adult female muscle and embryos were sampled from fish inhabiting Se laden (11.9 – 38.9 µg/L) waters (Janz et al., 2014; Muscatello et al., 2006). Adult female northern pike reported muscle Se concentrations in the range of 16.6 - 38.3 µg Se/g muscle dm, and embryo Se concentrations between 31.3 - 48.2 µg Se/g embryo dm

(Muscatello et al., 2006). Larvae reared from these embryos exhibited increased incidences of morphological abnormalities during early life stage development that followed a dose-dependent trend (Muscatello et al., 2006). In a study with rainbow trout and brook trout (*Salvelinus fontinalis*), spawning fish were collected from a reference site and a Se contaminated site, Luscar Creek near Jasper, Alberta, Canada, and embryos from these two species at Luscar Creek reported mean Se concentrations of 9.9 µg Se/g embryo dm and 7.8 µg Se/g embryo dm, respectively (Holm et al., 2005). These embryo concentrations were significantly elevated in comparison to embryos from the reference site, but there were no reportable effects of Se induced toxicity during early life stage development in either species (Holm et al., 2005). These embryo concentrations are below the US EPA's recently updated Se criterion, which recommends that an embryo-ovary tissue concentration of 15.1 µg Se/g tissue dm is protective of egg-laying vertebrates (USEPA, 2016). Considering available Se toxicity data based on maternal transfer studies with ten embryo-larval coldwater fish species, a species sensitivity distribution (SSD) predicted a concentration of 20 µg Se/g egg or ovary dm to be protective of 95% of the fish species (DeForest et al., 2012). It should be noted that Se has an extremely steep dose-response curve that is unique among essential trace elements, and this narrow range of toxicity is consistent in most fish species (Lemly, 1993; Lemly, 1999; Lemly, 2004).

1.2.4 Toxicity in juvenile and adult fish

Juvenile life stages of fish species, such as Sacramento splittail (*Pogonichthys macrolepidotus*) and the white sturgeon (*Acipenser transmontanus*), have been demonstrated to display Se induced toxicities after chronic dietary exposure to Se (Teh et al., 2004; Zee et al., 2016). White sturgeon exposed to dietary SeMet concentrations of 22.4 and 104.4 µg Se/g food dm reported decreased hepatosomatic indices, and increased frequency and severity of edema, including ocular edema causing protruding eyes that in certain cases led to a loss of equilibrium (Zee et al., 2016). Sacramento splittail reported a greater incidence of deformities and mortalities after 3-months of exposure at dietary Se concentrations of 26.0 and 56.7 µg Se/g diet dm (Teh et al., 2004). The presence of ocular cataracts is a notable effect of chronic exposure to elevated dietary Se concentrations that has been reported in rainbow trout and largemouth bass (*Micropterus salmoides*) (Lemly, 2002; Pettem et al., 2018).

The sublethal effects of Se exposure can cause changes in triglyceride, glycogen and cortisol concentrations in fish sampled from contaminated field sites; however, the mechanisms

for these changes have not been determined and these effects could be a result of complex contaminant mixtures in these sampling locations rather than strictly Se toxicity (Bennett and Janz, 2007; Drieger et al., 2010; Kelly and Janz, 2008). Metabolic changes due to Se exposure have been hypothesized to negatively influence swim performance in fathead minnow consuming sublethal dietary Se concentrations of less than 26.5 µg Se/g diet dm (McPhee and Janz, 2014). A coldwater species, rainbow trout, were fed a SeMet-spiked diet of 47.8 µg Se/g diet dm for 60-days and displayed glucose intolerance, but also an adaptive increased cardiac function (Pettem et al., 2018). The enhanced cardiac function contradicts similar research conducted in adult zebrafish (*Danio rerio*), a small-bodied neotropical species, which suffered decreased cardiac output after exposure to dietary SeMet (Pettem et al., 2017). It has been argued that coldwater fish species are more tolerant to Se, or have evolved mechanisms to cope with elevated Se concentrations in their environment, compared to fish that inhabit warmer waters, however more research in this area is required (Chapman, 2007).

1.3 Fathead minnow (*Pimephales promelas*) as model species in aquatic toxicology

1.3.1 Biology

Fathead minnows are small-bodied fish (6 - 7.4 cm adult body length) that live around 2 - 3 years. A member of the Cyprinidae family, fathead minnows are fast developing omnivores that are present in nearly all aquatic habitats across North America (Andrews, 1970; Ankley et al., 2006). This species is tolerant to a wide range of water quality characteristics including dissolved oxygen, pH, turbidity, alkalinity, hardness, conductivity and temperature (Andrews, 1970). Fathead minnows are a prey item of larger bodied fish and are a commonly used fish model for predator-prey relationships (Mathis and Smith, 1993). The species prefers habitat with cover and shallow water (less than 3m depth), and will inhabit both lentic and lotic habitats (Hood and Stocek, 2005). They form shoals as juveniles but become territorial when breeding. Adult fathead minnow become sexually mature at around 5 months of age and in natural habitats their breeding season begins in early July as water temperatures increase (Andrews, 1970; Ankley et al., 2006). Sexual dimorphism is most prevalent during this time and males develop a gold banding pattern over the midsection of their dark bodies. In addition, males develop a dorsal pad and extended tubercles on their head which are used to arouse females, and to prepare the substrate where she will lay her eggs (Andrews, 1970). Fathead minnows are intermittent,

asynchronous breeders that can produce thousands of eggs over a breeding season (Andrews, 1970; Scott and Crossman, 1998).

Husbandry of fathead minnow is simple and they are an established model species that can be continually cultured in a laboratory (Ankley et al., 2006). They are easily bred by placing males and females together in an aquarium, often in a 1:2 ratio, along with a breeding tile to act as a substrate during the breeding process. In laboratory settings, fathead minnows can be bred in any season allowing the testing of specific life stages to be planned and carried out year-round. Embryos are adhesively deposited on to the underside of the breeding tile and can be easily collected for use in a variety of toxicological tests or for rearing of culture fish for future research.

1.3.2 History in toxicology

Studies utilizing the fathead minnow as a model organism began in the 1950s. Since then, fathead minnows have become one of the most widely used fish models for regulatory ecotoxicology in North America (Ankley et al., 2006). This species has been identified as one in which toxicity data is predictive of chemical effects in other fish species, and the fathead minnow has a long history of use in acute and chronic studies (Besser et al., 2005; Miracle et al., 2003; Sappington et al., 2001). The reproductive and developmental physiology of fathead minnows is well characterized and allows researchers and regulators to use this species in an array of standardized short and long-term bioassays. A 7-day larval survival and growth test with fathead minnow is part of the US EPA's suite of tests in their whole-effluent monitoring program, and Environment and Climate Change Canada (ECCC) has an established protocol for the same bioassay (ECCC, 2011; USEPA, 2002).

The OECD has established guidelines for a Fish Short Term Reproduction Assay (Test. No. 229) for use with the fathead minnow (OECD, 2012). The short-term reproductive test was developed to characterize the effects of chemical exposure on reproduction, and therefore I followed this guideline, with slight modifications such as extending the exposure period, to meet my specific purpose of investigating the maternal transfer of Se and subsequent effects in embryo-larval offspring of fathead minnow.

1.3.3 Early life stage development

The development of fathead minnow embryos and larvae is rapid in comparison to salmonid species, with fathead minnows reaching swim-up stage at approximately 7 dpf whereas salmonid species often take multiple weeks, and in some cases months (USEPA, 1996). Fathead minnow embryos develop through 32 defined embryonic stages and hatch within 3 - 5 dpf (USEPA, 1996). The embryos have a clear and transparent chorion allowing for observation of embryonic development. After hatching, fathead minnow larvae are approximately 5mm in length, possess a functioning lateral line, an open mouth with movable jaws, and a streamlined yolk-sac (USEPA, 1996). Active larvae fully absorb their yolk sac around 48-hours post-hatch and feed on live food immediately. Fathead minnows develop through three larval stages (protolarval, mesolarval and metalarval) before entering the juvenile phase, which is defined by complete fin development, and which occurs at approximately 18 days post-hatch (USEPA, 1996).

1.3.4 Toxicity of selenium to fathead minnow

Fathead minnow have been shown to accumulate Se from contaminated aquatic systems but there is less evidence of field-based Se toxicities occurring in this species at the population level (Lemly, 1985; Schultz and Hermanutz, 1990). For example, in Belews Lake in North Carolina, USA, fathead minnow was one of three species, out of twenty total, that were unaffected by increased Se concentrations in the water and food-chain of this lake, and fathead minnows maintained a large reproducing population throughout the contaminated period (Lemly, 1985). Ogle and Knight (1989) fed adult fathead minnow a diet composed of 25% SeMet, 25% selenate and 50% selenite for 98 days Se at concentrations of 15 µg Se/g food dm and reported no impact of Se exposure on growth; however, the authors reported consumption of Se at concentrations of 20 µg Se/g food dm and 30 µg Se/g dm significantly inhibited growth. Furthermore, the maternal transfer of Se from adults fed this same Se laden diet containing up to 30 µg Se/g food dm did not have any effect on embryo hatchability or larval survival until 14 dpf, and there were no reported effects of dietary treatment on fecundity in the breeding adults (Ogle and Knight, 1989). At concentrations above 40 µg Se/g food dm, adult fathead minnow were reported to reduce feeding and reproduction, and consumption of Se at concentrations of 80 and 160 µg Se/g food dm prompted the development of severe edema within 24 hours (Ogle and Knight 1989). The only study investigating embryo-larval fathead minnow exposed adults and

embryo-larval offspring in artificial streams containing 10 µg Se/L (Schultz and Hermanutz, 1990). The results from this study did not report dietary Se exposure concentrations, were highly variable, and only determined deformities if they were present in the form of lordosis and/or edema (Schultz and Hermanutz, 1990). Therefore, it is difficult to make any conclusions regarding the effects of Se toxicity in early life stage fathead minnow from this study, and it also presents a knowledge gap in the sensitivity of embryo-larval fathead minnow to Se exposure via maternal transfer.

1.4 Methods to study maternal transfer

1.4.1 Field based collection

Sexually ripe fish inhabiting aquatic systems with elevated Se concentrations can be captured and used to study maternal transfer using non-lethal artificial fertilization techniques. The milt and eggs of these fish can be collected in the field and then be transported to an aquatic facility, where the eggs can be fertilized, and the embryo-larvae reared until swim-up to mimic the natural maternal transfer and exposure to Se that would happen in the wild. The observation of embryos from fertilization until hatch and through yolk sac absorption can help researchers determine potential species-specific effects of elevated Se concentrations within embryos because of maternal transfer. Field based collection of fish is resource and labor intensive, and depending on the species under investigation, can be very difficult to carry out. For instance, Se contaminated sites are often located nearby remote mining operations that might not be accessible by road. Furthermore, sampling of contaminated field sites could exacerbate the already on-going effects in that system, which is more apparent for species of concern that are part of a threatened or endangered population, and one must consider the complex nature of aquatic food webs which will influence bioaccumulation and subsequent maternal transfer in these species. However, this is the only method of studying maternal transfer that provides a holistic *in situ* look at potential early life stage Se induced toxicities in a species and site-specific manner, which is valuable information for risk assessors, academics and industry. Also, regulatory compliance monitoring often requires *in situ* sampling for assessing Se contamination.

1.4.2 Dietary exposure

Reproductive assays using a dietary exposure performed in the laboratory allow for more control of the dose being administered, water quality and chemistry, and a more comprehensive

experimental design. In asynchronous spawning small-bodied fish such as the fathead minnow or zebrafish, reproductive assays are simple to perform and these assays can provide valuable information at multiple levels of biological organization. However, in many synchronous spawning species such as rainbow trout or white sturgeon, reproductive assays are not as logistically possible and are often unable to be performed without the aid of large scale aquatic facilities that can house the brood stock used in the study (Pilgrim, 2012). Simply put, the size, life history and reproductive strategy of fish can render them an ideal or unideal species to work with for laboratory reproductive assays, or for field-based sampling.

1.4.3 Embryo microinjection

Previous research has demonstrated that embryo microinjection is a useful technique for the uniform delivery of compounds to fish embryos as a representative method for maternal transfer that can be used to study teratogenesis (Hu et al., 2008; Schubert et al., 2014; Thomas and Janz., 2016; Walker et al., 1992; Walker et al., 1994; Walker et al., 1996). This technique has also been applied in a wide-variety of research within the realm of molecular and biotechnology (Zhang and Yu, 2008). Embryo microinjections have many advantages – they can be used to investigate toxicities of maternally transferred chemicals and their metabolites over a range of concentrations, and the relatively high throughput allows for an appropriate level of statistical power to be achieved (Walker et al., 1996). The most valuable aspect of microinjection is that consistent, uniform doses of a compound can be repeatedly delivered into embryos or oocytes, and this type of controlled dosing is not as easily achieved using field-based or reproductive assays interested in F_1 generation toxicity.

Embryo microinjections have been performed in Japanese medaka (*Oryzias latipes*) embryos to investigate maternal transfer of azaspiracid, a lipophilic phycotoxin and its effect on cardiac function in early life stage development (Colman et al., 2005). Triphenyltin, a contaminant of concern in the Yangtze River of China, has been injected into Chinese sturgeon (*Acipenser sinensis*) and Siberian sturgeon (*Acipenser baerii*) embryos to study potential effects on development of early life stages in two non-model species (Hu et al., 2008). There have also been injection studies in birds such as quail, frogs such as the African clawed frog (*Xenopus laevis*), and other model fish species such as rainbow trout or zebrafish (Franci et al., 2018; Maldifassi et al., 2016; Thomas and Janz, 2016; Walker et al., 1994).

Microinjection has been used to study the effects of SeMet exposure in embryo-larval zebrafish (Thomas and Janz, 2016). Embryos were injected with environmentally relevant concentrations of SeMet and a dose-dependent response in developmental toxicities was observed (Thomas and Janz, 2016). These results were comparable to a previous maternal transfer study in zebrafish and provided the first insight as to the utility of SeMet microinjections for studying maternal transfer in a Se ecotoxicology context (Thomas and Janz, 2014; Thomas and Janz, 2016). Fathead minnow embryos exhibit a similar physiology and rapid development as zebrafish allowing them to be used for microinjection studies in similar fashion. This could provide an interesting comparison of effects in early life stage fathead minnow and zebrafish after exposure to SeMet via embryo microinjection, as both species belong to the same family (Cyprinidae) of fish but inhabit different habitats and climates. More importantly, extending this method into fathead minnow could establish embryo microinjection of SeMet as an acceptable proxy for maternal transfer in a North American freshwater fish, and would provide an additional line of evidence for using this method to study the effects of maternal transfer in other species of early life stage fish that might be of greater ethical or regulatory concern within North America.

1.5 Research rational, objectives and hypothesis

1.5.1 Research goals

The overall goal of this study was to compare the biological response of early life stage fathead minnow to *in ovo* Se exposure via two different exposure routes, dietary maternal transfer and embryo microinjection. There are knowledge gaps for North American freshwater fishes and their early life stage species-specific sensitivity to Se due to difficulty in performing maternal transfer studies in certain species. Embryo microinjection could be a valuable method for performing these studies, and could be used to study non-model species, or species where dietary maternal transfer studies are unreasonable to perform, and could provide novel information regarding species-specific differences in response to Se toxicity in these fishes. However, there is need to optimize and further validate egg injection methods in species native to North American freshwater systems before it can be utilized as a standardized approach for assessing Se toxicity across diverse species. This study utilized the model fish and ecologically relevant fathead minnow to determine the effects of *in ovo* exposure to SeMet through two routes of exposure, maternal transfer and embryo microinjection, to characterize how the response is

comparable or different at similar embryo Se concentrations. This research provides an additional line of evidence regarding the use of embryo microinjection to study the biological effects of *in ovo* SeMet exposure, and whether this methodology can be extended to non-model species that might otherwise be unfeasible to investigate because of their location, longevity or late sexual maturation.

1.5.2 Objectives and hypotheses

The central objective and hypothesis of my thesis is:

To assess the effects of maternal transfer and microinjection *in ovo* exposure routes to determine if embryo microinjection is a useful proxy for studying Se maternal transfer in *Pimephales promelas.*

H_0: The toxicities of Se exposure in early life stage P. promelas will not be significantly different among treatment groups after in ovo exposure via maternal transfer and microinjection.

The general objectives of my research and associated hypotheses are:

1) Characterize the effects of dietary SeMet exposure in adult *Pimephales promelas* on reproduction by calculating the cumulative mean embryo production per female and mean clutch size per female.

H_{01}: There are no significant differences in fecundity of P. promelas among treatment groups over the 28-day dietary exposure to graded SeMet concentrations.

2) Determine if the maternal transfer of Se differs among control and dietary treatment groups in adult *Pimephales promelas* after exposure to dietary SeMet by quantifying total Se in embryos.

H_{02}: There are no significant differences in measured Se concentrations among embryos collected from breeding P. promelas after dietary exposure to graded SeMet concentrations.

3) Determine the effects of *in ovo* SeMet exposure in embryo-larval *Pimephales promelas* via maternal transfer and microinjection using hatchability, survival and deformity analysis.

H_{03}: There are no significant differences among treatment groups in embryo-larval toxicities exposed in ovo to graded SeMet concentrations via maternal transfer.

H_{04}: There are no significant differences among treatment groups in embryo-larval toxicities exposed in ovo to graded SeMet concentrations via embryo microinjection.

CHAPTER 2

IN OVO EXPOSURE OF FATHEAD MINNOW (PIMEPHALES PROMELAS) TO SELENOMETHIONINE VIA MATERNAL TRANSFER AND EMBRYO MICROINJECTION: A COMPARITIVE STUDY

PREFACE

The objective of Chapter 2 was to compare the effects of maternal transfer and microinjected selenomethionine to provide additional insight for using this methodology to study the maternal transfer of selenium in egg-laying vertebrates. This objective was met by determining the effects of dietary selenomethionine exposure on reproduction and the subsequent effects in F_1 generation fathead minnow until swim-up. This data was then used to inform a comparative study where microinjection of selenomethionine was the dosage route rather than natural maternal transfer, and similar endpoints in the F_1 generation were assessed. This chapter was prepared as a manuscript for submission to *Aquatic Toxicology* and the title is as follows: *In ovo* exposure of fathead minnow to selenomethionine via maternal transfer and embryo microinjection: A comparative study. The manuscript was prepared under joint authorship with Derek Green, Kerstin Bluhm, Katherine Raes, David Janz, Karsten Liber and Markus Hecker.

Author contributions:

Taylor Lane (University of Saskatchewan) designed and helped perform the maternal transfer and microinjection experiments, conducted animal husbandry, prepared samples for ICP-MS analysis, helped prepare the exposure diet, helped with microinjection method development, analyzed data, prepared all figures, and drafted the manuscript.

Derek Greek (University of Saskatchewan) helped perform the maternal transfer experiment, helped prepare the exposure diet, worked on microinjection method development, helped perform the microinjection experiment, and provided input on the manuscript.

Kerstin Bluhm (University of Saskatchewan) helped design and perform microinjection experiments, worked on method development for the microinjection experiment, helped with animal husbandry, and provided input on the manuscript.

Katherine Raes (University of Saskatchewan) helped design and perform the maternal transfer experiment, helped with animal husbandry, helped prepare the exposure diet, and prepared samples for ICP-MS analysis.

David Janz (University of Saskatchewan) provided scientific input regarding the experimental designs, data interpretation, provided resources for ICP-MS sample preparation, and commented on and edited the chapter.

Karsten Liber (University of Saskatchewan) provided scientific input regarding the experimental designs, data interpretation, provided resources for the exposure, and commented on and edited the chapter.

Markus Hecker (University of Saskatchewan) provided scientific input regarding the experimental designs, data interpretation, commented on and edited the chapter, supervised Taylor Lane, and obtained funding for the research.

2.1 Abstract

Selenium (Se) is an essential trace element of concern that is known to contaminate aquatic ecosystems as a consequence of releases from anthropogenic activities. Selenium is of particular toxicological concern for egg-laying vertebrates as they bioaccumulate Se through the diet and deposit excess Se to embryo-offspring via maternal transfer, a process which has been shown to result in significant teratogenic effects that can potentially impact populations. The purpose of the present study was to determine and compare the *in ovo* effects of Se exposure on early development of a standard laboratory model fish species native to North American freshwater systems, the fathead minnow (*Pimephales promelas*), through two exposure routes, maternal transfer and microinjection. For maternal transfer studies, fathead minnow breeding groups (3 females: 2 males) were exposed to diets containing Se-background levels (1.18 µg Se/g food, dry mass [dm]) and environmentally relevant concentrations of selenomethionine (SeMet; 3.88, 8.75 and 29.6 µg Se/g food dm) for 28 days. Embryos were collected at different time points throughout the study to measure Se concentrations and to assess teratogenicity in embryos. While exposure to dietary Se did not negatively affect fecundity among treatment groups, the lowest treatment group (3.88 µg Se/g food dm) produced on average the most embryos per day, per female. Furthermore, the maternal transfer of excess Se occurred rapidly upon onset of exposure, reaching steady-state after approximately 14 days. Embryo Se concentrations increased in a dose-dependent manner and were significantly different among treatment groups. The greatest concentrations of maternally transferred Se significantly increased the total proportion of deformities in embryo-larval fathead minnows, but did not impact hatchability or survival. In a second study, fathead minnow embryos were injected with SeMet at concentrations of 0.00 (vehicle control), 9.73, 13.5 and 18.9 µg Se/g embryo dm. Microinjection of SeMet did not affect hatchability but significantly increased the proportion of deformed embryo-larval fish in a dose-dependent manner. There was a greater proportion of deformed fathead minnows at embryo Se concentrations of 18.9 µg Se/g embryo dm when exposed via microinjection versus maternal transfer, which illustrated a more pronounced effect in toxicity when exposed via microinjection; however, the findings suggest that both exposure routes induced similar developmental toxicities in early life stage fish at Se concentrations between 9.7 and 13.5 µg Se/g embryo dm. Overall, this study demonstrated that microinjection has utility for studying the effects of Se in embryo-larval fish, and that microinjection of SeMet represents a

promising method for the study of early life stage Se exposure in long-lived or endangered species of ecological relevance which cannot be feasibly evaluated in maternal transfer studies.

2.2 Introduction

Certain aquatic systems are prone to increased loading of the essential trace element selenium (Se) which can originate from natural sources and anthropogenic activities (Janz et al., 2014; Luoma and Presser, 2009). In particular, activities such as coal combustion, uranium mining and milling, and agricultural irrigation are known to introduce inorganic species of Se into aquatic environments through effluents or agricultural drainage (Brandt et al., 2017; Lemly, 1999; Muscatello et al., 2006). While all animal classes require Se for proper cellular function, many organisms are susceptible to the toxicological effects of this element due to its narrow range between essentiality and toxicity (Avery and Hoffman, 2018; Haratake et al., 2015; Mangiapane et al., 2014). In Se contaminated aquatic systems, Se has been shown to bioaccumulate within food webs, and prey items of fish have been reported to have Se concentrations that can exceed 70 µg Se/g food dry mass (dm), whereas background concentrations are often in the range of 1 - 2 µg Se/g food dm (Hamilton, 2004). Selenium can occur in different forms in aquatic ecosystems, with selenite and selenate being the predominant inorganic Se species found in the water column as soluble oxyanions (Friesen et al., 2017; Simmons and Wallschläger, 2005). Once inorganic Se enters surface waters, it is bioconcentrated by a consortium of primary producers (e.g. phytoplankton, periphyton) and microorganisms (e.g. bacteria, fungi), and is biotransformed into various organic species (Conley et al., 2013; LeBlanc and Wallschläger, 2016). Of these organic species, selenomethionine (SeMet) has been reported to constitute 60 - 80% of the dietary Se species present in aquatic food webs and is the primary species of concern in Se bioaccumulation and toxicity (Friesen et al., 2017; Janz et al., 2014).

Consuming elevated dietary SeMet is of specific concern for oviparous (egg-laying) vertebrates such as fish, birds, reptiles and amphibians that offload excess amounts of Se via maternal transfer into the egg yolk, and which has been shown to result in teratogenic effects in the offspring (Covington et al., 2018; Holm et al., 2005; Kuchapski and Rasmussen, 2015; Lemly, 1993; Masse et al., 2015; Ohlendorf, 2002; Van Dyke et al., 2014). Oviparous vertebrates, such as fish native to North American freshwater aquatic systems, can be particularly at risk from exposure to SeMet because of the intensive activities associated with the resource extraction of metals (e.g. uranium) and fossil fuels (e.g. coal) in these regions (Janz et

al., 2014). It is therefore important to establish the sensitivity to SeMet exposure of fish species native to these, where the degree of hazard is often site-specific, and which can be difficult to assess because of logistical, biological and ethical reasons (Janz et al., 2010; Lemly, 1985; Lemly, 1995).

In fish, the maternal transfer of Se to eggs occurs during vitellogenesis, when SeMet is incorporated into yolk sac proteins indiscriminately in place of the amino acid, methionine (Kroll and Doroshov, 1991; Mangiapane et al., 2014). Offspring are exposed to SeMet as they utilize yolk sac proteins for growth and development from fertilization until swim-up, which are the most sensitive stages in the life of a fish. Larvae exposed to excess SeMet concentrations during this period are known to display a suite of spinal, fin, and craniofacial abnormalities, as well as an increase in the occurrence of edema being observed in several field and lab studies (DeForest et al., 2011; Holm et al., 2005; Janz et al., 2014; Lemly, 2018; Thomas and Janz., 2016). While it is generally accepted that maternal transfer is the most relevant route of SeMet exposure in fish and other oviparous animals, studying the effects of maternal transfer of Se is difficult in situations where a specific species cannot be collected from a contaminated site, or when the longevity and reproductive cycle of a species makes conducting controlled maternal transfer studies not feasible. This is particularly of concern in long-lived fishes such as some sturgeon species that often have threatened, vulnerable, or endangered populations. For example, several sturgeon species such as green sturgeon (*Acipenser medirostris*), Atlantic (Gulf) sturgeon (*Acipenser oxyrhynchus deostoi)* and the endangered white sturgeon (*Acipenser transmontanus*) are known to accumulate elevated concentrations of Se from their natural environments, and are species for which no maternal transfer experiments can be conducted under controlled conditions because of ethical reasons and the fact that they do not become reproductively active until about 25 years of age (De Riu et al., 2014; Gundersen et al., 2017; Linares-Casenave et al., 2015).

One method that has shown promise to bridge this gap and enable researchers to study early life stage toxicity of SeMet in species that otherwise might go unstudied is embryo microinjection. Previous research has demonstrated that embryo microinjection is a useful technique for the delivery of teratogenic compounds to fish embryos, and that effects can be representative of normal maternal transfer (Hu et al., 2008; Schubert et al., 2014; Thomas and Janz, 2016; Walker et., 1992; Walker et al., 1994; Walker et al., 1996). However, to date, only one microinjection study with zebrafish (*Danio rerio*), a common laboratory fish model, has

investigated the toxicity of *in ovo* SeMet exposure using both microinjection and maternal transfer as routes of exposure (Thomas and Janz, 2016). Zebrafish share similarities with the fathead minnow (*Pimephales promelas*) in that both species belong to the Cyprinidae family of fishes, they are established toxicology model organisms that spawn asynchronously and produce translucent embryos, and their development from embryo to swim-up larvae occurs rapidly (Ankley et al., 2006; Hill et al., 2005). Nevertheless, the two species differ in the ecosystems and climates they are found in, with zebrafish naturally inhabiting warm-water, neotropical areas and the fathead minnow being widely distributed throughout North American freshwater systems including northern ecosystems that have been shown to have elevated Se concentrations (Hill et al., 2005; Hood and Stocek, 2005). Because of this, the fathead minnow has greater ecological relevance in comparison to the zebrafish for studying the effects of Se exposure in fish species that inhabit North American freshwater ecosystems. To our knowledge, no embryo injection studies investigating the effects of *in ovo* Se exposure have been carried out in a species native to northern freshwater systems. Fathead minnows also represent an important link between lower and higher trophic levels in aquatic food webs (Potthoff et al., 2008). Furthermore, the similarities in embryo physiology and development between zebrafish and fathead minnow render the fathead minnow as a useful model organism for embryo microinjection studies in a fish species that is representative of North American ecosystems.

The main objective of this study was to provide additional insight on the use of microinjection to simulate and study SeMet maternal transfer in a fish species native to North American freshwater systems, the fathead minnow. This was done by comparing the effects of Se exposure through two different exposure routes, maternal transfer and embryo microinjection, in early life stage fathead minnow. The study design also provided the opportunity to investigate the effects of dietary Se exposure in adult female fathead minnows with regards to reproduction and maternal deposition of Se to embryos.

2.3 Methods

2.3.1 Test species

All experimental and fish culture procedures performed in this study were approved by the Animal Research Ethics Board at the University of Saskatchewan (Protocol #20130142) and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Fish were

from an in-house stock population of fathead minnows cultured at the Aquatic Toxicology Research Facility (ATRF), at the Toxicology Centre, which originated from a commercial supplier (Aquatic Research Organisms Inc., Hampton, USA). Sexually mature (>6 months old) fathead minnows were randomly selected and sorted by sex to establish breeding groups consisting of 2 males and 3 females in each 21 L aquaria. Two halves of PVC pipe were placed in each aquarium to act as a breeding substrate from which embryos were collected. The temperature (°C), pH, dissolved oxygen (%) and conductivity (µS/cm) were measured throughout the study using a YSI Professional Plus probe (YSI Incorporated., Yellow Springs, OH, USA). Additionally, total ammonia, hardness (mg $CaCO_3$/L) and alkalinity (mg $CaCO_3$/L) were measured using commercial test kits (Mars Fishcare, Chalfont, PA, USA; LaMotte Company, Chestertown, MD, USA). Tanks were kept at a temperature of 25°C ± 1°C, a pH of 8.0 ± 0.5 and were illuminated at 800 - 1000 lux in a 16h:8h light/dark cycle as per OECD Test Guideline 229 (OECD, 2012). Water renewals of >60% occurred daily to help maintain water quality using carbon-filtered, bio-filtered City of Saskatoon municipal tap water. Measured water quality parameters are presented in the supplementary materials (Table A.1) and were within the acceptable range for fathead minnow toxicity testing. Fish were fed twice daily with bloodworms (Bio-Pure Blood Worms, Hikari Sales Inc., Haywards, CA, USA) until satiation. Any excess food present in aquaria after feeding was siphoned out.

2.3.2 Adult reproductive assay

A dietary exposure study was performed with 55 fathead minnow breeding groups in order to investigate the effects of dietary SeMet exposure on reproduction, maternal transfer, and development of the F_1 generation.

Frozen bloodworms were freeze dried (Dura-Dry™ MP, FTS Systems, Stone Ridge, NY, USA), homogenized, and spiked with one of three nominal concentrations of Se (3, 9 and 27 µg Se/g food dm), 100% in the form of SeMet (Seleno-L-methionine (>98% purity), Sigma-Aldrich, Oakville, ON, Canada) dissolved in deionized water. Diet preparation followed previously established methods for spiking freeze-dried bloodworms for feeding to fathead minnow (McPhee and Janz, 2014). The control diet was not spiked and only contained natural background Se concentrations. Representative dietary samples (n=3 - 5) for each respective treatment were collected for total Se analysis. The prepared diet had measured total Se concentrations for the four respective treatments as follows: Control, 1.18 ± 0.18; Low, 3.88 ±

0.34; Medium, 8.75 ± 0.80; High, 29.6 ± 4.46 µg Se/g food dm as determined by inductively coupled plasma-mass spectrometry (ICP-MS) as described below.

To establish baseline egg production, fish were subjected to a 9-day pre-exposure phase during which reproductive output (average number of eggs per female per day) was recorded daily. At the end of the pre-exposure phase, tanks were ranked from best to worst reproductive output. The four best egg-producing aquaria were randomly assigned to one of each of the four treatment groups; followed by assignment of one of each treatment groups to the next best four egg-producing aquaria, and so on until all tanks were assigned a treatment group. The goal of this assignment method was to achieve equal baseline fecundity across each Se treatment before the exposure phase began. Each exposure treatment (3.88, 8.75 and 29.6 µg Se/g food dm) was assigned to 13 breeding groups (n=13) and the control treatment (1.18 µg Se/g food dm) was assigned to 16 breeding groups (n=16).

Breeding groups were fed twice daily (5% body weight/daily ration) with either unspiked (control) or SeMet-spiked frozen bloodworms and bred for 28 days. The exposure period was extended beyond the guidelines of OECD Test No. 229 to allow more time for maternal transfer of Se to embryos to occur. Breeding tiles were checked for embryo production before each feeding and at the end of each day, and embryos were collected upon notice of deposition. After quantification of egg numbers, if a clutch size was large enough, a random subsample of embryos (n=25 - 75) were collected and stored at -80ºC for total Se analysis. Furthermore, random subsamples of embryos (n=10 - 40) were collected from producing tanks for three consecutive days beginning on days 14, 21 and 26 for evaluation of hatchability, survival until swim-up, and deformity analysis.

2.3.3 Microinjection experimental design

Fathead minnow breeding and husbandry was performed as described above. Viable embryos were collected and distributed equally across each injection treatment group to control for clutch-to-clutch variation across breeding groups (Marentette et al., 2013). Prior to injection, SeMet was weighed and dissolved in nanopure water containing 5mM HEPES buffer (pH 7.2) to generate SeMet injection solutions for nominal embryo Se treatment concentrations of 10, 15, and 20 µg Se/g embryo dm after injection, as determined through a preliminary dose-range-finding study (Fig. B.1). The microinjection equipment used in this study were the Eppendorf Femtojet express (Eppendorf, Hamburg, Germany) and the Drummond Nanoject III (#3-000-

207, Drummond Scientific Company, Broomall, PA, United States of America). Both microinjectors followed a similar calibration method to deliver the desired volume. Each embryo was injected with approximately 3.0 nL of solution into the yolk prior to early gastrulation (6 hours post-fertilization [hpf]). Two control groups were included in this study: 1) A negative control group, which were embryos that did not go through the microinjection process and were unexposed to SeMet; and 2) a vehicle control, in which embryos were injected with the same volume of exposure solution (3.0 nL) that did not contain SeMet. For each treatment and control group, eight replicates of 10 embryos were injected and assessed for hatchability, survival and deformities. Also, for each treatment group, eight replicates of 20 embryos were injected and stored at -80ºC for total Se analysis. After injecting an entire treatment, embryos were randomly distributed to a vial for ICP-MS analysis or a Petri dish for evaluation of hatchability, survival until swim-up, and deformities.

2.3.4 Embryo-larval rearing and assessment

Embryos from both maternal transfer and microinjection experiments were reared in plastic Petri dishes at 24 ± 1°C containing 100 mL of carbon-filtered, bio-filtered, City of Saskatoon municipal tap water, of which 50 mL was renewed daily. Embryos were incubated through hatch and until complete yolk sac absorption (swim-up stage) to determine hatchability, survivability and the frequency of deformities. The swim-up stage is when Se exposure from yolk sac proteins should be complete, so maximum exposure and associated effects were anticipated to have occurred at that time. At this time, larvae were euthanized in buffered tricaine methanesulfonate (MS-222; 250 mg/L, pH 7.4), preserved in 10% buffered formalin for 16 hours, and transferred to 70% ethanol for storage until deformity analysis. Daily observations of embryos and larvae, and the preservation of larvae at swim-up were performed as described previously (Thomas and Janz, 2014).

2.3.5 Deformity analysis

Preserved larval fathead minnow were used for the deformity analysis at swim-up, which occurred 7 dpf. All preserved larvae were assessed for malformations in a blinded fashion, where the treatment group was unknown throughout each assessment, using a Zeiss Stemi 508 microscope (Carl Zeiss Canada, Toronto, ON, Canada). Images were captured using Zeiss Axiocam 105 (Carl Zeiss Canada, Toronto, ON, Canada) with ZEN lite imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany). Each larval fish was examined for morphological

deformities in four categories: craniofacial, spinal curvatures, finfold and edema using previously established methodology (Holm et al., 2005; Lemly, 1997; McDonald et al., 2010; Thomas and Janz, 2014). Furthermore, the severity of each deformity was determined using a numerical value of 0 - 3, with 0=no deformity, 1=mild deformity, 2=moderate deformity, and 3=severe deformity. The scale used for assessing the severity of deformities was a Graduated Severity Index (GSI) and was based on previously established guidelines (Janz et al., 2010; McDonald et al., 2010). Total percentage of deformities for any specific category was calculated by dividing the number of larval fish with that specific deformity present by the total number of larval fish assessed within a respective replicate. Further details regarding the criteria for deformity analysis can be found in Appendix E.

2.3.6 Selenium quantification and digestion

All total Se measurements in dietary and pooled embryo samples were conducted using ICP-MS (8800 ICP-MS Triple Quad, Agilent Technologies, Santa Clara, CA, USA) operated in collision cell mode at the Toxicology Centre, University of Saskatchewan, following previously established methods. All tissue Se measurements occurred in solution after digestion procedures were complete. Prior to digestion, experimental diet samples (n=3 - 5 per dietary treatment) and pooled embryo samples (10 - 75 embryos per sample) were freeze dried, homogenized, weighed, and transferred into individual polytetrafluoroethylene (PTFE) vials for digestion. Samples were digested with high purity, 69% nitric acid (1 mL) and high purity, 30% hydrogen peroxide (0.66 mL) (Sigma-Aldrich, St. Louis, MO, USA) using a MARS-5 microwave digestions system (CEM Corporation, Matthews, NC, USA) following previously established methods (Markwart et al., 2019). Digested samples were filtered (0.45 μm pore size, polyethersulfone membrane), diluted to approximately 2% HNO_3, and stored at 4°C until ICP-MS analysis was performed.

The instrumental certified reference material for Se analysis was "1640a – Trace Elements in Natural Water" (National Institute of Standards and Technology, Gaithersburg, MD, USA). The mean (±SD) for Se analysis was 101 ± 0.96% of the certified reference value. TORT-2 (lobster hepatopancreas) from NRC Canada (Institute for Environmental Chemistry, Ottawa, Canada) was used as the certified standard reference material for tissue and measured 106 ± 3.5% (mean ± SD).

2.3.7 Statistical analysis

All data analyses were performed using R v3.5.1 (R Core Team, 2018). Data were tested for normality via the Shapiro-Wilk test. Homogenity of variance was tested using Levene's test. Data that were not normally distributed were transformed via Box-Cox transformation (Box and Cox, 1964) for normalization. All figures contain non-transformed data. Likelihood ratio tests (LRT) were used for all tests of statistical significance differences in total Se concentrations in embryos, embryo hatchability, mortality and deformities of early life stage fathead minnow among the control and treatment groups, within both respective exposure routes. The LRT assesses whether the removal of a variable causes a significant decrease in model fit. P values (α=0.05) denote a significant increase in deviance when a respective variable was removed. For the graduated severity index (GSI) data, data that met the assumptions for parametric analysis was tested using one-way ANOVA followed by Tukey's post hoc test; whereas data that could not be transformed to meet the assumptions were analyzed using the Kruskal-Wallis one-way ANOVA by ranks test was used followed by Dunn's multiple comparisons post hoc test. Test probabilities are two-tailed throughout. A linear model was used to determine whether Se accumulated in embryos over time via maternal transfer. Here, we analyzed if the mean embryo Se concentration from a clutch of embryos was affected by the dietary treatment the parental fish received, represented by 'treatment', and the independent variable, 'day', which describes the number of days of dietary exposure to Se (interaction 'treatment' × 'day'). To assess potential differences in fecundity among treatment groups, we used a linear model to analyze if the dependent variable, 'fecundity' (cumulative average number of eggs/female/day), was influenced by the dietary treatment the parental fish received ('treatment') and the number of days of dietary exposure, represented as 'day' (interaction 'fecundity' × 'day'). The analysis of deformity proportions was based on binomial data (deformed or not deformed); therefore, we used a generalized linear model (GLM) that fit the model using iteratively reweighted least squares. As data displayed overdispersion, a quasibinomial distribution was assumed. The GLM analyzed whether the proportion of deformed fathead minnow larvae ('proportion', response variable) was affected by the relationship between embryo Se concentration ('dose', explanatory variable) and the route of exposure ('route', covariate), and the specific interaction 'dose' × 'route' was analyzed to compare effects of maternal transfer and microinjection exposure routes. Slopes

generated in respective linear and generalized linear models were compared using one-way ANOVA.

2.4 Results

2.4.1 Selenium concentrations and maternal transfer

Water quality conditions were consistently acceptable throughout the experiments and the full suite of measured parameters is summarized in the supplemental materials (Table A.1). The mean total Se concentrations of the fish diet for the four respective treatments were as follows: Control, 1.18 ± 0.18; Low, 3.88 ± 0.34; Medium, 8.75 ± 0.80; High, 29.6 ± 4.46 µg Se/g food dm. The corresponding mean total Se concentrations in embryos sampled from the maternal transfer study were very similar to the dietary treatment received, at nearly an approximate trophic transfer factor of 1 from diet to embryo (Table 2.1); and Se concentrations in embryos from the microinjection study were in reasonable agreement, although slightly higher at the low and medium Se treatments and lower at the high Se treatment. Both were in good agreement with their respective nominal concentrations (Table 2.1). In embryos that received Se via microinjection of SeMet, there was a significant difference in embryo Se concentration among microinjection treatment groups, except between the negative and vehicle controls (LRT, df=4, χ^2=-36.464, $p<0.001$, Table 2.1). The period of dietary Se exposure from days 0 - 13 shared a significant and positive relationship between the number of days of exposure and Se accumulation in embryos (LRT, df=1, χ^2=-9.230, $p<0.001$). For the rest of the exposure (days 14 - 28) there was a nonsignificant relationship between the number of days of exposure and Se accumulation in embryos, suggesting steady-state accumulation was reached for this time period (LRT, df=1, χ^2=-0.018, $p=0.274$, Fig. 2.1). However, mean embryo Se concentrations during days 14 - 28 of the exposure period were significantly different among treatment groups, suggesting that embryos accumulated different amounts of Se as a function of the dietary treatment the maternal fish received (LRT, df=3, χ^2=-4620.8, $p<0.001$, Fig. 2.1).

Table 2.1. Embryo Se concentrations determined from the maternal transfer and microinjection studies. Maternal transfer embryo Se concentrations were from embryo samples collected between days 14 - 28 of exposure. Different letters represent a significant difference (α=0.05) in Se concentration among treatment groups within either respective exposure route. Lower case letters represent the maternal transfer treatment groups; Uppercase represent the microinjection treatment groups. Data are presented as mean ± SEM.

	Maternal Transfer		**Microinjection**	
	Embryo Selenium Concentration (µg Se/g embryo dry mass[dm])			
Treatment	**Nominal**	**Actual**	**Nominal**	**Actual**
Negative Control	0	1.96 ± 0.20^{a}	0	1.95 ± 0.11^{A}
Vehicle Control	--	--	0	2.09 ± 0.06^{A}
Low	3	4.97 ± 0.21^{a}	10	9.73 ± 0.41^{B}
Medium	9	10.93 ± 0.56^{b}	15	13.49 ± 0.73^{C}
High	27	28.39 ± 1.60^{c}	20	18.90 ± 0.86^{D}

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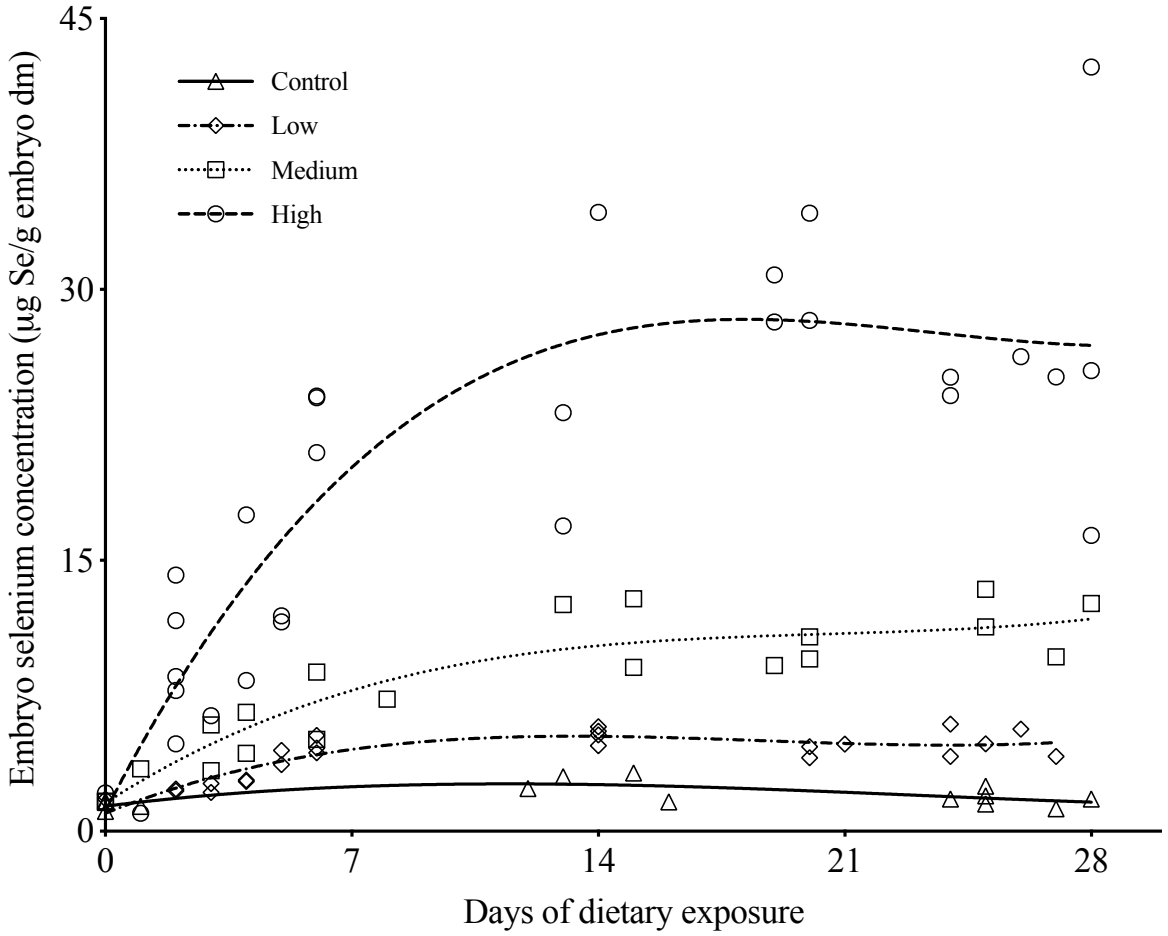

Figure 2.1. Concentrations of total Se in embryos collected from adult *P. promelas* after exposure to one of four Se dietary treatments over the course of 28 days. The four treatment groups were Control (1.18 µg Se/g food dm), Low (3.88 µg Se/g food dm), Medium (8.75 µg Se/g food dm), and High (29.58 µg Se/g food dm). Each plotted point represents a mean Se concentration measured from an individual clutch within a respective treatment. The line for each treatment was fit using a third order polynomial ($Y=B_0 + B_1*X + B_2*X^2 + B_3*X^3$).

2.4.2 Reproductive output

Exposure to dietary Se did not have an impact on reproductive output of any of the adult fish in the maternal transfer study as demonstrated by the lack of significant difference in mean clutch size among treatments during the 28-day exposure period (LRT, df=3, χ^2 = -1.400, p=0.299; Fig. C.1). There was a significant relationship between number of dietary exposure days and the average cumulative number of embryos produced per female per day (LRT, df=1, χ^2= -4.268, p < 0.001, Fig. C.2) and dietary exposure to SeMet among treatment groups did not affect the slope (LRT, df=3, χ^2= -0.982, p=0.334) nor the intercept (LRT, df=3, χ^2= -1.127, p=0.273) of this relationship.

2.4.3 Early life stage assessment

Exposure of embryos through maternal transfer of Se did not have a significant impact on hatchability (LRT, df=3 χ^2=-9.875, p=0.303, Fig. D.1B) and over 89% of embryos successfully hatched within any respective treatment group. Survival of fathead minnow larvae from fertilization until swim-up was also not significantly impacted by the maternal transfer of Se (LRT, df=3, χ^2=-11.374, p=0.529, Fig. D.1A) and over 86% of larvae survived within any given treatment group. In contrast, microinjection of SeMet did have a significant effect on the survival of fathead minnow larvae until swim-up in the high treatment (LRT, df=4, χ^2=-20.009, p<0.001, Fig. D.1A), but likewise, did not cause a significant effect on hatchability among treatment groups (LRT, df=4, χ^2=-3.229, p=0.520, Fig. D.1B).

Representative images of selected morphological abnormalities are shown in Fig. 2.2 (spinal and edema) and Fig. 2.3 (craniofacial and finfold). The assessment of morphological abnormalities revealed a significant difference among treatment groups in the proportion of total deformities (any deformity, any severity) in larval fathead minnow exposed via maternal transfer (LRT, df=3, F=7.034, p<0.001, Fig. 2.4) and via embryo microinjection (LRT, df=4, F=11.228, p<0.001, Fig. 2.4). In larval samples from the microinjection study, we observed significant differences in the proportion of each type of deformity among treatment groups within all respective deformity categories, which included: finfold (LRT, df=4, F=7.230, p<0.001, Fig. 2.5A); spinal (LRT, df=4, F=8.886, p<0.001, Fig. 2.5B); craniofacial (LRT, df=4, F=7.029 p<0.001, Fig. 2.5C); and, edema (LRT, df=4, F=3.881 p=0.010, Fig. 2.5D).

For the microinjection exposure route, regression analysis revealed a significant positive relationship between embryo Se concentration and the percentage of deformities present (LRT,

df=1, F=34.499, p<0.001, Fig. 2.6). Likewise, the maternal transfer exposure route also had a concentration dependent effect on the proportion of deformities present (LRT, df=1, F=10.901, p=0.005, Fig. 2.6). Notably, we observed that microinjection of SeMet increased the frequency of deformities (y = 2.307x + 0.112) more so than did maternal transfer (y = 1.910x + 0.034) at similar Se concentrations, and the slopes were significantly different between regression lines of the exposure routes (ANOVA, p<0.001, Fig. 2.6). Morphological abnormalities within each respective category of deformities were also evaluated using a GSI and is summarized in Fig D.2. There were no significant differences in the severity of deformities among treatment groups within either exposure route for finfold abnormalities (maternal transfer: KW, df=3, χ^2=1.195, p=0.754; microinjection: KW, df=4, χ^2=9.136, p=0.058, Fig. D.2A) and edema (maternal transfer: KW, df=3, χ^2=1.870, p=0.600; microinjection: KW, df=4, χ^2=5.922, p=0.205, Fig. D.2D). For craniofacial abnormalities, there was only a significant difference in the severity among treatment groups within microinjection exposed fish (KW, df=3, χ^2=11.89, p=0.008, Fig. D.2C). Moreover, there was a significant difference in the severity of spinal deformities among treatment groups exposed via maternal transfer (ANOVA, df=3, $F_{3,15}$=5.831, p=0.008, Fig. D.2B) and microinjection (KW, df=4, χ^2=14.04, p=0.007, Fig. D.2B).

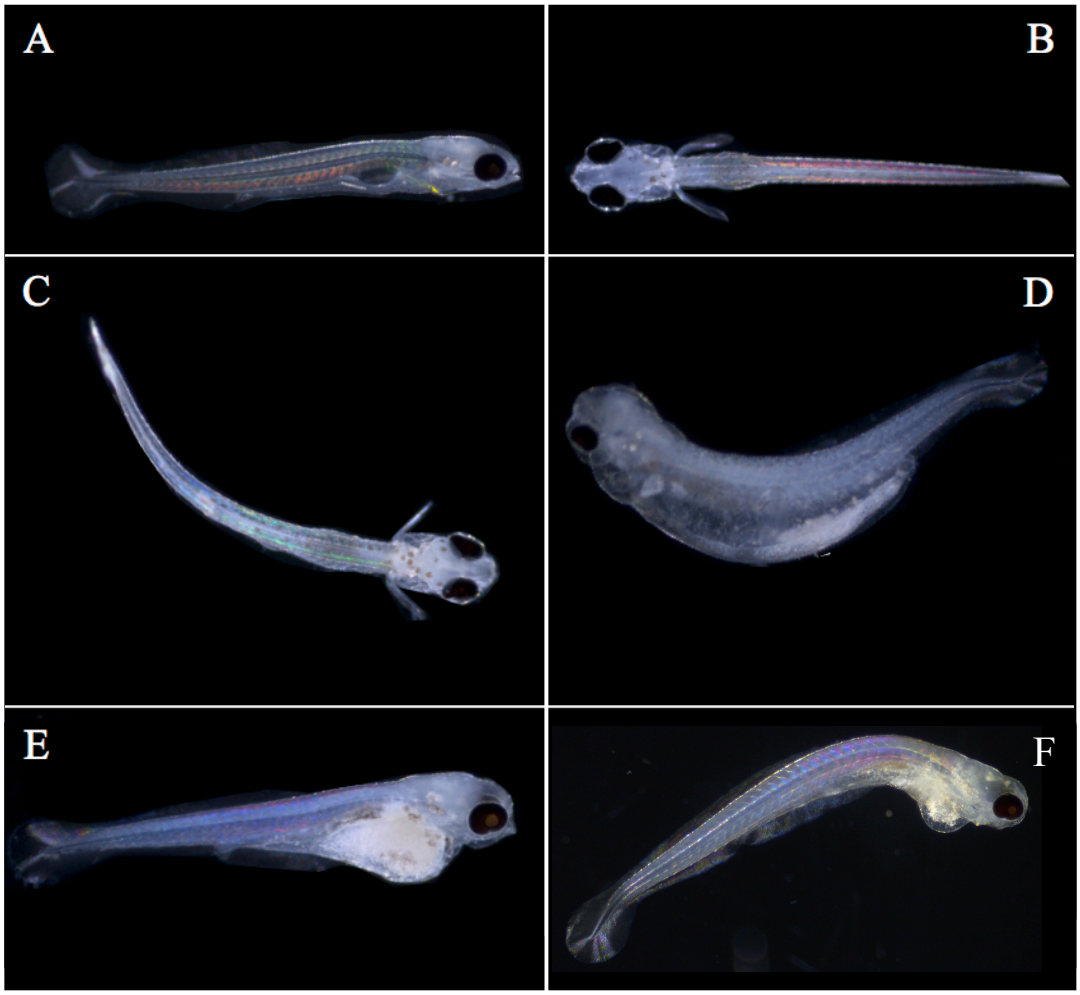

Figure 2.2. Representative whole body images of normal (A and B) and morphologically abnormal (C, D, E, and F) *P. promelas* photographed at swim-up stage during deformity analysis. Images A and B depict normal fish from lateral and dorsal viewpoints, respectively. Image C is a deformed fish with scoliosis and finfold abnormalities. Image D displays pericardial and yolk sac edema, and the spinal abnormality, lordosis. Image E depicts a fish with edema and deformed craniofacial structure. Image F is a fish with kyphosis and pericardial edema.

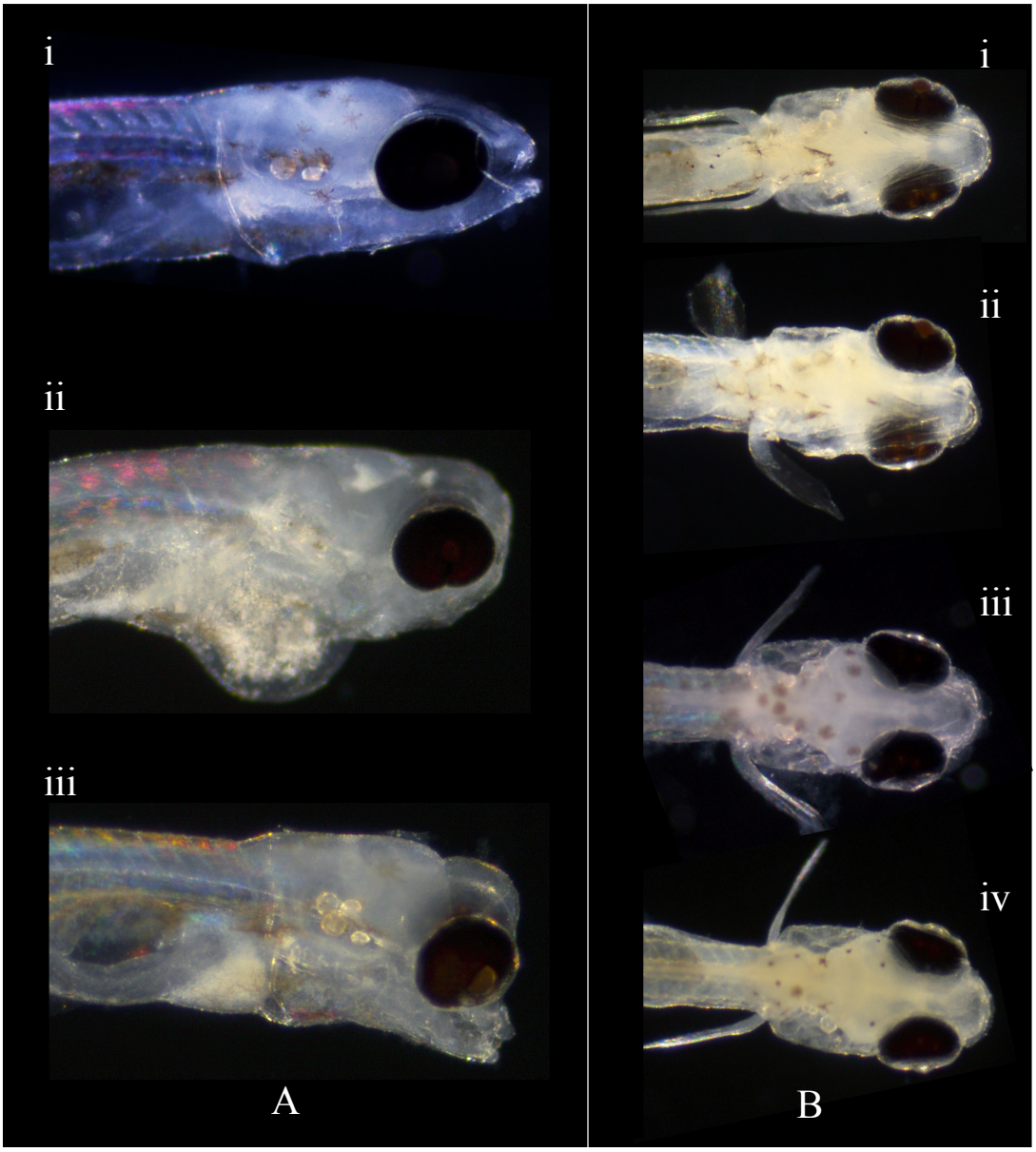

Figure 2.3. (A) Representative features of craniofacial development which depict normal development (i), absent jaw (ii), and deformed jaw and craniofacial structure (iii) in *P. promelas* at swim-up. (B) Representative images of normal finfold development (i), abnormal finfold curvature (ii), and misaligned finfold development (iii & iv) in *P. promelas* at swim-up.

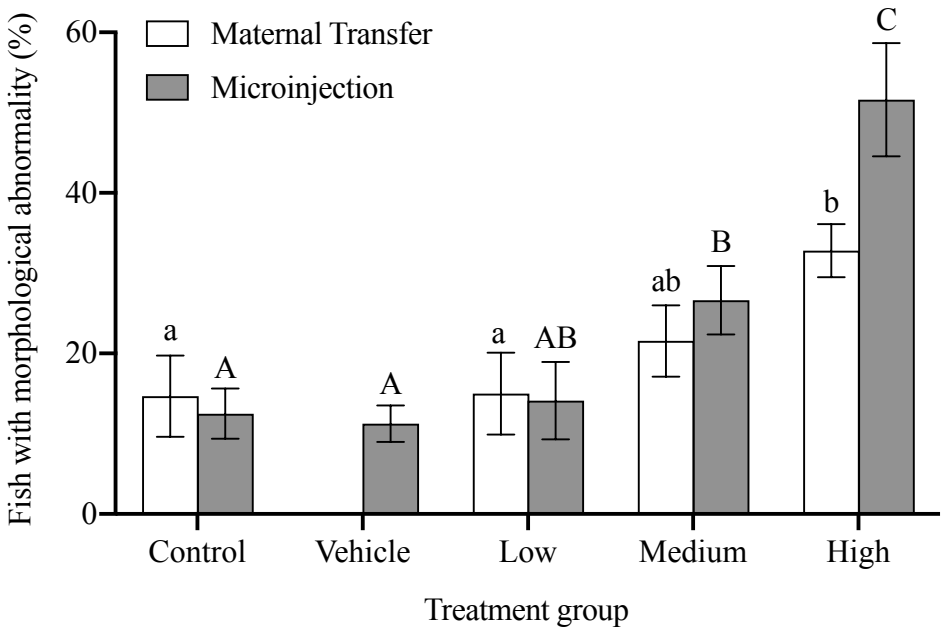

Figure 2.4. Percentage of *P. promelas* with any morphological deformity present at swim-up. Different letters represent a significant difference (α=0.05) in the proportion of deformities among treatment groups, within each exposure route. Uppercase letters represent significant differences within the microinjection exposure treatment groups. Lowercase letters represent significant differences within the maternal transfer exposure treatment groups. Data are presented as mean ± SEM.

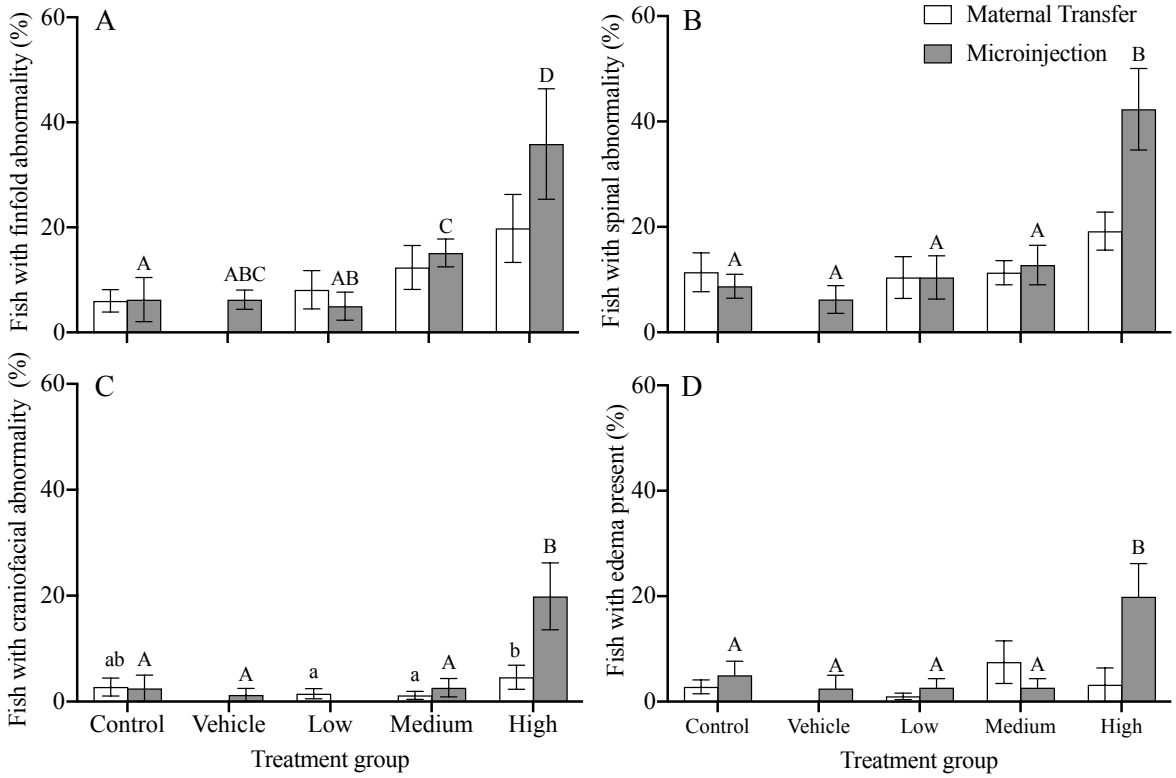

Figure 2.5. Percentage of *P. promelas* at swim-up with (A) finfold abnormality, (B) spinal abnormality, (C) craniofacial deformity, and (D) edema present. Different letters represent a significant difference (α=0.05) in the proportion of deformities among treatment groups, within each exposure route. Uppercase letters represent significant differences within the microinjection exposure treatment groups. Lowercase letters represent significant differences within the maternal transfer exposure treatment groups. In panels where no letters are present there were no significant differences among treatment groups. Data are presented as mean ± SEM.

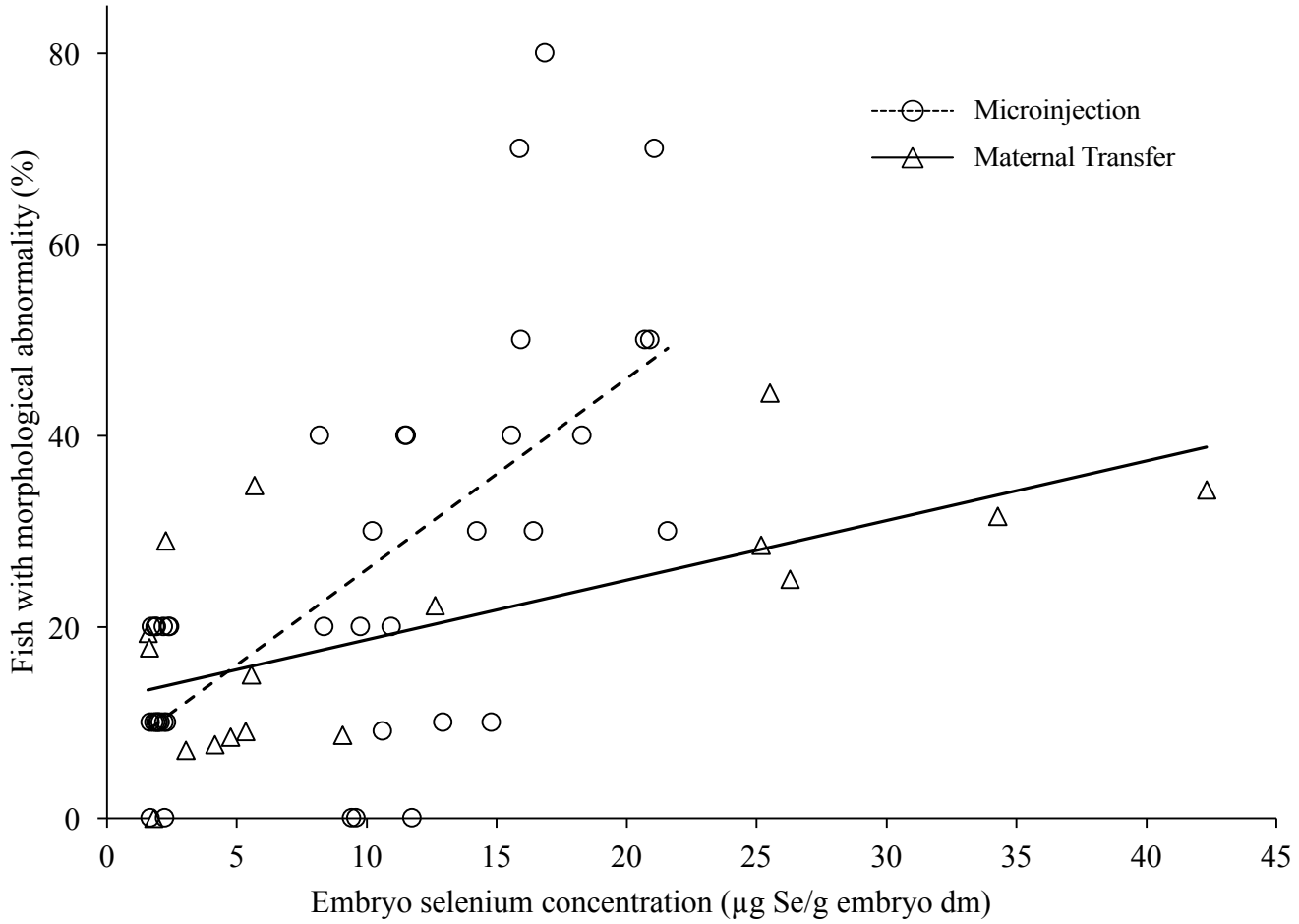

Figure 2.6. Regression analysis depicting the relationship between embryo selenium concentration and the proportion of *P. promelas* with a morphological abnormality (any deformity) at swim-up. Each plotted point is representative of a clutch of embryos that were randomly subsampled between days 14 and 28 to measure total Se concentration and for deformity analysis. The fitted lines are least squares regression lines and were significantly differently between the exposure routes (ANOVA, $p<0.001$).

2.5 Discussion

This study revealed significant differences in the sensitivity of fathead minnows to SeMet exposure as a function of embryo-larval life stage and route of delivery. It was demonstrated that dietary exposure to SeMet at environmentally relevant concentrations did not affect fecundity in adult fathead minnows, nor caused any reductions in hatchability or survival until swim-up in offspring. However, increases in teratogenic malformations were observed in embryos exposed through both maternal transfer or microinjection, and microinjection decreased survival until swim-up at the highest embryo Se concentration. Most notably, at Se concentrations of 18.9 µg Se/g embryo dm, microinjection of SeMet increased the frequency of deformities in early life stage fathead minnow to a greater extent than in fish exposed through maternal transfer, suggesting that embryos were slightly more sensitive when injected at this concentration compared to those exposed via maternal transfer at concentrations of 28.4 µg Se/g embryo dm. The response to embryo Se exposure in the form of total deformities (any deformity, any severity) and individual categorical deformities (spinal, craniofacial, finfold and edema) were similar at concentrations of 9.7 to 13.5 µg Se/g embryo dm regardless of exposure route.

2.5.1 Reproduction and dietary selenium exposure

Fecundity of adult female fathead minnows was not affected by elevated concentrations of dietary Se and appeared to follow a supra-nutritional trend with the lowest exposure dose (3.88 ± 0.34 µg Se/g food dm) showing the greatest embryo production. A similar trend, where supra-nutritional Se supplementation has been shown to be beneficial to the organism, has been described before in several aquaculture studies (Berntssen et al., 2018; Lee et al., 2016). It is generally accepted that exposure to elevated levels of dietary Se does not impact fecundity, nor does the maternal transfer of Se reduce fertility or hatchability rates in the F_1 generation of exposed parents, as shown in several fish species including brown trout (*Salmo trutta*), zebrafish, cutthroat trout (*Oncorhynchus clarkii*), northern pike (*Esox lucius*), white sucker (*Catostomus commersoni*), rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis)* and Dolly Varden char (*Salvelinus malma*) (Covington et al., 2018; Holm et al., 2005; Kennedy et al., 2000; McDonald et al., 2010; Muscatello and Janz, 2009; Thomas and Janz, 2014).

Ogle and Knight (1989) observed no significant effect on fecundity when exposing fathead minnows to a diet composed of 25% SeMet, 25% selenate, and 50% selenite at total Se concentrations as high as 29.5 µg Se/g food dm. Our study, which utilized a similar dietary Se

concentration (29.6 ± 4.46 µg Se/g food dm), administered 100% in the form of SeMet, is in agreement with the observations by Ogle and Knight (1989) on hatchability and survival in embryo-larval fathead minnow. We recognize the fact that in a natural aquatic system, fish consume a diet that contains a variety of Se species, but previous research has shown that SeMet species are an important form of Se that can compose greater than 60% of the Se speciation within an aquatic food-web where Se contamination is present, and this SeMet is highly bioavailable and trophically transferred among aquatic organisms (Janz et al., 2014). Ingested SeMet is less prone to biotransformation than inorganic Se species and can be directly assimilated into methionine-containing proteins in a dose-dependent fashion (Behne et al., 1991). The diet adult fathead minnows were exposed to in this study would presumably have greater bioavailability of SeMet than a diet containing a mixture of organic and inorganic Se species, thereby rendering it an effective and useful approach for investigating Se maternal transfer in fathead minnow. Furthermore, the concentration of Se that undergoes maternal transfer is altered by the amount of SeMet that is available to be incorporated into methionine-containing proteins, and therefore using a diet that is spiked strictly with SeMet provides an optimal exposure scenario for inducing maternal transfer.

The dietary Se concentrations used in this study were selected based on environmentally relevant concentrations and were consistent with previous field and laboratory studies which reported sublethal Se toxicities at concentrations comparable to the prepared diets used in this work (Covington et al., 2018; Hamilton, 2004; Lemly, 1993; Lemly, 2018; McPhee and Janz, 2014). A dietary Se concentration of 40 µg Se/g food dm, which is greater than those tested here, has been reported to impair growth, feeding and spawning in fathead minnows (Ogle and Knight, 1989). In this context, the range of nominal dietary Se concentrations and Se composition (e.g. 100% SeMet) selected for the current study were considered suitable for examining the maternal transfer of Se in fathead minnow without introducing lethality in the spawning adult fish.

2.5.2 Maternal transfer of selenium

Reproductively active female fathead minnow efficiently and rapidly transferred Se to eggs during oogenesis in a dose-dependent fashion. Selenium concentrations of fathead minnow embryos were significantly and positively correlated with the dietary treatment the maternal fish received, and embryo Se concentrations reached a steady-state of approximately a 1:1 ratio to the dietary Se concentration received. Selenium has been demonstrated to be incorporated into

ovarian follicles during vitellogenesis (Kroll and Doroshov, 1991). Vitellogenesis is a key event in oocyte development regarding maternal transfer of Se to the embryo, because this is when reserves of yolk proteins, derived from the precursor vitellogenin, are deposited into oocytes (Sullivan and Yilmaz, 2018; Tyler and Sumpter, 1996). Vitellogenin is a precursor to yolk proteins and is critical as a carrier off carbohydrates, lipids, minerals, fat-soluble vitamins and hormones required for normal embryo-larval development, and is also the primary source of amino acids (Lubzens et al., 2017; Sullivan and Yilmaz, 2018). It is hypothesized that female fathead minnow exposed to elevated levels of dietary Se efficiently shuttle this compound via sulfur containing proteins in vitellogenin to developing ovarian follicles (Janz et al., 2010; Kroll and Doroshov, 1991). Biochemically, the enzyme methionyl-tRNA acylase does not discriminate between methionine and SeMet, as both compounds have a similar molecular structure with the only difference being the sulfur atom in methionine being replaced with a Se atom in SeMet (Janz et al., 2010; Schrauzer, 2000). This structural similarity results in the integration of SeMet into methionine-containing proteins, in a dose-dependent fashion, and our results suggest this occurs rapidly in the ovaries of fathead minnows upon dietary exposure to elevated concentrations of SeMet, prompting maternal transfer of SeMet to embryos.

2.5.3 Comparison of maternal transfer and microinjection exposure routes

The main goal of this study was to compare the effects of Se exposure in early life stage fathead minnow between two different exposure routes, maternal transfer and microinjection, to provide additional insight regarding the use of microinjection as a proxy for maternal transfer of Se. Teratogenic effects often do not begin to manifest until after hatch when fishes begin increasing use of yolk sac components for growth and development (Holm et al., 2005). This study concurs with that observation, as neither maternally transferred nor microinjected SeMet impacted hatchability of fathead minnow embryos at Se concentrations of up to 28.4 ± 6.50 µg Se/g embryo dm (maternal transfer) and 18.9 ± 2.44 µg Se/g embryo dm (injection), while significant increases in developmental malformations had manifested in larvae sampled at swim-up. Furthermore, the embryo microinjection of SeMet at concentrations of 18.9 ± 2.44 µg Se/g embryo dm decreased survival of fathead minnow until swim-up. To our knowledge, there is only one other SeMet microinjection study that has been performed in fish embryos, and therefore, Thomas and Janz (2016) offers an important point of comparison because of similarities in embryo Se concentrations to the current study. Contrary to our observations on

fathead minnow hatchability, however, Thomas and Janz (2016) reported that microinjection of SeMet into zebrafish embryos at Se concentrations of 18.7 ± 1.0 µg Se/g embryo dm significantly reduced hatchability. This could be due to species-specific differences in their susceptibility to Se toxicity based on the rate of pre-hatch development and amino acid usage (Finn and Fhyn, 2010). For instance, zebrafish hatch between 48 - 72 hours (at 28°C) and fathead minnow embryos hatch after approximately 96 – 120 hours (at 25°C) (Hill et al., 2005). The quicker pre-hatch development in zebrafish could make this species more prone to Se toxicity, especially to freely available SeMet, because the more rapid development would allow less time to cope with Se exposure. Selenomethionine is known to generate superoxide radicals when broken down because of redox cycling of its two main metabolites, methylselenol and selenide anion (Palace et al., 2005; Spallholz et al., 2004). Faster rates of pre- and/or post-hatch development could metabolize greater concentrations of SeMet during protein catabolism, and thereby intensify the production of reactive oxygen species over a shorter period of time, rendering these organisms more vulnerable to oxidative stress.

Palace et al. (2004) indicated that a susceptible stage of embryonic development to Se toxicity is before production of the enzyme superoxide dismutase (SOD) in the liver of fish. Presumably, this could be a period of development where coping with oxidative stress in the form of superoxide radicals is minimal or non-existent. Zebrafish develop a visible liver within 24 hpf, which undergoes rapid growth until fully developed, and SOD activity has been demonstrated in zebrafish larvae sampled at 96 hpf (Shi et al., 2011; Wilkins and Pack, 2013). In fathead minnow, the liver is first observed approximately 36 hpf, around the onset of blood circulation, where it extends into the yolk sac throughout development and does not approach complete development until approximately 120 hpf (USEPA, 1996). While slight, these differences in liver and overall early life stage development are potential reasons for the observed disparities in pre-hatch tolerance to elevated Se concentrations observed between zebrafish and fathead minnow.

Survival until swim-up in microinjected zebrafish was reported by Thomas and Janz (2016) to significantly decrease at *in ovo* concentrations of 11.0 µg Se/g embryo dm. The current study also detected a significant decrease in survival until swim-up after microinjection, but at slightly higher concentrations of 18.9 ± 2.44 µg Se/g embryo dm, and with a lower cumulative mortality rate at similar Se concentrations compared to the Thomas and Janz (2016) zebrafish

study. While fathead minnows appear to be slightly less sensitive to microinjection than zebrafish, it should be noted that the toxicity threshold for most North American fish species is approximately 20 µg Se/g embryo dm and there are species-specific differences with regards to Se toxicity in early life stage fish (DeForest et al., 2011). The underlying mechanisms of these species-specific differences are unclear, but they might be a function of embryo composition (e.g. lipid, protein, free amino acid, ion and glycogen content), in additional to differences in developmental rates (Finn and Fhyn, 2010; McDonald et al., 2010; Singleman and Holtzman, 2014).

Deformity analysis of fathead minnow larvae at swim-up revealed that maternal transfer and microinjection both increased the frequency of deformed larvae in a dose-dependent fashion, and which agrees with earlier studies that found an increased proportion of deformed larvae hatched from embryos with elevated Se concentrations (Covington et al., 2018; Holm et al., 2005; Muscatello et al., 2006; Thomas and Janz, 2016). Maternal transfer of Se to embryos at concentrations of 3.9 ± 1.87 µg Se/g embryo wet weight (ww) (~10.6µg Se/g embryo dm) were reported by Schultz and Hermanutz (1990) to have significantly increased the percentage of fathead minnow larvae that developed edema (24.6 ± 36.1%) or the spinal abnormality, lordosis (23.4 ± 20.8%), but the degree of variability and lack of a described procedure for conducting deformity analysis make their study less interpretable, and the authors did not report the total percentage of deformed fish. Our study found that the most common type of morphological malformations were finfold and spinal, which in most cases were approximately two-fold more common than craniofacial abnormalities or edema, regardless of Se concentration or exposure route. Although quantifying the frequencies of individual types of deformities is useful to compare exposed fish to natural background rates of specific types of deformities in control groups, quantifying of the total proportion of deformed fish provides a more ecologically realistic perspective on the potential for population level effects related to Se toxicity. Following an index developed by Lemly (1997) for Se induced teratogenesis to assess population level effects, our data suggests that at the highest maternal transfer embryo Se concentration (28.4 ± 6.50 µg Se/g embryo dm) greater than 20% of the larval fathead minnow population would likely succumb to mortality because of developmental abnormalities, and a substantial impact on the larval population could be anticipated. Under natural conditions, fish endure a multitude of stressors, such as predation, disease, and changes in environmental conditions, many of which

can lead to mortalities or reduced recruitment of healthy juveniles into the adult population. Therefore, the effects of Se exposure in conjunction with these natural stressors are indicative of increased potential for adverse population level effects to occur (Brandt et al., 2017; Lemly, 1999).

An important finding of the present study was that microinjection of SeMet resulted in a greater proportion of deformed fish at swim-up, at lower embryo Se concentrations, in comparison to fish exposed through maternal transfer. This difference in potency between the two exposure routes is likely a function of bioavailability of Se within the yolk sac (e.g. protein-bound vs. free amino acid) (Finn and Fhyn, 2010; McDonald et al., 2010; Thomas and Janz, 2016). When SeMet is microinjected into an embryo it is not protein-bound, but is available in free form which can be used in protein synthesis or undergo metabolic alterations without protein catabolism being required (Finn and Fhyn, 2010). This differs from maternal transfer where Se deposition in the embryo will occur primarily as protein-bound SeMet, and studies have shown that protein-bound amino acids are predominantly used post-hatch; however, there still will be other forms of Se present, such as selenocysteine (protein-bound), inorganic Se (mainly selenite) and free SeMet, although in considerably lower amounts than protein-bound SeMet (Finn and Fhyn, 2010; Rigby et al., 2014).

Interestingly, while the frequency of deformed fathead minnows increased in a dose-dependent fashion, there was little evidence of deformities becoming more severe at increasing Se concentrations. The severity of deformities present in fathead minnows exposed via maternal transfer was variable across all treatment groups and within all categories of deformities. In contrast, there appeared to be a trend towards greater severity of deformities at the highest concentration of microinjected SeMet (18.9 ± 2.44 µg Se/g embryo dm), which could be in part due to the combination of increased SeMet bioavailability and metabolism of SeMet. This is an issue for injected SeMet because it is freely available, but not for maternal transfer because it is predominantly protein-bound, and this disparity could be reason for a more noticeable dose-dependent trend in the severity of deformities (Rigby et al, 2014; Thomas and Janz, 2016).

2.5.4 Conclusions

In summary, dietary exposure to elevated levels (3.88 - 29.6 µg Se/g food dm) of SeMet in reproductively active fathead minnows did not affect negatively affect fecundity, and the lowest dose (3.88 ± 0.34 µg Se/g food dm) produced the greatest number of eggs per female,

suggesting a potential supra-nutritional benefit of dietary SeMet supplementation. The maternal transfer of Se did not induce changes in hatchability or survival until swim-up, but there were increased frequencies of deformities present in the offspring of fathead minnow from parents fed a diet containing 29.6 ± 4.46 µg Se/g food dm. This increased the proportion of deformed F_1 generation fathead minnows, and which could have implications at the population level as there would likely be less survival and thereby decreased recruitment of fish into the population. When SeMet was microinjected into fathead minnow embryos at concentrations of 18.9 ± 2.44 µg Se/g embryo dm, there was a greater increase in the proportion of deformities at Se concentrations lower than those of embryos exposed through maternal transfer (28.4 ± 1.60 µg Se/g embryo dm). With this said, microinjection provides a similar estimate of the toxicity of maternally transferred Se in embryos and larvae at concentrations in the range of 9.73 ± 0.41 µg Se/g embryo dm to 13.49 ± 0.73 µg Se/g embryo dm. Thus, our study provides an additional line of evidence which suggests that microinjection of SeMet is a useful technique for studying maternal transfer of Se, but the increased bioavailability and potency of injected SeMet must be considered at concentrations nearing 20 µg Se/g embryo. It is hypothesized that differences between exposure routes are due to microinjection of SeMet not being protein-bound within the yolk sac, and that microinjection could represent a 'worst-case scenario' where a developing fish must cope with the most bioavailable and toxic form of Se (e.g. free form SeMet) during its most sensitive period of development in the early life stages. Considering the toxicokinetic mechanisms that dictate Se maternal transfer, microinjection is a viable route of exposure as it can efficiently deliver SeMet into the yolk sac region and is a useful dosing method to investigate the toxicity of Se in fish embryos as a surrogate for maternal transfer. This technique could enable the study of Se toxicities in non-model fish species that might otherwise go unstudied because of logistics or ethical reasons, such as long-lived, rare or endangered species, and has the potential to be a useful tool for cross-species assessments of such toxicities.

CHAPTER 3

3.0 GENERAL DISCUSSION

3.1 Thesis rationale and overview

There is growing concern surrounding the essential trace element selenium (Se) because of its potential to contaminate aquatic ecosystems (Janz et al., 2014; Lemly, 2004; Young et al., 2010). Selenium is introduced to the environment through anthropogenic and natural sources where it can bioconcentrate at the base of aquatic food webs and bioaccumulate through dietary means within aquatic organisms at higher trophic levels (Presser and Luoma, 2009). All animal classes are susceptible to the toxic effects of increased Se accumulation because of the narrow margin between essentiality and toxicity (Haratake et al., 2015; Mangiapane et al., 2014). Furthermore, egg-laying vertebrates are at notable risk because of the maternal transfer of Se during vitellogenesis (Janz et al., 2010). This process leads to increased levels of Se exposure in embryo-larval offspring as they absorb their yolk sac during early life stage development (Holm et al., 2005; Janz et al., 20120). Exposure to Se during early life stage development is known to increase the proportion of deformities of early life stage fish and can impact the overall fitness or survival of larval populations (Lemly, 1999; Lemly, 1997). The adverse effects associated with Se contamination have been extensively described in field studies dating back to the 1980s, and as recently as 2018 (Lemly, 1985; Lemly, 2018; Ohlendorf, 2002).

While field-based research provides evidence for Se induced toxicities across multiple case-studies, laboratory studies exposing juvenile or adult fish to dietary Se in the form of selenomethionine (SeMet) have established a variety of toxic effects such as altered oxidative stress response, impacts on the cardiovascular system, changes in behaviour, and influences on physiological swimming performance (McPhee and Janz, 2014; Naderi et al., 2018; Pettem et al., 2018; Zee et al., 2016). Nevertheless, the most detrimental effects of Se toxicity are a result of maternal transfer, which is difficult to study because it is resource, time and logistically intensive. Furthermore, laboratory exposures require fish to be exposed through the diet until they can be spawned in-culture, which is logistically, and to some degree, ethically problematic in species that have long reproductive cycles. Hence, the primary goal of this thesis research was to establish and validate an embryo injection model to simulate the maternal transfer of Se in a

fish species native to North American freshwater ecosystems, the fathead minnow (*Pimephales promelas*). This methodology could support future maternal transfer studies in non-model fish species, but could also be extended into other egg-laying vertebrates that might be at risk of over accumulating and offloading Se into embryonic offspring. To meet this goal, the effects of *in ovo* Se exposure in early life stage fathead minnow were compared through two different exposure routes: i) dietary SeMet exposure leading to maternal transfer; and, ii) embryo injection of SeMet as a proxy for maternal transfer.

My thesis research determined that embryo microinjection of SeMet can induce developmental effects in early life stage fathead minnow that are comparable to effects from exposure through maternal transfer at concentrations in the range of 9.73 ± 0.41 µg Se/g embryo dry mass (dm) to 13.49 ± 0.73 µg Se/g embryo dm. In both routes of exposure an increase in the proportion of deformed fathead minnow was observed in a dose-dependent fashion. However, it is important to note that fish exposed via microinjection responded to SeMet exposure with more frequent deformities and greater mortalities at concentrations of 18.9 ± 0.86 µg Se/g embryo dm in comparison to the highest embryo concentrations via maternal transfer (28.39 ± 1.60 µg Se/g embryo dm), likely as a function of bioavailability differences within the yolk sac leading to comparable but not analogous toxicities. Dietary SeMet exposure did not negatively impact fecundity of breeding adult fathead minnow, and maternal transfer began efficiently upon exposure with embryo Se concentrations reaching steady-state after approximately 14-days of exposure. The research presented here addresses knowledge gaps regarding maternal transfer in fathead minnow and provides an additional line of evidence for using microinjection as a proxy for maternal transfer in fish species native to North American freshwater systems.

3.2 Studying the maternal transfer of selenium

Maternal transfer studies are challenging to perform in many fish species because of varying reproductive strategies in fish, logistical reasons and ethical concerns. For instance, performing a maternal transfer study in rainbow trout (*Oncorhynchus mykiss*), which is a species used in multiple regulatory tests and an accepted model organism, is extremely time and resource intensive (Pilgrim, 2012). Rainbow trout spawn after approximately 2 - 3 years of development and it is simply not feasible to expose and maintain fish for this long of a time-period in a laboratory setting, and therefore one must utilize the support of a fishery or hatchery with the appropriate aquaculture conditions to conduct such studies (Pilgrim, 2012). Even with these

difficulties, researchers have been able to generate information about the maternal transfer of Se in rainbow trout, and in other species where laboratory studies are difficult such as brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), northern pike (*Esox lucius*) and white sucker (*Catostomus commersonii*) by performing artificial fertilization using gametes of male and female fish from field sites known to have elevated concentrations of Se in the water and food-chain (Covington et al., 2018; Holm et al., 2005; Muscatello et al., 2006). This is valuable information as it provides direct site- and species-specific insight into the potential health status of adult fish, and of larval populations that would be the offspring for that year. However, Se is likely not the only stressor in the environment which these fish inhabit and therefore other compounds (e.g. PAHs and/or metals), which are often part of the complex mixtures that include Se and contaminate aquatic habitats, and this could contribute to the overall observed effects in embryo-larval offspring sampled within these locations.

Performing maternal transfer studies in the laboratory offers researchers the opportunity to look directly at the effects of Se maternal transfer in the offspring by exposing parental fish to strictly Se through the diet. Researchers have questioned the environmental relevance of spiking a diet with SeMet because in a natural environment fish would consume a diet composed of multiple Se species including other forms of organic (e.g. selenocysteine) and trace amounts of inorganic selenium (e.g. selenite and/or selenate) (Rigby et al., 2014). While this is true, the biochemistry of Se maternal transfer suggests that a diet spiked with only SeMet could be an effective method for inducing the overall maternal transfer of Se in egg-laying vertebrates. Furthermore, multiple studies have used a SeMet-spiked diet to expose fish and have generated data which suggests that this type of spiked-diet exposure prompts a dose-dependent response across multiple levels of biological organization (McPhee and Janz, 2014; Thomas and Janz, 2014; Pettem et al., 2017; Pettem et al., 2018). With this said, future research focusing on dietary Se exposure could utilize a diet that has naturally accumulated Se from lower trophic levels, to simulate a simple food chain, and examine if the effects of maternal transfer differ when parental fish are exposed to a naturally elevated Se diet rather than an SeMet-spiked diet.

The fathead minnow proved to be a useful fish species for studying the maternal transfer of Se. The asynchronous breeding pattern of sexually mature fathead minnow allowed for the quantification of fecundity rates and total embryo Se concentrations throughout the entirety of the study. This would not be possible in a synchronous spawning fish, as all ovarian follicles

develop at the same rate, and they do not produce multiple small clutches. A previous study investigating the maternal transfer of methylmercury in fathead minnow avoided sampling embryos during the first 5-days of exposure to ensure that any embryos collected and observed were exposed throughout all stages of oogenesis (Bridges et al., 2015). I did not perform observations on hatchability, survival or morphological abnormalities of fathead minnows collected during the first 5 days of exposure, but rather collected embryos during this time to quantify embryo Se concentrations. I observed that the maternal transfer of excess Se occurs rapidly when reproductively active female fathead minnow are exposed to elevated concentrations of dietary Se. There was a noticeable increase in Se deposition that was measured in embryos from SeMet treatment groups the day after the exposure was initiated, and this continued for approximately 14 days of the exposure until embryo Se concentrations appeared to plateau in a 1:1 ratio within each respective dietary treatment. To my knowledge, this is the first study to examine this process in embryos and provides valuable insight regarding the proficiency of Se maternal transfer in asynchronous spawning fish. From this time point on, there were no significant changes in embryo Se concentration within treatment groups, however embryo Se concentrations were significantly different in dose-dependent fashion among treatment groups. Therefore, it was during this period that embryos were collected for assessing the effects of Se exposure on early life stage development.

When sexually mature and reproductively active, female fathead minnow have ovarian follicles at all developmental stages (Leino et al., 2005). It is only during the vitellogenesis stage of ovarian follicle development that the maternal transfer of Se occurs (Kroll and Doroshov, 1997; Lubzens et al., 2017). Yet, fish species have a variety of reproductive strategies where ultimately the duration of vitellogenesis is variable and could influence the potential for Se incorporation (Janz et al., 2010). For instance, in synchronous spawning species, the dietary intake of Se immediately prior to spawning might have less impact on embryo Se concentrations as long as vitellogenesis has completed. Bull trout (*Salvelinus confluentus)* are a species that inhabit large, clean rivers but migrate into small tributaries, some of which are known to contain elevated levels of aqueous and dietary Se, to spawn. This might not directly affect the reproducing adult bull trout, nor the F_1 generation as a result of maternal transfer, but potential effects might manifest in the swim-up larvae which would consume dietary prey items from within the Se contaminated tributary. This could induce the bioaccumulation of Se in bull trout

during after swim-up and could potentially disrupt normal development into the juvenile life stage. Dietary intake leading up to vitellogenesis in adult fish could impact embryo Se concentrations, as would Se accumulation in the liver and muscle could be mobilized and subsequently transported into yolk sac proteins as a depuration strategy, or simply as an indiscriminate biochemical process. A specific focus on species-specific duration of vitellogenesis, and the amount of mobilized tissue Se during vitellogenesis, could provide further insights regarding species differences in rates of embryo Se deposition for fish.

3.3 Maternal transfer vs. embryo microinjection exposure routes

3.3.1 Embryo selenium concentrations

Measured Se concentrations from both experiments in Chapter 2 were significantly greater in treatment groups compared to control groups, and in both exposure routes, a dose-dependent response was evident in the form of morphological abnormalities. In the maternal transfer study, fathead minnow fed a diet spiked with SeMet displayed embryo Se concentrations that reached an approximate 1:1 ratio with the dietary treatment the fish received. This agrees with previous Se research that suggests the incorporation of Se into tissues is dose-dependent and is more prevalent in tissues undergoing higher rates of protein synthesis (Covington et al., 2018; Holm et al., 2005; Janz et al., 2010; Kupchaski and Rasmussen, 2015).

Embryo microinjection of SeMet into the yolk region of fathead minnow proved to be an effective exposure route. In preliminary injection work, it was observed that embryos dosed at 24.7 ± 1.73 µg Se/g embryo dm were prone to morality rates of 65.8% (Fig. B.1); therefore, it was decided that the highest nominal treatment group would be 20 µg Se/g embryo dm to avoid mass mortality within this treatment group. The medium treatment group in the microinjection study was targeted at 15 µg Se/g embryo dm. This injection concentration was selected for a direct comparison to the US EPA 2016 Freshwater Selenium Criterion for the Protection of Aquatic Life value for egg/ovary fish tissue of 15.1 mg Se/ kg tissue dm. The low (9.73 µg Se/g embryo dm) and medium (13.49 µg Se/g embryo dm) treatment groups in the microinjection study had an embryo Se concentration that bracketed the medium (10.9 µg Se/g embryo dm) treatment group in the maternal transfer study, and which are comparable concentrations slightly below the US EPA's 2016 Selenium Criterion egg/ovary tissue guideline. The Se concentrations used for dosing embryos via microinjection study were selected to cover a range of environmental and regulatory relevant concentrations while also being comparable to the embryo

Se concentrations measured in the maternal transfer study. One limitation of the microinjection study was that embryo concentrations greater than 20 µg Se/g embryo dm caused mass mortality before swim-up and did not allow for a direct comparison of deformities in swim-up larvae at concentrations that matched the maternal transfer study. Overall, the selection of embryo Se concentrations for microinjection exposure were below the threshold for embryo mortality according to preliminary microinjection work.

3.3.2 Embryo-larval effects

In Chapter 2, I observed that the maternal transfer of elevated concentrations of Se did not cause any reductions in hatchability or survival of fathead minnow larvae from fertilization until swim-up. However, in fish sampled at swim-up and analyzed for deformities, there was a clear increase in the frequency of deformed fish that followed a dose-dependent trend. This observation concurs with previous studies investigating the effects of Se maternal transfer after exposure in embryo-larval offspring across a range of fish species, and is indicative of Se exposure (Covington et al., 2018; DeForest et al., 2011; Holm et al., 2005; Kennedy et al., 2000; Muscatello et al., 2006; Ogle and Knight, 1989; Thomas and Janz, 2014).

In the comparative study using microinjection as the dosage route, there was no effect of the injection process or Se exposure on hatchability at concentrations of 18.9 µg Se/g embryo dm or less. In congruence to the maternal transfer study, there was a clear dose-dependent response in the frequency of deformed fish at swim-up. Using regression analysis, it was also observed that deformed fish exposed via microinjection occurred more frequently, but at slightly lower doses of Se compared to the maternal transfer study as per the significant difference in the slopes of the exposure route regression lines. This difference in response could occur because of a key difference in potential bioavailability that exists between the injection and maternal transfer exposure routes, and this might have led to slightly different toxicities observed in embryo-larval fathead minnow, and it occurred more prominently in embryo Se concentrations of 18.9 µg Se/g embryo dm or greater. In a normal, healthy fish embryo there are multiple species of protein-bound and free form Se present, and although free form amino acids are still available in trace amounts (e.g. <1%) it is the protein-bound forms that compose greater than 58% of proteins (Rigby et al., 2014). When using microinjection, the introduction of free form SeMet would increase the amount of free form amino acid within the yolk sac in comparison to the natural

composition, and this free form SeMet could be more readily metabolized, thereby increasing potential for oxidative stress within the organism.

I observed that microinjection of SeMet resulted in a greater proportion of deformed fish at slightly lower Se concentrations than in fish from the maternal transfer study. However, the type of deformities (e.g. finfold, spinal, craniofacial or edema abnormality) that were present in fathead minnow followed a similar pattern regardless of exposure route. Finfold and spinal deformities were approximately 2-fold more common than the prevalence of craniofacial and edema malformations, and in all cases, there appeared to be a dose-dependent trend regarding the frequency of deformities within each respective category that was evaluated. When a deformity was present I also assessed how severe the deformity was to investigate if the severity of deformities followed a dose-dependent trend. However, this was not evident and the severity of deformities within each individual category was quite variable across treatment groups, regardless of exposure route, and there was no clear trend in the severity response.

3.4 Future considerations for selenium maternal transfer research

The discoveries from this thesis contribute an additional line of evidence into the use of embryo microinjections as a proxy for studying the maternal transfer of Se in fish native to North American freshwater habitats. Microinjection is emerging as a useful method for inducing a response in embryo-larval organisms to Se exposure, but the difference in bioavailability between free form amino acids and protein-bound amino acids will remain a key dynamic that differentiates SeMet exposure via embryo microinjection and maternal transfer. When using microinjection of SeMet, one must consider that this will be a dosage of free form amino acids that are likely more bioavailable, thereby increasing the potential of this compound to be metabolized. Furthermore, as outlined in Chapter 2, a key time-period in embryo-larval development with regards to Se induced oxidative stress is after the onset of liver development but before activity of the enzyme superoxide dismutase (SOD) begins (Palace et al., 2004). This makes sense as the metabolism of SeMet can generate superoxide radicals and organisms will cope with this form of oxidative stress through SOD enzymes which scavenge the superoxide radicals and act as antioxidants (Palace et al., 2004). It could prove useful for researchers to investigate how early in embryonic development SOD enzymes are active across multiple species, how this activity changes in pre-hatch and post-hatch development, and whether Se exposure alters the overall oxidative response. Fish species have different developmental rates

from fertilization until swim-up, and studying how these differences influence the response to Se exposure during pre- and post-hatch development is a current knowledge gap that could potentially be fulfilled using microinjection.

Future studies could also use microinjection to investigate specific aspects (e.g. oxidative stress response or morphogenesis) of the organismal response across multiple levels of biological organization in embryo-larval fish species that are of concern in Se contaminated environments. Using molecular techniques such as transcriptomics and targeted gene expression, researchers could characterize toxicity pathways in early life stage teleosts that are phylogenetically distant to determine if these pathways are conserved or have evolved differently amongst fishes. These are species such as northern pike, rainbow trout, and white sturgeon (*Acipenser transmontanus*) which have greater ecological and cultural relevance in comparison to standardized model organisms such as zebrafish (*Danio rerio*) or fathead minnow, and reside in upper trophic levels which makes them more prone to Se accumulation. In the case of white sturgeon, this is a long-lived and endangered species, and microinjection is a potential exposure method that can be used to generate data concerning the response of this species to SeMet exposure during early life stage development, which otherwise would be implausible to study in a maternal transfer context. Future use of microinjection could prove to be a useful tool for cross-species comparisons of early life stage Se toxicity and an effective method for the generation of mechanistic data in potentially any egg-laying vertebrate species of concern.

A current limitation of Se based research, including maternal transfer research, is the lack of readily available and valid analytical techniques to accurately characterize and quantify Se speciation in tissue. The complex speciation of Se is known to compose more than 50 species, however, identifying which species are present within fish tissue and to what degree is difficult. As analytical methods for determining Se speciation within tissues improve, researchers will have more opportunity to gain understanding of where certain Se species compartmentalize within fish tissues and how this might influence toxicity. While potentially resource intensive, understanding the compartmentalization, mobilization and transport of Se within an embryo-larval fish from fertilization until swim-up could provide information regarding the onset of certain developmental abnormalities. Research has shown that Se will accumulate in the eye lens of zebrafish, and which has been demonstrated to produce cataracts in juvenile rainbow trout after dietary Se exposure (Choudhury et al., 2015; Pettem et al., 2018). I did not observe any

cataract like effects of dietary SeMet exposure in adult fathead minnow, nor did I observe any cataract like effects in swim-up fathead minnow during the deformity analysis. However, there could be potential defects in eye development during the early life stages which might be more evident in behavioural assessments once the fish have developed into juvenile or adults, such as during shoaling or predator response events.

3.5 Conclusion

My thesis research was part of a larger overall project called the Ecological Risk Assessment of Selenium (ERASe), which focused on characterizing the potential effects of Se throughout various trophic levels of aquatic food webs using field and lab-based studies. For example, the ERASe project included research on the bioconcentration of two important inorganic Se species, selenite and selenate, in diverse periphyton communities; laboratory based simple aquatic food web exposures investigating the trophic transfer of Se from primary producers(algae)-primary consumers(invertebrate)-secondary consumers(fish); and mesocosm based studies performed at the IISD Experimental Lakes Area which investigated the effects of increased aqueous inorganic Se concentrations and aquatic food web trophic transfer of Se under realistic ecological conditions. It has been accepted that the most sensitive aspect of Se toxicity are the effects of Se maternal transfer in embryo-larval offspring. Therefore, my thesis research, as part of the ERASe project, focused on developing and validating an embryo injection method as a proxy for Se maternal transfer to support future studies in non-model fish species.

My thesis research was two-fold and studied the effects of SeMet exposure on reproduction and maternal transfer in fathead minnow. First, I investigated the potential effects of increased concentrations of dietary Se exposure on reproduction, maternal transfer and the F_1 generation. Secondly, I explored the use of embryo microinjections as a proxy for the maternal transfer of Se by comparing the response of embryo-larval fathead minnow in both exposure routes. The results suggest that exposure to elevated concentrations of dietary Se did not have a negative effect on fecundity and that a supra-nutritional dosage might be beneficial to reproduction. Dietary exposure to elevated Se induced rapid maternal transfer of excess Se to embryos, and which reached an approximate steady-state after 14 days of exposure. Embryo-larval fathead minnow exposed to elevated concentrations of Se through maternal transfer did not show reductions in hatchability or survival, but there was a clear response in the form of larval morphological abnormalities at swim-up that followed a dose-dependent trend. In the

comparative study, embryo-larval fathead minnow exposed to SeMet via microinjection did not show reductions in hatchability. However, fish exposed through this exposure route demonstrated slightly lower survival rates and I observed a similar trend in the proportion of deformed fish to the maternal transfer study at concentrations in the range of 9.73 – 13.5 µg Se/g embryo dm. Notably, fathead minnow larvae displayed a greater proportion of deformities at embryo Se concentrations of 18.9 µg Se/g embryo dm when exposed via microinjection versus embryo Se concentrations of 28.4 when exposed via maternal transfer, likely as a function of Se bioavailability resulting in slightly greater toxicities.

Using this information, it could be useful to establish a correction factor for the difference in toxicity between maternal transfer and microinjection in fathead minnow. Then, as future research using microinjection as a proxy for maternal transfer is completed in species for which the early life stage sensitivity to Se has been established in field- or lab-based maternal transfer studies (e.g. rainbow trout or white sturgeon), correction factors could be determined in these species which differ in phylogeny and life history. This could provide further insight regarding sensitivity differences between the microinjection and maternal transfer exposure routes across diverse fish species. Ultimately, the goal of this research was to investigate embryo microinjection as a method which could provide risk assessors and regulators with a more accurate technique than simply applying a safety factor which can be used to set specific and protective guidelines for species of concern. Embryo microinjection is a promising tool in this context and its use in non-model species will be vital to fully determine its application. To conclude, my thesis research provides an additional line of evidence into the use of embryo microinjections as a surrogate for maternal transfer when studying Se related toxicities in early life stage fish native to North American freshwater systems.

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APPENDICES

Appendix A: Water quality data.

Table A.1. Water quality parameters measured throughout the maternal transfer study. Data represent the mean ± SD of water samples taken throughout the study.

Treatment	Temperature (ºC)	pH	Dissolved Oxygen (%)	Ammonia (mg/L)	Hardness (mg/L)	Alkalinity (mg/L)	Conductivity (µS/cm)
Control (1.18 µg Se/g food dm)	25.0 ± 0.3	8.14 ± 0.1	86.9 ± 12.5	0.4 ± 0.4	162.3 ± 18.9	141.9 ± 10.2	516.2 ± 10.8
Low (3.88 µg Se/g food dm)	25.0 ± 0.3	8.07 ± 0.1	89.9 ± 9.6	0.4 ± 0.2	147.6 ± 10.0	142.1 ± 8.7	519.9 ± 6.3
Medium (8.75 µg Se/g food dm)	24.9 ± 0.3	8.00 ± 0.1	79.9 ± 13.4	0.4 ± 0.2	145.9 ± 9.0	142.0 ± 5.7	513.4 ± 25.1
High (29.58 µg Se/g food dm)	25.0 ± 0.4	8.05 ± 0.1	90.0 ± 9.4	0.4 ±0.2	151.2 ± 19.8	143.5 ± 9.8	515.2 ± 11.3

71

Appendix B: Preliminary SeMet microinjection dose-range-finding trial.

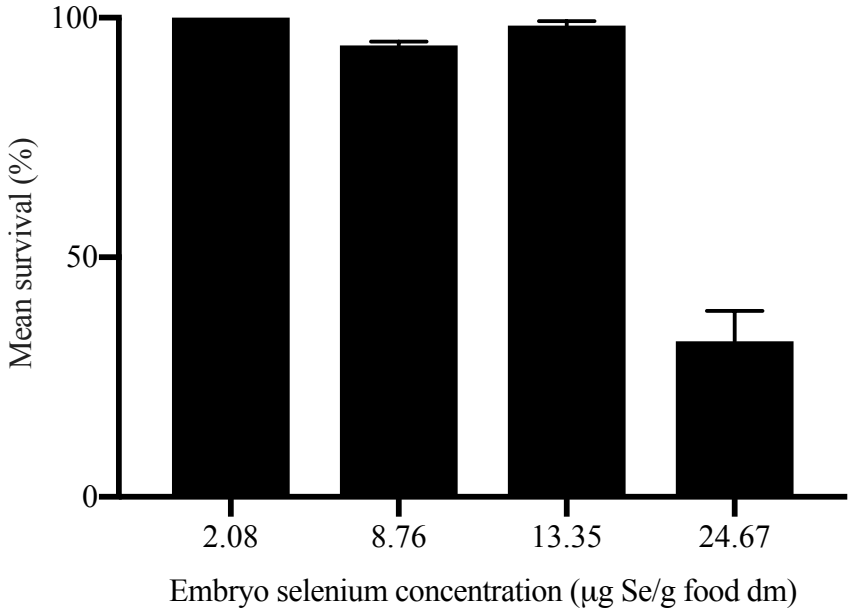

Figure B.1. Mean survival of *P. promelas* until 4 dpf after embryo microinjection of SeMet during preliminary dose-range-finding study. Data represent the mean ± SEM of replicate (*n*=4) samples of 30 injected embryos.

Appendix C: Fecundity of female *P. promelas* during 28 days of dietary exposure to SeMet.

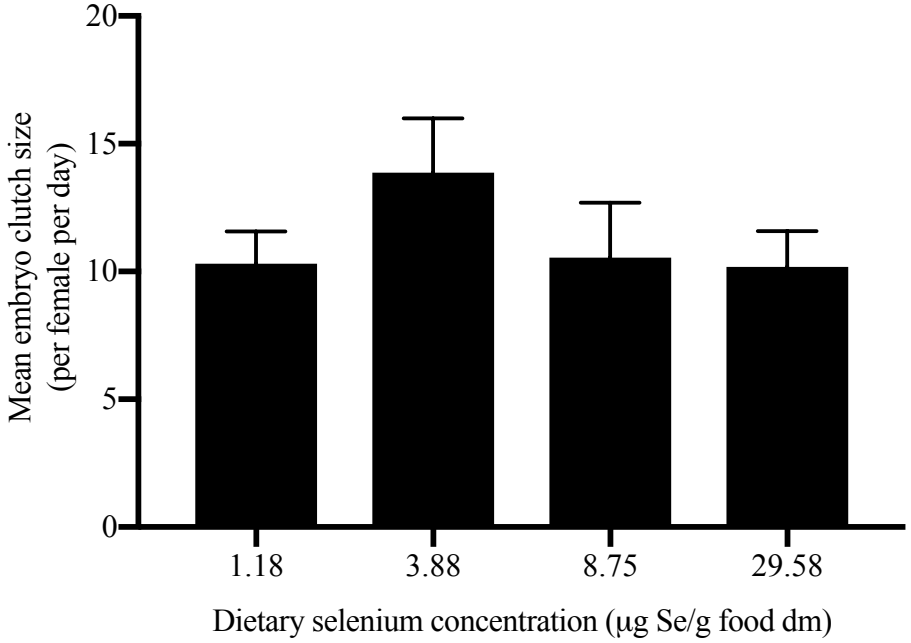

Figure C.1. Mean clutch size of embryos produced daily per female *P. promelas* during 28 days of dietary exposure to SeMet. Data represent the mean ± SEM.

Appendix C: Fecundity of female *P. promelas* during 28 days of dietary exposure to SeMet.

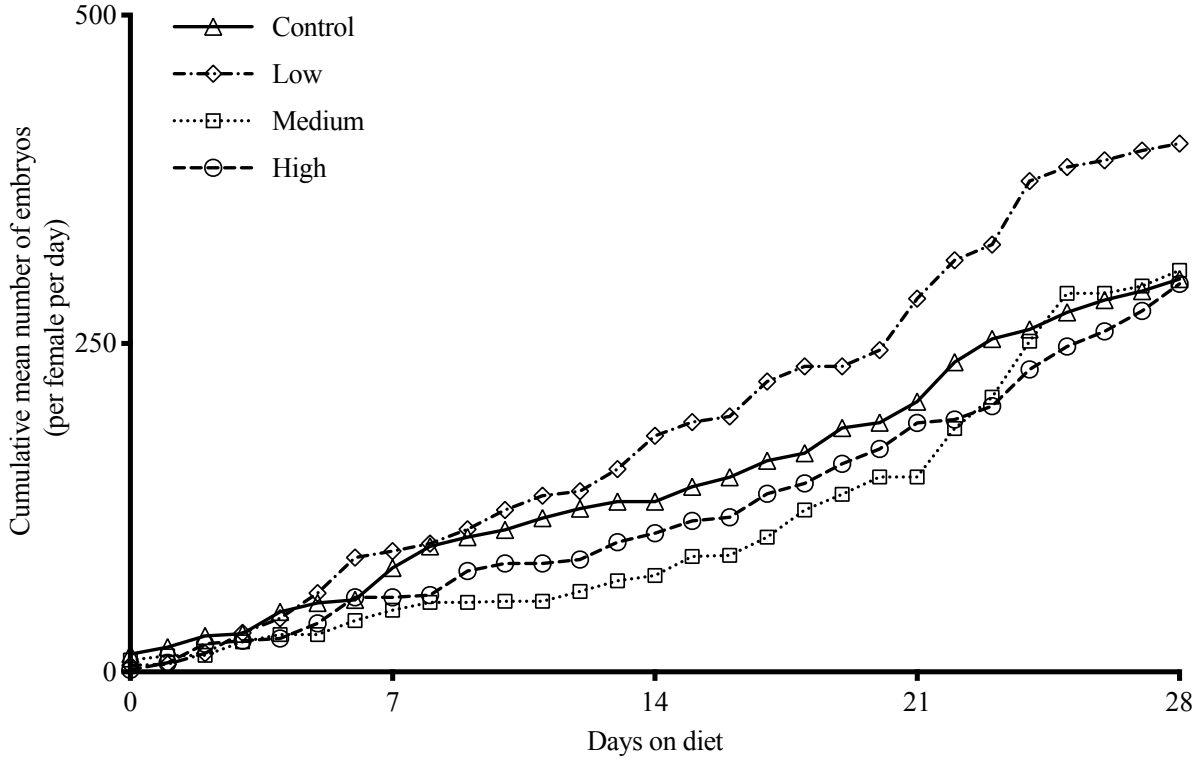

Figure C.2. Cumulative number of mean embryos produced by *P. promelas* breeding groups during 28 days of dietary exposure to SeMet. The four treatment groups were Control (1.18 µg Se/g food dm), Low (3.88 µg Se/g food dm), Medium (8.75 µg Se/g food dm), and High (29.6 µg Se/g food dm). Each plotted point represents the mean number of embryos produced per female, per day, within a respective treatment.

Appendix D: Embryo-larval developmental endpoints.

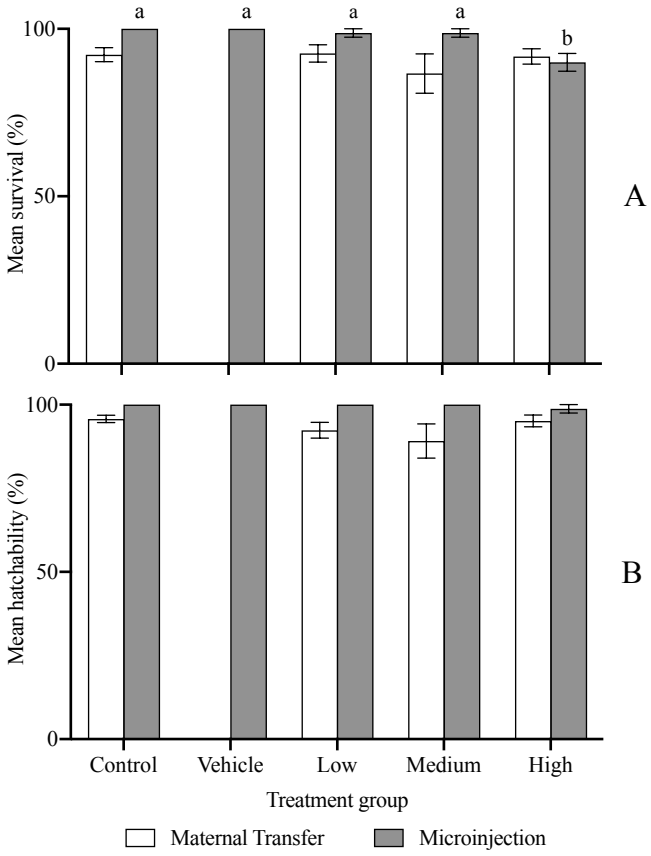

Figure D.1. (A) Percentage of *P. promelas* alive at swim-up after *in ovo* exposure to SeMet. (B) Percentage of *P. promelas* that successfully hatched after *in ovo* exposure to SeMet. Different letters represent a significant difference (α=0.05) in the proportion of surviving fish among treatment groups. In panels where no letters are present there were no significant differences among treatment groups. Data are presented as mean ± SEM.

Appendix D: Embryo-larval development endpoints.

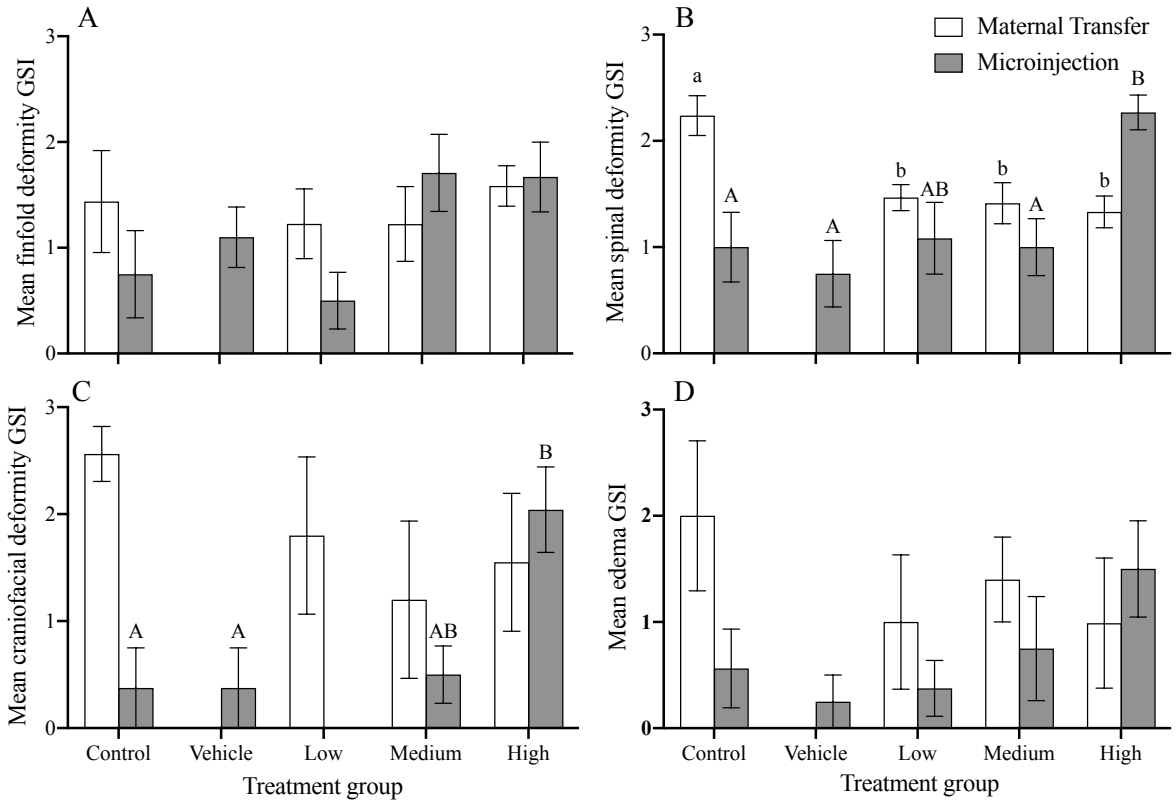

Figure D.2. Graduated severity index (GSI) values determined for each category of deformity in larval *P. promelas* after *in ovo* exposure to SeMet. (A) finfold deformities; (B) spinal deformities; (C) craniofacial deformities; (D) edema. Different letters represent a significant difference (α=0.05) in mean GSI scores among treatment groups, within of each exposure route. Uppercase letters represent significant differences within the microinjection exposure treatment groups. Lowercase letters represent significant differences within the maternal transfer exposure treatment groups. In panels were no letters are present there were no significant differences among treatment groups. Data are presented as mean ± SEM.

Appendix E: Criteria for deformity analysis

The evaluation of deformed swim-up *P. promelas* was performed in a blinded fashion by covering identification labels on vials containing preserved fish. Therefore, during deformity analysis the treatment the larvae had received was unknown. Each vial containing preserved swim-up *P. promelas* were analyzed one at a time, and each individual larva from within a vial were analyzed one at a time. Larvae were always kept in 70% ethanol solution and were transported using a 5 mL transfer pipette which never physically touched the larvae to ensure that larvae were not physically damaged during the transfer process. Larvae were inspected from lateral, dorsal and ventral views at various magnifications to ensure appropriate observations of each specimen. Each swim-up larva was investigated individually for deformities within each of the four following categories: i) spinal curvatures were assessed for kyphosis, a convex curvature in the thoracic region of the spine; lordosis, a concave curve of the lumbar region of the spine; scoliosis, a lateral or S-shaped curve of the spine; and stunted trunk and/or tail development; ii) edema of the yolk sac and pericardium; iii) craniofacial abnormalities including absence of or malformed jaw, and ocular microphthalmia (reduced eye size); and iv) finfold thickness, curvature and orientation. Deformities were assessed for frequency (presence or absence) and for severity using a graduated severity index (GSI) as per recommendations by Janz et al. (2010) and McDonald et al. (2010). The GSI used scores of 0 – 3 to assess the severity of individual deformities. A score of 0 was given to fish displaying normal development. A score of 1 was given to slight deformities within a respective category that were deemed to unlikely impair fish movement or feeding. A score of 2 was given to moderate deformities that were likely to impair fish movement or feeding. A score of 3 described a severe deformity that was likely to greatly impair fish movement or feeding.