ADVERSE HEALTH EFFECTS OF DIETARY SELENOMETHIONINE ON JUVENILE WHITE STURGEON (*ACIPENSER TRANSMONTANUS*)

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ABSTRACT

Sturgeon are an ancient family of fish which have remained essentially unchanged for 200 million years, rendering them physiologically distinct from the more modern teleosts. Of the 26 known species of sturgeons all are likely endangered. North American populations have been declining steadily since the 1800s due to factors such as overharvesting, habitat alterations and increasing pollution. White sturgeon (*Acipenser transmontanus*), endemic to Western North America, are the largest freshwater fish on the continent. Protecting white sturgeon is of interest because nearly all Canadian populations are endangered and they are culturally and economically important. Factors such as great size, longevity, position in the food chain and benthic life style render white sturgeon particularly susceptible to bioaccumulation of toxicants. They are known to be among the most sensitive species to pollutants such as metal ions, dioxin-like compounds and endocrine disrupters. However, little is known about their susceptibility to other priority contaminants such as selenium (Se). Selenium, in its organic form selenomethionine (SeMet) has become a contaminant of particular concern as it is a known toxicant that efficiently bioaccumulates and biomagnifies in the food chain. It is also of interest as Se is an essential micronutrient that becomes toxic at only marginally greater than optimal doses. Current elevated concentrations of SeMet in white sturgeon prey, with predicted increases in anthropogenic releases, have made it a contaminant of concern for this species. It is hypothesized that increased releases of Se to aquatic environments have contributed in part to sturgeon declines; however, to date little is known about its specific effects on this species. Therefore, the purpose of the present study was to investigate the sensitivity of three year old white sturgeon to dietary SeMet and to link physiological effects to key molecular events of toxicity and to elucidate the mechanism of toxicity. Specifically, this thesis focused on oxidative stress in liver tissue as a hypothesized primary mechanism of toxicity. For 72 days sturgeon were given either a control diet of 1.4 µg Se/g feed or a diet spiked with SeMet (5.6, 22.4 or 104.4 µg Se/g feed dry mass). These doses corresponded to an uptake necessary for proper health, two environmentally relevant exposures, and a worst-case scenario for industrial Se release, respectively. A subsample of fish was taken at day 10 to investigate molecular endpoints. Within 10 days of exposure, pathological effects were observed in fish given the high dose. Occurrence of severe edema causing exophthalmos

developed within 15, 23 and 52 days in high, medium and low dose group fish, respectively. There was a 54% and 22% occurrence of lethal effects in the high and medium dose groups, respectively. Se accumulated in a dose dependent manner and reached equilibrium in high dose fish after approximately 40 days. Growth, liver weight and hepatosomatic index were all significantly lower in the high dose group. Histology of 72 day liver samples showed a significant and dose dependent increase in melanomacrophage aggregates and decrease of energy stores and cell size. Food avoidance was also observed in sturgeon exposed to the high dose. To investigate oxidative stress, 10 day liver samples were tested for changes in gene expression coding for glutathione peroxidase (GPx), superoxide dismutase, catalase, glutathione S-transferase, apoptosis inducing factor and caspase 3, using real-time PCR. Only GPx was significantly induced. Day 72 liver samples were tested for the presence of lipid hydroperoxides but there were no significant differences between dose groups and controls, which shed doubt on oxidative stress being the main driver of toxicity. Taken together the data makes a strong case for the sensitivity of white sturgeon to Se accumulation and indicates a general suppression of health due to toxic levels of exposure. However, in contrast to other fish species exposed to Se, oxidative stress is not likely the main mechanism of toxicity in white sturgeon. Findings from the present study could be used for the risk assessment of sturgeon to anthropogenic Se in aquatic ecosystems.

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LIST OF ABBREVIATIONS

AIF – apoptosis inducing factor

ANOVA – analysis of variance

BC – Bonferroni correction

Cas3 – caspase 3

CAT - catalase

dm – dry mass

Eq – equation

Fig - figure

GPx – glutathione peroxidase

GSH – glutathione (reduced form)

GSSG – glutathione disulfide (oxidized form)

GST – glutathione S-transferase

H & E – hematoxylin and eosin

KW – Kruskal Wallis

LHP – lipid hydroperoxide

LPO – lipid peroxidation

MDA – malondialdehyde

MMA – melanomacrophage aggregates

MOA – mechanism of action

PAS – periodic acid Schiff

qPCR – real time polymerase chain reaction

ROS – reactive oxygen species

SD – standard deviation

Se – selenium

SEM – standard error of the mean

SeMet – selenomethionine

SOD – superoxide dismutase

TBARS – thiobarbituric acid reactive substances

TTF – trophic transfer factor

USEPA – United States Environmental Protection Agency

PREFACE

This thesis is presented in ‘manuscript’ style following the parameters set by the College of Graduate Studies and Research. Chapter 1 of this thesis is a general introduction, Chapter 4 is a general conclusion, and Chapters 2 and 3 are organized as manuscripts for publication in scientific journals. Thus, there is some repetition between the introduction and the materials and methods sections in each chapter. Although the publications are coauthored I undertook the leadership role in the conceptualization, data collection and analysis, and writing of each paper. Chapter 2 has been accepted for publication by Environmental Toxicology and Chemistry and has been released online ahead of print, Dec. 3, 2015 DOI: 10.1002/etc.3320. Chapter 3 has been submitted for publication to Ecotoxicology and Environmental Safety.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Sturgeon Life-History

Sturgeon are an ancient family of ray-finned fish that have changed little over hundreds of millions of years (LeBreton et al., 2004; Billiard & LeCointre, 2001; Moyle & Cech, 2004). Sturgeon evolutionarily branched off from other families of fish approximately 200 million years ago (Doroshov, 1985) and are physiologically quite different from the more common modern day bony fishes (Bolker, 2004). They are a distinctive family with prehistoric body forms, an almost entirely cartilaginous skeleton and rows of large scutes, under scaleless skin, for protection (Moyle & Cech, 2004; LeBreton et al., 2004). Depending on the species, they have the potential to live for over 100 years and can reach enormous sizes. There are 26 species of sturgeon (Acipenseridae) in the world today with 9 species native to North America (Vecsei & Peterson, 2004).

White sturgeon (*Acipenser transmontanus*) are the largest freshwater fish in North America (Wilson & McKinley, 2004) and have been known to grow over 6 meters in length and 800 kg in weight (Doroshov, 1985) so references to leviathan are understandable. They are endemic to western North America (Linville, 2006) and found in the Columbia, Fraser and Sacramento River basins (Moyle, 2002). They can be extremely long lived, reaching up to 100 years of age. They exhibit late sexual maturation, with first spawns occurring between 10-12 years of age for males and 15-32 years of age for females (Doroshov, 1985; Doroshov et al., 1997). White sturgeon are a benthic species, spending most of their time near the river bottom searching for prey. Juvenile sturgeon eat benthic invertebrates, crustaceans and bivalves while adults primarily feed on molluscs, shrimp, amphipods, and other young or dead fish (Gessner & Hochleithner, 2001; Linville, 2006; Silvestre et al., 2010). They are considered a highly valuable species from a cultural, recreational and economic perspective. Some American states manage sport or commercial sturgeon fishing harvests (Linville, 2006; Billiard & LeCointre, 2001), while only limited recreational catch and release is allowed on the Canadian Columbia and Fraser Rivers.

1.2 History of Sturgeon Fishing in North America

Sturgeon species across North America were a staple for many First Nations groups. They were a significant food item comparable to buffalo on the plains (Holzkamm & Waisberg, 2004) and a source of staple goods such as oil, isinglass and tough skin for construction material, thus becoming a cultural icon (Holzkamm & Waisberg, 2004; Butterworth & Leonard, 2004). While catching sturgeon was often necessary for survival it was a very difficult task with the larger species and often involved a great struggle, with fishermen or boats being hauled around until the fish tired and could be dragged ashore (Holzkamm & Waisberg, 2004). There are stories of some bravados from the Yurok tribe of Northern California attempting to ride white sturgeon much like rodeo cowboys on broncos, with minimal landing success (Saffron, 2002). It appears that for thousands of years sturgeon populations remained healthy and were self-sustaining, as first contact Europeans found rivers teaming with these fish. Intensive settler sturgeon fishing began in the late 1800s, and by the 1880s this was nicknamed the "caviar rush". Increasing European demand for caviar by the upper and middle classes had quickly depleted old world continental stocks and companies began looking to the New World to fill the void (Saffron, 2002; Holzkamm & Waisberg, 2004). Due to the sturgeon's life history, river systems in both Europe and North America were decimated within 30 year spans (Holzkamm & Waisberg, 2004).

The North American caviar rush began on the more populated East Coast and moved West as once teeming rivers were fished out. On the Delaware River, USA, fishermen averaged 65 sturgeon per haul in the 1870s, 30 per haul in the 1880s and only 8 per haul in 1899 (Saffron, 2002). In Lake Erie the total catch in 1885 was 5 million lbs and only 200,000 lbs a decade later (Saffron, 2004). The St John River, New Brunswick had a total catch of 602,500 lbs in 1880 and 16,264 lbs in 1886 (Holzkamm & Waisberg, 2004). The Columbia River began to be fished commercially in 1888, and the white sturgeon catch peaked in 1892 at 5.5 million lbs total catch (Holzkamm & Waisberg, 2004) with an average fish weight of 150 lbs (Binkowski & Doroshov, 1985). By 1895 the average fish weight was 50-60 lbs and the total harvest was 73,000 lbs (Holzkamm & Waisberg, 2004). First Nations chiefs near Chilliwack BC, then relegated to reserves, complained to the government in 1894 that they would surely starve from a lack of the

essential white sturgeon if settlers continued their rapacious ways. Typical to the time, they were ignored and in 1902 the Harrison River (a tributary of the Fraser River) was fished out and no longer commercially viable (Saffron, 2004). With European and North American sturgeon on the verge of extinction the Caspian Sea has become the main producer of the world's caviar (Saffron, 2002).

Alongside intensive fishing pressure sturgeon have also faced pollution and intensive habitat alteration (Bolker, 2004; Saffron, 2004). Installation of hundreds of river dams affected river flow regimes and temperatures which are key sturgeon spawning triggers. Increased sediment deposition rates covered spawning gravel beds and may have buried benthic organisms needed for food (Jaric & Gessner, 2012; Saffron, 2002; Wilson & McKinley, 2004; Auer, 2004). Damming also effectively cut off the highly migratory sturgeon from traditional spawning sites and landlocked many fish creating separate populations and preventing ocean access (Auer, 2004). Log drives, pulp and paper mills, mining operations and their discharges, flood control and river channelization changed many North American rivers causing habitat loss (Auer, 2004). Because of all these pressures sturgeon population numbers around the world have drastically declined over the past 100 years (Holzkamm & Waisberg, 2004; Saffron, 2002)

1.3 Sturgeon Populations Today

According to the International Union for Conservation of Nature (IUCN), of the 26 species of sturgeon worldwide, 16 are critically endangered, 7 are near threatened - endangered and only 3 are species of least concern (IUCN, 2004). The IUCN considers white sturgeon generally to be a species of least concern due to large global numbers and reasonably strong populations in the Sacramento-San Joaquin and Columbia-Snake river basins. However, five subpopulations in the Columbia and Fraser River systems are considered vulnerable, endangered or critically endangered (IUCN, 2004; Upper Columbia River and Nechako River - critically endangered, Kootenai River and Upper Fraser River – endangered, Fraser regional - vulnerable). The province of British Columbia has its own red list ranking system, under which white sturgeon are generally considered imperiled (2nd highest rank) and four populations are considered critically imperiled (highest rank) (British Columbia Government, 2004). The federal Committee on the Status of Endangered Wildlife in Canada (COSEWIC) has classified Canadian

white sturgeon as vulnerable since 1990 (UCWSRIa, 2012) and endangered since 2003 (Fisheries and Oceans Canada, 2016). Four of the populations (Upper Columbia, Kootenay, Nechako, Upper Fraser) have been listed as endangered under the federal Species at Risk Act since 2006 (Fisheries and Oceans Canada, 2016).

The transboundary white sturgeon population in the Upper Columbia River, which runs between British Columbia, CAN and Washington, USA, and which was the focus of the present study, is listed as critically endangered by the IUCN (2004), as endangered by COSEWIC (2003) and in Schedule 1 of SARA (2006), and as critically imperiled on BC's red list (since 1993). In 1994 white sturgeon harvests on the Canadian portion of the Columbia River were banned and First Nations voluntarily stopped sustenance harvests. Although white sturgeon are not officially recognized as endangered by the state of Washington, in 2002 the fishery was closed with prompting from State and US Tribal fish managers (UCWSRI a, 2012). In 2005 there were approximately 3,100 fish remaining in the transboundary Upper Columbia population, 1,100 individuals on the Canadian side of the border and 2,000 from south of the border to the Grand Coulee Dam, WA (UCWSRI b, 2012). The Upper Columbia White Sturgeon Recovery Initiative (UCWSRI) has worked to protect this particular population since 2000 by developing both a short and long term recovery plan that involves over 25 stakeholders (i.e. federal and provincial governments, industry, First Nations, fisheries) (UCWSRI c, 2012).

Since the mid-1990s sturgeon have been included in a new focus on historic fish stock rehabilitation (Auer, 2004). Natural resource agencies, and multiple levels of government, have since developed sturgeon protection and rehabilitation plans that include fishing restrictions, habitat restoration, aquaculture, restocking programs, genetic registries, and increased public awareness (Auer, 2004; Van Eenennaam et al., 2004; Fisheries and Oceans Canada, 2016; British Columbia Government, 2004; UCWSRI b, 2012). The shift in conservation practices from protecting populations to protecting large tracks of landscape and whole ecosystems has also benefitted sturgeon which are highly migratory. Despite their increased protection white sturgeon still face some worrying pressures. These include poaching, climate change, continuing habitat alteration, pollution and the competing needs of increasing human populations for water, energy and food resources (Van Eenennaam et al., 2004).

1.4 Selenium as a Contaminant of Concern in the Environment

Se is a metalloid or non-metal element that has a chemical behaviour similar to that of sulfur (Maher et al., 2009). It is primarily found in rocks and soils (Lemly, 2002b), but is not evenly distributed throughout the Earth's crust. Cretaceous marine sedimentary rocks, marine shales, black shales, phosphate rock, coal and crude oils are major sources of Se, while igneous rock and limestone are minor sources (Janz, 2012; Maher et al., 2009). Most fresh- and salt-water environments have around 0.01-0.1 µg Se/L but can have up to 5-50 µg Se/L when in contact with highly seleniferous shale deposits (Janz, 2012). Concentrations in the aquatic environment may increase as a result of natural geological processes and/or anthropogenic disturbances such as irrigation runoff and mining in seleniferous soils. Se is used in many consumer products such as coloured glass, antidandruff shampoo, electronics, xerography, fungicides, pharmaceuticals and multivitamins/supplements and consequently may end up in municipal landfill leachate and municipal effluents (Chapman, 2009; Lemly, 2002b; Janz, 2012; Maher et al., 2009). However, the greatest contributors to Se contamination in surface waters are mining and smelting operations, fossil fuel by-product waste disposal and agricultural irrigation runoff (Lemly, 2002b; Chapman et al., 2009; Janz, 2012; Maher et al., 2009). Some notable case studies of industrial contamination illustrate this point: Belews Lake, NC, USA was a cooling reservoir for a coal fired plant from 1974-1986 and the subsequent contamination (150-200 µg Se/L inputs) wiped out 19 of the 20 resident fish species (Lemly, 1985; Lemly, 2002a). Thousands more fish and water birds were poisoned when agricultural drainage via the San Luis Drain (300 µg Se/L inputs) terminated at the Kesterson National Wildlife Refuge, CA, USA; pond concentrations averaged 122 µg Se/L (Saiki & Lowe, 1987; Lemly, 2002b). Tributaries of the Colorado River, CA which receive irrigation runoff can contain up to 400 µg Se/L (Saiki & Lowe, 1987). Se contamination has been identified as a concern in countries around the globe (Lemly, 2002b; Chapman et al., 2009).

Se can be found in elemental, inorganic (ex: selenate, selenite), organic (ex: selenocysteine, selenomethionine amino acids) or methylated forms. Since it is fairly reactive, biochemical intermediates may also be found as organisms accumulate, metabolize, biotransform and excrete Se (Maher et al., 2009). Se most commonly enters the aquatic ecosystem as inorganic species where it is taken up by primary producers such as microphytes and bacteria.

These microorganisms biotransform inorganic Se to its organic species, selenomethionine (SeMet), an amino acid analog that can be incorporated into proteins (Fan et al., 2002; Janz et al., 2009). These low trophic level organisms can tolerate extremely high Se body burdens, bioconcentrating Se 10^2- to 10^6-fold above water concentrations without apparent harm (Chapman et al., 2009; Maher et al., 2009; Stewart et al., 2009). Therefore, although regulatory limits for Se concentrations in water (5 µg/L in the USA, USEPA, 2014; 1 µg/L in Canada, CCME, 2015) may not be breached, the initial large bioconcentration poses a great risk to higher trophic organisms such as sturgeon (Fan et al., 2002).

SeMet accumulates in the food chain via dietary exposures and is found in organisms at all trophic levels (Bakke et al., 2010; Linville, 2006). The amount of Se bioaccumulation and sensitivity in an organism varies by species and is dependent on factors such as food choice, physiology, and association with sediment (Maher et al., 2009). Multiple studies have shown that fish with access to the sediment accumulate greater amounts of Se than those held above (Hamilton, 2004). Fish may consume contaminated detritus intentionally or accidentally ingest sedimentary particles with adsorbed Se (Hamilton, 2004; Fan et al., 2002). This is an especially pertinent exposure pathway for benthic species, such as sturgeon.

1.5 Selenium Toxicity in Fish

Selenium presents a paradox as it is both an essential micronutrient for vertebrates and a poison depending on the concentration (Mayland, 1994; Chapman, 2009). Previous studies have shown that to maintain normal growth and function a minimum of 0.1-0.5 µg Se/g feed dry mass (dm) is required in the diet of Atlantic salmon (*Salmo salar*) and fingerling channel catfish (*Ictalurus punctatus*) (Gatlin & Wilson 1984, Poston et al., 1976; Hodson & Hilton 1983; Lemly 1997) and 0.7 µg Se/g feed in the diet of grouper (*Epinephelus malabarious*) (Stewart et al., 2009). However at concentrations greater than 3 µg Se/g (dm) dietary exposure, toxic effects such as decreased growth, pathological changes in organs, edema, popeye (exophthalmos), mortalities, reproductive failure and teratogenesis have been observed in various fish species (Lemly, 1997; Lemly, 2002a; Sorensen et al., 1984; Hamilton, 2004; Misra et al, 2012; Finley, 1985). According to Lemly (2002b), dietary Se concentrations should not exceed 3 µg/g if health is to be maintained in the most sensitive species such as rainbow trout (*Oncorhynchus mykiss*),

chinook salmon (*Oncorhynchus tshawytscha*) and bluegill (*Lepomis macrochirus*) (Lemly, 2002b; Hamilton, 2004). Toxic effect thresholds of Se in whole body, muscle, liver and egg/ovary tissue of 4 µg/g, 8 µg/g, 12 µg/g and 10 µg/g (dm) respectively, have been recommended for freshwater fish by Lemly (2002b). DeForest et al. (2012) recommended whole body, ovary, and dietary limits of 6 µg/g, 17 µg/g, and 11 µg/g (dm) respectively, for cold water anadromous fishes. The British Columbia Ministry of the Environment updated the provincial Water Quality Guideline for Se in 2014 to include fish tissue concentrations of 4 µg/g, 4 µg/g and 11 µg/g (dm) for whole body, muscle/muscle plug and egg/ovary respectively. The USEPA (2015) is moving towards tissue thresholds of 8.0 µg/g, 11 µg/g and 15.8 µg/g (dm) in whole body, muscle and egg/ovary respectively.

Wild caught fish with elevated Se concentrations in tissues, most likely from dietary SeMet, have exhibited a host of pathological effects. In Belews Lake, NC, USA, a highly Se contaminated water body, green sunfish (*Lepomis cyanellus*) exhibited pathological alterations in many internal organs including swelling of gill lamellae leading to decreased respiratory capacity, liver and kidney failure, and a change to blood composition, which suggested physiological stress and a change in overall health (Lemly, 1993; Lemly, 2002a). Other fishes in the lake exhibited severe pericarditis and myocarditis, damaged egg follicles in ovaries, hatchling and larval deformities, cataracts of the lens and cornea, and protruding eyeballs due to internal edema (Lemly, 1993; Lemly, 2002a). Chronic Se exposure also leads to various skeletal and internal organ pathologies in adult and juvenile fishes (Lemly, 2002a; Janz et al., 2009).

1.6 Toxicity of Se in White Sturgeon

White sturgeon are believed to be particularly at risk to Se toxicity as they spend most of their lives in contact with the sediment, feed on prey that can accumulate large amounts of Se, are very long-lived and have been found to be among the most sensitive species to other pollutants including metal ions, dioxin like compounds and endocrine disrupters (Vardy et al, 2011; Vardy et al., 2012; Doering et al., 2012; Doering et al., 2014; Dwyer et al, 2005). Studies conducted in San Francisco Bay, CA, USA found that Se concentration in clams (*Potamocorbula amurensis*), a preferred prey of white sturgeon in the area, ranged from 5 – 20 µg/g (dm), averaging 15 µg/g (dm) at some sites (Linville et al., 2002; Linville, 2006; Luoma & Presser,

2006), and a study conducted by the U.S. Department of the Interior and U.S. Geological Survey predicted that Se concentrations in clams could reach >100 µg/g (dm) under certain Se loading and climate conditions (Luoma & Presser, 2006). Furthermore, elevated levels of Se have been found in white sturgeon tissues. In the San Francisco Bay Delta concentrations of Se of 20.8 ± 4.11 µg/g, 10.2 ± 1.93 µg/g and 21.8 ± 2.07 µg/g (dm) (mean ± SEM) have been reported in ovary, muscle and liver tissues of vitellogenic females, respectively (Linares-Casenave et al., 2014). Ovarian tissues of white sturgeon from the Kootenay River, BC, CAN contained Se concentrations of up to 12 µg/g (average = 1.76 ± 2.02 µg/g) (Kruse, 2000). These concentrations exceeded toxic effect thresholds for Se set by the British Columbia Ministry of the Environment (2014) and those proposed for fish by Lemly (2002b), and they approached levels suggested by DeForest et al. (2012).

Previous dietary SeMet studies conducted with juvenile white sturgeon have shown that SeMet accumulates in kidneys, liver, gill, muscle and blood plasma (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). Activity and growth were adversely affected when fish were exposed to dietary levels ≥ 40 µg Se/g (dm). At dietary doses ≥ 20.5 µg Se/g (dm) histopathologies were observed in kidney, liver, muscle and gill tissues although findings have been inconsistent (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). Decreased whole body protein and lipid, as well as decreased glycogen in the liver were also observed at these doses (Tashjian et al., 2006; De Riu et al., 2014). Diminished energy stores could lead to a reduced ability to forage, avoid predators, swim upstream, and navigate fish ladders around dams (Tashjian et al., 2006; De Riu et al., 2014; Cocherell et al, 2010).

1.7 Potential Mechanisms of Toxic Action for Selenomethionine

Multiple mechanisms of action have been proposed for SeMet toxicity. It was initially thought that because cellular enzymes are unable to distinguish between methionine and SeMet when synthesizing proteins, that these substitutions lead to improper protein structure, function and abnormal cellular biochemistry (Lemly, 2002a; Spallholz & Hoffman, 2002), but new evidence suggests this hypothesis is not plausible due to the location of Se in the molecule (Janz et al., 2009). A pathology that is believed to occur due to distorted membrane proteins is edema in the head and body cavity, caused by disrupted cell permeability (Ellis et al., 1937; Lemly,

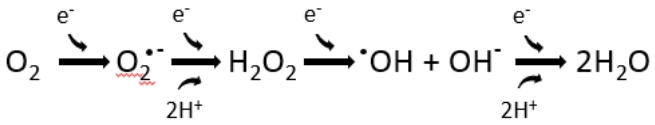
2002a; Lemly, 1993). Another hyposthesized mechanism of action is that excess organic Se compounds are detoxified via methylation metabolism that produces hydrogen selenide as an intermediate metabolite. If the metabolic pathway is interrupted and hydrogen selenide (H_2Se) accumulates, liver damage would be expected as it is a known hepatotoxin (Spallholz & Hoffman, 2002).

A third, and possibly dominant, mechanism of Se toxicity is oxidative stress (Palace et al., 2004; Miller, 2006; Spallholz & Hoffman, 2002; Hoffman, 2002; Janz et al., 2009). Hypothesized pathways of Se causing oxidative stress include: 1) metabolism of SeMet to methylselenol (CH_3Se^-) either via the trans-sulfuration pathway or directly via methionase, which initiates redox cycling and generates reactive oxygen species (ROS) that then damage, bind or otherwise inhibit important enzymes and proteins (Pacini et al., 2013; Spallholz & Hoffman, 2002), 2) Se species react with glutathione (GSH) creating ROS (Miller, 2006), and 3) lipid hydroperoxide radicals may cause tissue damage or upset cell permeability (Miller, 2006). Spallholz & Hoffman (2002) suggested that selenate (SeVI) and SeMet do not generate ROS; however, Palace et al. (2004) showed that rainbow trout embryos exposed to SeMet generated increased levels of superoxide radicals. Miller (2006) stated that teratogenicity in fish is mediated by oxidative stress. Oxidative stress leads to increased cellular damage, and potentially organ damage, as well as increased physiological stress (O'Toole & Raisbeck, 1997; Palace et al., 2004). These mechanisms of action could account for the number of pathologies observed in both embryos and adults.

1.8 Oxidative Stress and Antioxidant Defense

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the body's antioxidant protection and repair capabilities with the consequence of damage to cellular components including DNA, proteins and/or lipid membranes (Martinez-Alvarez et al., 2005; Pacini et al., 2013; Kelly et al., 1998). This can be caused by increased ROS production, decreased antioxidant defense, decreased ability to repair damages, or all three. Examples of ROS include superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\cdot$), singlet oxygen (O_2^-) and nitrogen oxide radical ($NO\cdot$) (Kelly et al., 1998). Redox cycling is a process in which a parent molecule, such as methylselenol (CH_3Se^-), is reduced by a single electron

forming a reactive intermediate or radical. This radical then transfers an electron to molecular oxygen forming a superoxide radical and regenerating the parent molecule. The superoxide radical can then set off a cascade of ROS forming reactions (Eq. 1.1) (Cohen & d'Arcy Doherty, 1987).



…… (Eq. 1.1)

1.8.1 ROS Damage

Cumulative oxidative damage can lead to apoptotic cell death, tissue degeneration, and mutagenicity (Skrha, 2012; Mates, 2000; Miller, 2006; Martinez-Alvarez et al., 2005). ROS, mainly hydroxyl radicals, can damage DNA by removing or damaging the nitrogenous bases or by breaking the sugar-phosphate backbone causing fragmentation, ring opening or hydroxylation. Typically, after the first oxidation, secondary reactions such as DNA-protein molecule cross-linking are what create lasting lesions. Double DNA strand breaks are often mutagenic or lethal for the cell. ROS can also mediate changes in gene expression (Kelly et al., 1998). Oxidation of proteins can cause fragmenting, modify protein function, or increase susceptibility to proteolytic degradation. Different proteins have differing sensitivities to oxidative attack but it appears that most have evolved structures that protect sensitive areas as misfolded proteins are more susceptible to oxidation (Droge, 2002).

Cell membrane damage occurs when ROS react with membrane polyunsaturated fatty acids in a process called lipid peroxidation (LPO) (Kelly et al., 1998). The weaker carbon-hydrogen bonds of an unsaturated fatty acid make it a target for an ROS to abstract a hydrogen atom. The more unsaturated sites on the lipid chain there are, the more susceptible it is to LPO. LPO is a self-propagating process where an ROS such as singlet oxygen, hydroxyl or superoxide radical abstracts a hydrogen from a methylene carbon thus creating a carbon centered lipid radical (L·) which is itself highly reactive. The procedure is propagated when oxygen (O_2) is added to the lipid radical producing a lipid peroxyl radical (LOO·) which can then abstract another hydrogen creating another lipid radical and a lipid hydroperoxide (LOOH). Alternatively

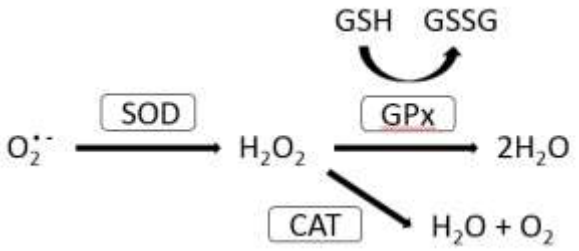
transition metals in the cell can break down lipid hydroperoxides creating lipid radicals. The LPO cycle is terminated when two radicals couple and form a nonradical or when an antioxidant, such as vitamin E, donates a hydrogen to a ROS, thus creating a neutral molecule (Kelly et al., 1998). Breaking these sorts of cascades and redox cycling, and repairing damage already done can be very resource costly for an organism.

1.8.2 Antioxidants

Besides being produced by xenobiotics, ROS are regularly produced as byproducts of various important cellular processes, such as energy production and cellular metabolism. To maintain redox homeostasis vertebrates have evolved a complex system of antioxidant defenses to prevent oxidative damage (Kelly et al., 1998; Livingstone, 2001; Valavanidis et al., 2005). This involves a suite of enzymatic and nonenzymatic antioxidant molecules that scavenge free radicals shortly after production, and oxidative damage that does occur in a healthy individual can be easily repaired with no lasting consequences (Valavanidis et al., 2005).

Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione s-transferase (GST). There are various other nonenzymatic, low molecular weight, scavenging antioxidants including reduced glutathione (GSH), vitamin E (α-tocopherol), vitamin C (ascorbic acid), vitamin K, ubiquinols, carotenoids, and uric acid (Miller, 2006; Martinez-Alvarez et al., 2005; Valavanidis et al., 2005; Kelly et al., 1998; Skrha, 2012; Mates, 2000). Each antioxidant converts a specific ROS to neutral molecules (Eq. 1.2). Superoxide dismutase (SOD) scavenges superoxide radical. There are three types of SOD: manganese SOD in the mitochondria, copper/zinc SOD in the cytosol, and extracellular SOD which is bound to the plasma membrane in the extracellular matrix. In this reaction a superoxide radical is converted to hydrogen peroxide and oxygen. Catalase (CAT), found mainly in the peroxisomes, converts hydrogen peroxide to water and oxygen. Glutathione peroxidase (GPx), a Se containing enzyme, reduces organic peroxides (e.g. lipid peroxide, hydrogen peroxide) in the cytosol and extracellular matrix to water and oxygen. GPx is important during low levels of oxidative stress, however CAT becomes more important for protecting against high levels of oxidative stress (Mates, 2000). Glutathione (GSH – reduced form; GSSG – oxidized form) is a nonenzymatic antioxidant that provides the reducing equivalents needed by GPx

catalyzed reactions (2GSH + ROOH → GSSG + ROH + H_2O). Glutathione reductase is important to GPx function as it reduces GSSG to regenerate GSH in an NADPH dependent reaction (Kelly et al., 1998; Skrha, 2012; Mates, 2000). Gluthathione S-transferase (GST) reduces lipid and hydroperoxides (Hayes & Pulford, 2008) and catalyzes the reaction of carcinogens, drugs and other xenobiotics by reduced glutathione (GSH) (Villanueva & Kross, 2012; Mates, 2000).



…..……. (Eq. 1.2)

1.8.3 Selenium and Oxidative stress

Various studies have detected Se induced oxidative stress by measuring antioxidant response and/or oxidation end products. Misra et al (2012) showed that rainbow trout hepatocytes develop an antioxidant response when cultured in SeMet spiked media over 24 hours. GPx, CAT, SOD, caspase 3 (Cas3) and caspase 7 activity were all significantly induced when cells were exposed to 1000 µM SeMet for 24 hours. Lipid peroxidation (OXI-TEK TBARS kit) was increased in a dose dependent manner at both 4 and 24 hrs. As well, the more SeMet present in the culture media the greater the GSSG:GSH ratio in hepatocytes which indicated that GSH was being oxidized in order to combat ROS. Using chemiluminescence produced in the presence or absence of SOD, Palace et al. (2004) showed that rainbow trout embryos exposed to SeMet produced superoxide radicals.

In a review by Hoffman (2002) it was concluded that dietary Se exposures in the laboratory (mainly SeMet) and in the wild resulted in oxidative stress or triggered antioxidant responses in various aquatic avian species at multiple life stages. Mallard ducklings (*Anas platyrhynchos*), wild avocet ducklings (*Recurvirostra Americana*) and mallard adults had increased malondialdehyde (MDA) levels measured as thiobarbituric acid reactive substances (TBARS), increased GSSG:GSH ratio and increased GPx activity in liver when given diets

spiked with SeMet. Wild willets (*Catoptrophorus semipalmatus*), American coots (*Fulica Americana*) and emperor geese (*Chen canagica*) also showed signs of oxidative stress with increases in GPx activity, GSSG:GSH ratios and concentrations of TBARS in the liver as well as elevated hepatic Se concentrations.

Multiple studies have exposed sturgeon species to organic Se via the diet and observed markers of oxidative stress. Pacini et al. (2013) fed Siberian sturgeon (*Acipenser baerii*) selenocysteine (SeCys) for 60 days and observed signs of oxidative stress in the liver. Increased MDA concentrations in the 20 µg/g dose at 60 days indicated an increase in LPO. There was a significant increase in GPx activity in the 20 µg/g dose by day 30 and in all doses greater than 5 µg/g by day 60. The changes in GPx activity were positively correlated with Se concentration in liver tissue. Glutathione reductase (which reduces GSSG back to GSH) activity was significantly increased in dose groups greater than 5 µg/g at day 60. There was no significant change in GST activity; however, it was positively correlated with Se levels in liver. There was an increase in SOD activity in liver in all treatment groups at day 30 and then a return to base levels by day 60.

De Riu et al. (2014), Linville (2006) and Tashjian et al. (2006) all exposed juvenile white sturgeon to similar dietary SeMet concentrations and although they did not test specifically for oxidative stress it was hypothesized to be a main mechanism of Se toxicity due to the type of pathologies observed. Oxidative damage has been implicated as the cause of histopathological liver changes (De Riu et al., 2014; Linville, 2006) and reduced energy reserves (Tashjian et al., 2006). It was suggested that the need to combat oxidative stress may explain depleted energy reserves and slowed growth rates observed (Tashjian et al., 2006). Cross-linking of actin filaments due to oxidative damage in muscle was proposed as a reason for decreased swimming activity (Tashjian et al., 2006). However, these hypotheses have not been empirically tested in white sturgeon.

1.9 Objectives and Hypotheses

The purpose of the present study was to link adverse health effects caused by dietary exposure of white sturgeon to SeMet with specific mechanisms of toxicity, with a focus on oxidative stress. Although reproductive failure and teratogenic deformities from maternal transfer of SeMet to eggs has become the primary endpoint of concern regarding Se toxicity and

ecological risk assessments (Janz et al., 2009; Lemly, 1993; Lemly, 1997), for the success of restocking programs it is important to understand the effects of SeMet exposure on juvenile fishes. While adverse health effects such as reduced growth, decreased swimming activity, histopathological liver lesions and decreased glycogen had been previously observed in juvenile white sturgeon given diets spiked with SeMet (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014), the causative mechanisms of these symptoms have remained unknown. Specifically, the present study exposed white sturgeon to a range of dietary doses known to be environmentally relevant and to cause adverse effects. General health effects were observed throughout the exposure, and liver tissue was examined for histopathological changes and tested for signs of oxidative stress (concentration of lipid hydroperoxides and changes to expression of apoptotic signalling genes) and antioxidant response (changes to expression of antioxidant genes). Increased knowledge of white sturgeon sensitivity to SeMet and the underlying mechanisms may assist risk assessors attempting to balance ecological health with anthropogenic activities expected to increase environmental Se concentrations. This was accomplished by addressing the following three specific objectives and associated null hypotheses:

Objective 1) Characterize the adverse health effects of subchronic dietary exposure of juvenile white sturgeon to SeMet as determined by survival, gross morphological and histological analyses. Dietary doses ranged over levels necessary for fish health (1 µg Se/g feed dry mass), two environmentally relevant doses (5 and 25 µg/g), and a predicted worst case scenario environmentally relevant dose (125 µg/g). It was predicted that if dietary SeMet was toxic to white sturgeon, then decreased survival, and morphological and histopathological changes would be observed. If the size of the dose mattered, then a dose dependent response was expected.

Null-Hypothesis 1 (Ho1): There are no statistically significant changes in survival, gross morphological and/or histopathological endpoints between fish exposed to increasing concentrations of dietary SeMet when compared to control fish.

Objective 2) Investigate the occurrence of oxidative stress and antioxidant response in the liver of juvenile white sturgeon exposed to dietary concentrations of SeMet representing a level necessary for proper health, two environmentally relevant exposures and a predicted worst case

scenario exposure. It was expected that if dietary SeMet caused oxidative stress in liver of white sturgeon, then an increase in oxidative endpoints (i.e. lipid peroxides and apoptotic signaling), or an increase in antioxidant response (i.e. expression of GPx, CAT, SOD, and GST genes) would be measureable in this tissue. If the size of the dose mattered, then a dose dependent response was expected.

Null-Hypothesis 2 (Ho2): There are no statistically significant changes in oxidative endpoints or antioxidant response in liver tissue of fish exposed to increasing concentrations of dietary SeMet compared to control fish.

Objective 3) Determine association between symptoms of Se toxicosis (i.e. survival, gross morphological and histological changes) and occurrence of oxidative stress in liver tissue. It was expected that if oxidative stress was a main driver of selenium toxicity, then incidence of tissue damage and occurrence of oxidative stress parameters would increase concurrently.

Null-Hypothesis 3 (Ho3): There are no correlations among symptoms of toxicity and oxidative stress parameters in juvenile white sturgeon exposed to increasing concentrations of dietary SeMet.

CHAPTER 2: ADVERSE HEALTH EFFECTS AND HISTOLOGICAL CHANGES IN WHITE STURGEON (*ACIPENSER TRANSMONTANUS*) EXPOSED TO DIETARY SELENOMETHIONINE[1]

[1]This chapter has been accepted for publication in Environmental Toxicology and Chemistry (10.1002/etc.3320) under joint authorship with Sarah Patterson (University of Saskatchewan), Danielle Gagnon (University of Saskatchewan), and Markus Hecker (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. Any edits required by the defense committee since publication have been indicated in footnotes. References cited in this chapter are listed in the references section of this thesis. Supplementary material submitted to the journal has been included in Appendix A.

2.1 Abstract

It has been shown that selenium (Se) released to the aquatic environment can have devastating effects on local wildlife. White sturgeon (*Acipenser transmontanus*) have a life history particularly susceptible to contaminants, and their protection is of interest as they are culturally and economically important, and many populations are classified as endangered. During the present 72 d dietary study, multiple signs of decreased health and Se lethality were observed. Juvenile white sturgeon were given diets containing 1.4mg, 5.6 mg, 22.4mg, or 104.4mg selenomethionine/g food (dry mass). Se accumulated in muscle and liver tissue in a dose dependent manner. Edema causing exophthalmos developed within 15 d and 23 d, and lethal effects occurred in 54% and 22% of fish in the high and medium dose groups, respectively. Growth and hepatosomatic index were significantly lower in the high dose group, which also had a high incidence of food avoidance. Histology of the liver revealed a dose dependent increase in melanomacrophage aggregates and decrease of energy stores, which indicated toxicity. These results indicate that white sturgeon are susceptible to the effects of Se accumulation over relatively short time periods. This stresses the need for continued sturgeon research, and studies looking into the environmental fate and regulation of released Se.

2.2 Introduction

Selenium (Se), a metalloid found naturally in varying concentrations in rocks and soil, is considered a contaminant of concern for higher level aquatic consumers because it can be highly persistent in the environment, bioaccumulative, and toxic (Lemly, 2002b). Concentrations of Se in aquatic environments may increase as a result of natural geological processes and anthropogenic disturbances. It is used in many consumer products and therefore may end up in municipal wastewater and landfill leachate. However, the greatest contributors to Se contamination in surface waters are mining and smelting operations, fossil fuel by-product waste disposal, and irrigation runoff from seleniferous soils (Lemly, 2002b; Chapman et al., 2009; Janz, 2012; Maher et al., 2009).

Se presents a toxicological paradox for aquatic organisms as it is both an essential micronutrient and a poison depending on the concentration (Chapman et al., 2009; Mayland, 1994). Fish require a minimum of 0.1- 0.5 µg Se/g feed in their diets to maintain normal growth and function (Gatlin & Wilson, 1984; Poston et al., 1976; Hodson & Hilton, 1983; Lemly, 1997). However, at dietary concentrations greater than 3 µg Se/g, toxic effects such as decreased growth, pathological changes in organs, edema, popeye (exophthalmos), mortalities, reproductive failure and teratogenesis have been observed (Lemly, 1997; Lemly, 2002a; Sorensen et al., 1984; Hamilton, 2004).

The most soluble and dominant form of Se in the water column is inorganic Se (selenite and selenate). Although relatively unavailable to fish, inorganic Se is readily taken up by primary producers and biotransformed to the organic form selenomethionine (SeMet), an amino acid analog (Maher et al., 2009; Fan et al., 2002; Janz et al., 2009). SeMet is a dominant form of Se found in tissues at all trophic levels and transfers efficiently through the food chain via dietary exposures (Lemly, 2002b; Bakke et al., 2010; Linville, 2006). Low trophic level organisms such as microphytes and bacteria[1] can tolerate high Se body burdens (Fan et al., 2002; Janz et al., 2009) which may then be efficiently transferred to higher trophic level consumers, such as fish and birds, that are less tolerant; although sensitivity to Se exposure is species specific[2] (Chapman et al., 2009; Maher et al., 2009; Fan et al., 2002). Another potentially important Se exposure pathway is via sediment. Multiple cage studies have shown that fish with access to sediments accumulate greater amounts of Se than those held in the water column (Hamilton, 2004). Contaminated detritus may be consumed intentionally, or Se adsorbed to sedimentary particles may be ingested accidentally, leading to elevated Se exposure and accumulation (Hamilton, 2004; Fan et al., 2002).

The amount of Se bioaccumulation and sensitivity of an organism varies greatly by species and is dependent on factors such as food choice, physiology, and association with sediments (Maher et al., 2009). White sturgeon (*Acipenser transmontanus*), a benthic species endemic to Western North America, are believed to be particularly at risk from exposure to Se as they spend most of their lives in contact with the sediment, feed on prey that can accumulate

[1] Published version: "Low trophic level organisms such as phytoplankton, zooplankton, cyanobacteria and protozoa can tolerate high Se body burdens (Chapman et al., 2009)…". This statement has been edited for greater accuracy.
[2] Published version: "although sensitivity to Se exposure is highly species specific". This statement has been edited for greater accuracy.

large amounts of Se, and are very long-lived (Linville, 2006; Moyle, 2002; Doroshov et al., 1997). White sturgeon are of particular interest to researchers and conservationists as they are a physiologically unique species, being evolutionary distinct from teleost fishes, and most populations are critically endangered (Fisheries and Oceans Canada, 2011; US Fish and Wildlife Service, 2015). Reasons for the decline of white sturgeon populations are not fully understood to date but factors such as overfishing, extensive habitat alteration and fragmentation, late sexual maturation, and pollution are considered main contributors to poor recruitment during past decades (Doroshov et al., 1997; Billard & Lecointre, 2001; LeBreton et al., 2004; Hildebrand et al., 1999; Doroshov, 1985). In this context, contamination of surface waters and sediments constitutes a particular concern as white sturgeon are among the most sensitive fish to other environmental pollutants, such as metal ions and dioxin-like compounds (Vardy et al., 2011; Vardy et al., 2012; Doering et al., 2012; Doering et al., 2014). Increased knowledge regarding the sensitivity of this species to priority contaminants such as SeMet is key to conducting meaningful ecological risk assessments, and has become a priority for North American governments and industries operating within white sturgeon habitats.

White sturgeon fry feed on zooplankton, periphyton and detritus, juveniles on benthic invertebrates, crustaceans and bivalves, and adults on mollusks, shrimp, amphipods, and other young or dead fish (Linville, 2006; Billard & Lecointre, 2001; LeBreton et al., 2004; Doroshov, 1985). Studies conducted in San Francisco Bay (CA, USA) found that Se concentration in clams (*Potamocorbula amurensis*), a preferred prey of white sturgeon, range from 5 – 20 µg/g dry mass (dm), averaging 15 µg/g (dm) in some areas (Linville, 2006; Linville et al., 2002; Luoma & Presser, 2006), and a study conducted by the U.S. Department of the Interior and U.S. Geological Survey predicted that Se concentrations in clams could reach >100 µg/g (dm) under certain Se loading and climate conditions (Luoma & Presser, 2006). Furthermore, elevated levels of Se have been found in white sturgeon tissues. In the San Francisco Bay Delta, concentrations of Se of 20.8 ± 4.11 µg/g, 10.2 ± 1.93 µg/g and 21.8 ± 2.07 µg/g (dm) (mean ± standard error of the mean [SEM]) have been reported in ovary, muscle and liver tissues of vitellogenic females respectively (Linares-Casenve et al., 2014). Ovarian tissues of white sturgeon from the Kootenay River (BC, Canada) contained Se concentrations of up to 12 µg/g (average: 1.76 ± 2.02 µg/g) (Kruse, 2000). These concentrations exceed the toxic effect thresholds of Se in whole body, muscle, liver and egg/ovary tissue of approximately 4 µg/g, 8 µg/g, 12 µg/g and 10 µg/g,

respectively, for freshwater fish as recommended by Lemly (2002b). Furthermore, according to Lemly (2002b) dietary Se concentrations should not exceed 3 µg/g if health is to be maintained in the most sensitive species such as rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*) and bluegill (*Lepomis macrochirus*) (Lemly, 2002b; Hamilton, 2004).

Previous dietary SeMet studies conducted with juvenile white sturgeon have shown that SeMet accumulates in kidneys, liver, gill, muscle and blood plasma (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). Activity and growth were adversely affected when fish were exposed to ≥ 40 µg Se/g (dm). At dietary doses ≥ 20.5 µg Se/g (dm) pathologies were observed in kidney, liver, muscle and gill tissues, although findings have been inconsistent among studies (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). Decreased whole body protein and lipid, as well as decreased glycogen in the liver, was also observed at these doses (Tashjian et al., 2006; De Riu et al., 2014). Diminished energy stores may lead to[3] a reduced ability to forage, avoid predators, swim upstream, and navigate fish ladders around dams (Tashjian et al., 2006; Cocherell et al., 2010).

The overall aim of the present study was to assess the toxicological effects of subchronic dietary SeMet exposure to juvenile white sturgeon raised from parental stock originating from the USA-Canada transboundary reach of the Columbia River. This Canadian population is landlocked and therefore geographically and genetically separated from the Sacramento River, (USA) population (Hildebrand et al., 1999), which has featured in all previous studies (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). The present study discusses 1) the characterization of subchronic toxicity of dietary SeMet to Columbia River juvenile white sturgeon; 2) the differences in histolopathological responses between various white sturgeon dietary SeMet exposures; and 3) previously unreported endpoints such as food avoidance, prevalence of edema, and mortality during the exposure. Future work using the same samples will include analysis of oxidative stress in the liver, changes in stress response (blood cortisol, glucose, and lactate concentrations), and alteration of gene expression patterns in the liver using Illumina next generation whole transcriptomic sequencing. Together these studies will provide a

[3] Published version: "Diminished energy stores may indicate a reduced ability to forage…". This statement has been edited for greater accuracy.

clearer understanding of the sensitivity of white sturgeon to dietary SeMet exposure and the underlying mechanism of toxicity.

2.3 Methods

2.3.1 Fish

Fertilized white sturgeon eggs, from wild brood stock, were obtained from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). Eggs were hatched and fish were raised under standardized conditions in the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, SK, Canada) (Vardy et al., 2012; Conte et al., 1988) until they were approximately 3 years old. Ten days after initiation of exposure[4] fish were 124 ± 44.5 g and 26.0 ± 2.8 cm fork length (mean ± standard deviation [SD]). To minimize handling stress, fish were not weighed and measured at the start of the present study. Fish were raised on commercial trout chow (Martin, Profishent Aquaculture Nutrition, 6PT; Elmira, ON, Canada). All procedures involving live animals were approved by the University of Saskatchewan's Animal Research Ethics Board (Animal Use Protocol #20070049).

2.3.2 Experimental Diets

SeMet concentrations in diets were chosen to represent the range of current environmentally relevant exposures experienced by some white sturgeon populations (5 and 25 µg Se/g) and a predicted worst-case exposure scenario (125 µg Se/g). Four diets were prepared: a control containing 1 µg Se / g feed, and three treatments containing 5, 25 and 125 µg Se/g feed (dm). Doses of seleno-L-methionine (SeMet) (Sigma-Aldrich; Oakville, ON, Canada) were dissolved in nanopure water and thoroughly mixed into fine grain commercial trout chow (Proform Aquaculture Feed, Aqua-Balance Trout 52:19 Starter #2 Crumble, Viterra Feed Products; Okatoks, AB, Canada) with a Hobart industrial mixer. The mixture was compacted into larger pellets using a Hobart extruder with a 33 mm hole size, and dried in an oven at 55°C

[4] Published version: “At ten days fish were 124…”. This statement has been edited for greater clarity.

for 18 hrs. No SeMet was added to the control diet since this commercial trout chow came with the trace amounts of Se necessary for fish health.

2.3.3 Exposure Study Design

Fish were exposed to SeMet through the diet for 72 days (control, low, medium dose groups) or 65 days (high dose group) under flow-through conditions in carbon filtered municipal water in accordance with loading densities recommended by ASTM International (2007). Due to high mortality rates the high dose treatment group was taken down a week early; however throughout the present study's report, final take downs of all groups will be referred to as day 72. Five fish were randomly assigned to seven replicate tanks per treatment group (35 fish/treatment). Photoperiod was kept at 16:8 hrs light:dark. Water quality was monitored daily and maintained in all tanks as follows (mean ± SD): temperature, 13.4 ± 0.4^{o}C; pH 7.5 ± 0.2; dissolved oxygen, 82 ± 6.4%. Alkalinity ($CaCO_3$ ppm), hardness ($CaCO_3$ ppm), nitrate, nitrite and ammonia were measured periodically and the results averaged 140 ± 9 ppm, 172 ± 10 ppm, 0.48 ± 0.2 ppm, less than the limit of detection (< LOD) and < LOD respectively.

All fish were transitioned to the control diet 2 weeks prior to initiation of exposure. Fish were fed 1.5% of their body weight daily, which is within an optimal range for sturgeon health (Hung & Lutes, 1987), six days a week. Approximately one hour after feeding, fish waste and uneaten feed were syphoned from tanks. Fish were monitored daily for changes in feeding behaviour, gross morphological changes and mortalities. The predominant gross morphological effect observed was edema, and fish were categorized based on edema severity as follows: normal (0) – normal appearance with eyes flush to the skull; slight (1) – eyes appeared slightly raised from the skull - a minor variation from the classified normal state; moderate (2) – definite protruding of eyes from skull; strong (3) – greater protruding of eyes from skull and often noticeable bloating of the abdomen; and severe (4) – severe protruding of eyes and severe abdominal bloating (Fig. 2.1). Fish in stage 3 or greater were considered to be moribund as no fish recovered from such a state during the present study and stage 3 edema impaired normal functioning such as eating and swimming proficiency. Fish in stage 4 were euthanized when loss of equilibrium occurred.

On day 72 and when classified as moribund, fish were euthanized with a sharp blow to the head, and weight and fork length were determined. Immediately thereafter blood was collected from the caudal vein and/or heart and centrifuged at 5000 rpm for 15 minutes. The resulting plasma was frozen at -80°C for use in a parallel study. The entire liver was removed[5] and weighed for hepatosomatic index (HSI) determination. Portions of liver, kidney, heart, gill, intestine, spleen, brain, eye and muscle were snap-frozen in liquid nitrogen and stored at -80°C. Another portion of each tissue was fixed in 10% buffered formalin for histopathological analysis. Additionally, a random subset of one fish per tank was sampled after 10 days of exposure. Fish were euthanized, processed and analyzed as described for the 72 day sampling. Condition factor (Eq. 2.1), hepatosomatic index (HSI; Eq. 2.2), and trophic transfer factor (Eq. 2.3) were calculated as follows:

Condition factor = (body weight/length^3) x 100 …… (Eq. 2.1)
HSI = (liver weight/body weight) x 100 …… (Eq. 2.2)
Trophic transfer factor = Se concentration in tissue / Se concentration in diet ……. (Eq. 2.3)

2.3.4 Se Analysis

Total Se was analyzed in feed, as well as in white sturgeon tissues collected at day 10, day 72, and from moribund fish throughout the study. Muscle and liver tissues were freeze-dried before extraction. Homogenized samples were cold-digested in Teflon vials (100 mg sample in 5 mL ultra-pure nitric acid and 1.5 mL hydrogen peroxide), concentrated on a hot plate (<75°C), reconstituted in 5 mL of 2% ultrapure nitric acid and stored at 4°C until analysis. Total Se concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS) following US EPA method ILM05.2D (Creed et al., 1994; McPhee & Janz, 2014; Wiseman et al., 2011). All samples were measured in triplicate, along with blanks, and Se recovery was determined using a certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada). The average method detection limit was 0.37 ± 0.29 µg/kg (mean ± SD). The average percent recovery of Se for instrumental and method certified reference material was 100

[5] Published version: "The entire liver was dissected and weighed…". This statement has been edited for clarity.

± 5.29% and 97 ± 8.66%, respectively. Analysis of each of the diets revealed that actual Se concentrations were 1.4 ± 0.06 µg/g, 5.6 ± 0.02 µg/g, 22.4 ± 0.37 µg/g and 104.4 ± 4.81 µg/g (mean ± SD) in the control, low, medium and high diets respectively.

Figure 2.1. Characteristic pictures of each edema category. From left to right: normal (0), slight (1), moderate (2), strong (3), and severe (4).

2.3.5 Histological Analysis

Excised tissues were fixed in 10% buffered neutral formalin (VWR International, West Chester, PA, USA) for 24 hrs and then transferred to and stored in 70% ethanol. Liver was chosen as the organ of interest for histopathological analyses (72 day samples only) as it is one of the major detoxifying organs, is involved in metabolism of xenobiotics (Passantino et al., 2014) and is one of the tissues where greatest Se accumulation occurs (Linville, 2006; Linares-Casenave et al., 2014; Tashjian et al., 2006). After paraffin embedding (Appendix C) histological sections were cut 5 µm thick on a Microm HM model 310 microtome (Germany), fixed to slides (Eukitt, Fluka mounting fluid, Sigma-Aldrich, St. Louis, USA) and stained with either hematoxylin and eosin (H&E), periodic acid Schiff's (PAS) or best carmine (Luna, 1968; Clark, 1981; Appendix C). These stains were chosen to highlight various aspects of the tissue. H&E is a routine stain for analysis of cellular structures. Best carmine stains glycogen granules bright red. PAS stains lipofuscin, which is a product of oxidative polymerization of polyunsaturated fatty acids and is found in melanomacrophages (Passantino et al., 2014). Melanin is a third component of melanomacrophages however due to its dark colouration it is visible without staining.

Slides were coded and a blind review was done for all analyses. Slides were qualitatively analyzed for vacuolar degeneration, apoptosis, necrosis and general cell health (H&E), size and frequency of melanomacrophage aggregates (MMAs) (H&E, PAS), and glycogen depletion (best carmine). Three views per fish (H&E) were analyzed at 10x and 40x magnification (Olympus BH-2 microscope, Japan) using the following scoring system: - = normal state, + = mild change, ++ = moderate change, and +++ = severe change. To quantify lipid depletion the surface area (μm^2) of 10 randomly choses parenchymal cells, of one fish per tank, were measured using Zeiss software (Munich, Germany) to obtain an average per dose group. To validate that the measurement of 10 cells was representative of the organ, the surface area of 50 randomly chosen cells were measured in a subset of samples (2 fish per dose group). The averages were comparable (Fig. C2.S1 & Table C2.S1).

Strong contrasts between PAS stained MMA components (lipofuscin and melanin) and other unstained structures allowed for quantitative analysis using ImageJ (IJ 1.46r) software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014). PAS stained slides were blinded and a targeted analysis

was done. Non-overlapping images were taken of the three most affected areas (i.e. with the most and/or largest MMAs) of one section per fish using a Zeiss Axio Observer.Z1 microscope (Goettingen, Germany) at 40x magnification (oil immersion). Since previous subjective analysis in H&E had shown trends towards increasing size and frequency of MMAs with increasing dietary Se it was decided that a targeted analysis was the most effective way of representing this trend. Using ImageJ, images were size calibrated, converted from colour to black and white RGB Stack, and thresholded so that only the dark MMAs were highlighted. Area (μm^2) of highlighted MMAs was then automatically measured. Best carmine stained slides were analyzed visually for glycogen content and scored qualitatively.

2.3.6 Statistics

Statistical evaluation of the data was conducted using IBM SPSS Statistics V20 (IBM Corp., Armonk, NY). Comparisons of weight, length and HSI at both the 10 day and 72 day time points were conducted using a one-way analysis of variance (ANOVA, $p = 0.05$) with a Tukey's Highly Significant Differences (HSD) post hoc test. Comparisons of Se concentrations in liver and muscle at day 72 and the size of MMAs were conducted with log-transformed data using a one-way ANOVA followed by Tukey's HSD post hoc test. All other data were analyzed using a Kruskal-Wallis (KW, $p = 0.05$) test followed by a Mann Whitney *U* (MU) post hoc test with a Bonferroni correction (BC) where necessary.

2.4 Results

2.4.1 Selenium Accumulation

Se concentrations in muscle showed a non-significant, dose dependent, increasing trend at day 10, while at day 72 Se concentrations in muscle were significantly different among treatment groups (ANOVA, $p < 0.001$), averaging 1.1 ± 0.1 µg/g, 5.3 ± 0.90 µg/g, 23.5 ± 3.38 µg/g, and 64.1 ± 17.02 µg/g (dm) (mean ± SD) in the control, low, medium and high treatment groups respectively (Fig. 2.2). Se concentrations in livers were significantly different (ANOVA,

$p < 0.001$) among treatment groups at day 72 with averages of 0.7 ± 0.28 µg/g, 2.9 ± 0.45 µg/g, 9.3 ± 1.42 µg/g and 91.7 ± 32.91 µg/g (dm) (mean ± SD) in the control, low, medium and high treatment groups respectively (Fig. 2.2). Based on data collected throughout the present study from high treatment group mortalities, it was estimated that Se concentrations in muscle reached a plateau between 60 and 70 µg/g (dm) around day 40 (Fig. 2.2 inset). Trophic transfer factors were 0.7, 0.9, 1.1 and 0.6 in muscle and 0.5, 0.5, 0.4, and 0.9 in liver in the control, low, medium and high treatment groups respectively (Table C2.S2).

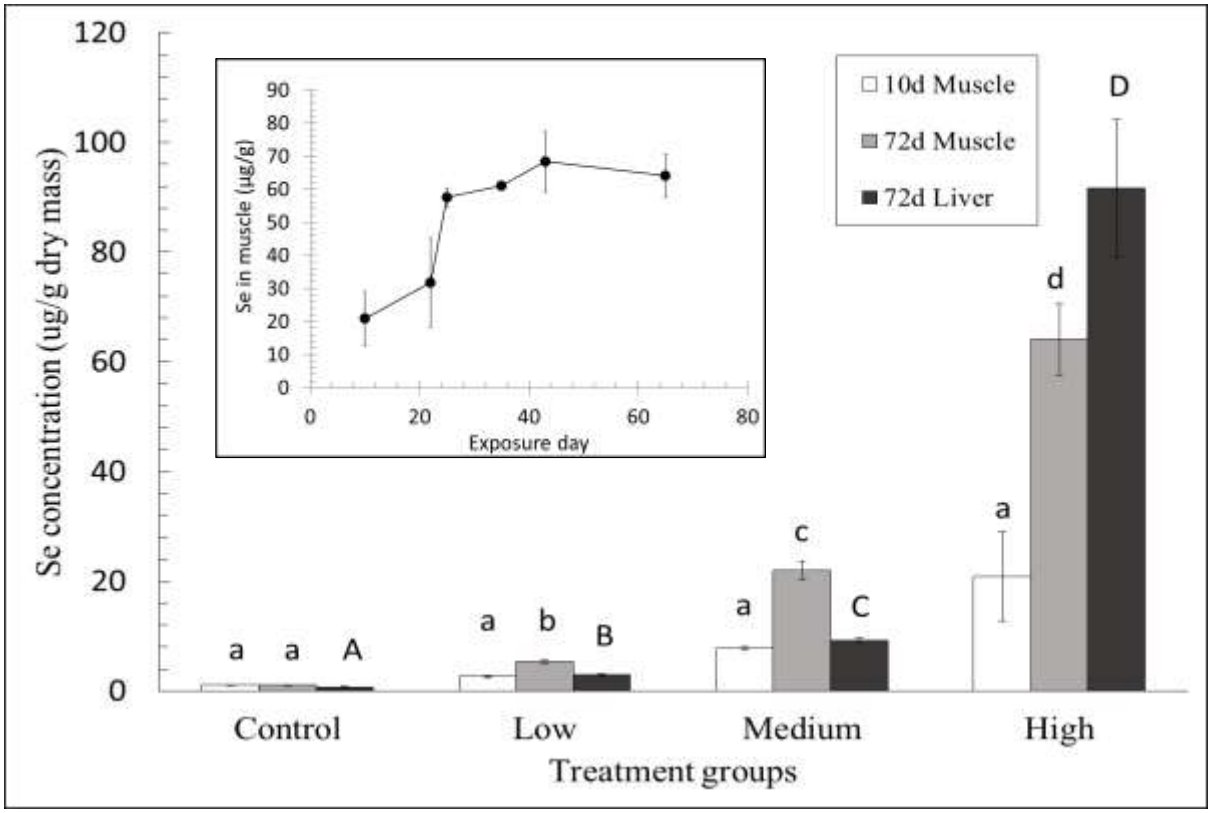


Figure 2.2. Se concentrations in liver and muscle tissues after 10 and 72 days of exposure (65 days for high treatment). Letters indicate statistical differences between dose groups and/or time points. Lower case letters for muscle tissue data and upper case for liver tissue data. Error bars indicate SEM. Inset: Accumulation of Se in muscle over time in the high dose group.

2.4.2 Feeding Behaviour

At initiation of the study, fish in all treatment groups devoured their feed in under 2 minutes. However by day 21 fish in all high dose tanks were showing clear signs of food avoidance. A common behaviour was for high dose fish to swim slowly over the feed as if they were hungry but then to leave it untouched and return to rest. Conversely, fish in control, low and medium dose tanks would actively search out feed for a few minutes even after it was entirely consumed. Regardless of the occurrence of food avoidance in the high dose group fish continued to accumulate Se over time (Fig. 2.2).

2.4.3 Mortality, Euthanizations and Edema

Over the course of the present study there were three mortalities and seven euthanizations (due to loss of equilibrium) in the high dose group. There were no mortalities or euthanizations in the other dose groups. High dose group mortalities/euthanizations averaged 36 ± 9.2% per tank (mean ± SD). All mortality/euthanized fish suffered from severe edema with an average of 27 ± 11.0 mL (mean ± SD) of measured abdominal fluid (Fig. 2.4 & Table C2.S3). Interestingly, there was no correlation between the level of edema and the concentration of Se in muscle and/or liver.

At take down, fish with an edema score of stage 3 or greater were considered moribund. At take down there were five and six moribund fish in the high and medium dose groups respectively. Moribund fish appeared to have less muscle mass compared to control fish sampled at the same time (Fig. 2.4). There were no fish in the low dose group with stage 3 edema or greater and no edema was observed in any of the control fish during the study. Occurrence of lethal effects was calculated by adding the number of fish within a treatment group that died/were euthanized with the number of fish with strong to severe edema (≥ stage 3), and dividing by the number of fish in the treatment group (excluding fish subsampled at day 10). There was a 54% (36% mortality/euthanization, 18% morbidity) and 22% (morbidity) occurrence of lethal effects in the high and medium dose groups respectively (n_{high} = 28, n_{medium} = 27).

Edema began to develop very early among fish in the high treatment group. By day 10, three of the seven sampled high dose fish had body cavities full of fluid (up to 50 mL, Table C2.S3). As the study progressed the number of fish affected in all treatment groups, as well as the severity of the popeye, increased in a dose dependent manner. At day 72 control, low, medium and high dose groups had 0%, 23 ± 18%, 41 ± 35% and 75 ± 25% (mean ± SD) incidences of stage 2 edema or greater in each tank, respectively (Fig. 2.3). At day 72 average edema severity scores were significantly greater in all dose groups compared to control (MU, all dose group p values ≤ 0.003 compared to control, BC, $p = 0.013$) and high dose fish had significantly greater edema severity scores than medium dose fish (MU, $p = 0.012$, Fig. C2.S2). Fish did not regain homeostasis from stage 2 edema or greater and in the medium and high treatment groups often worsened progressively unto death.

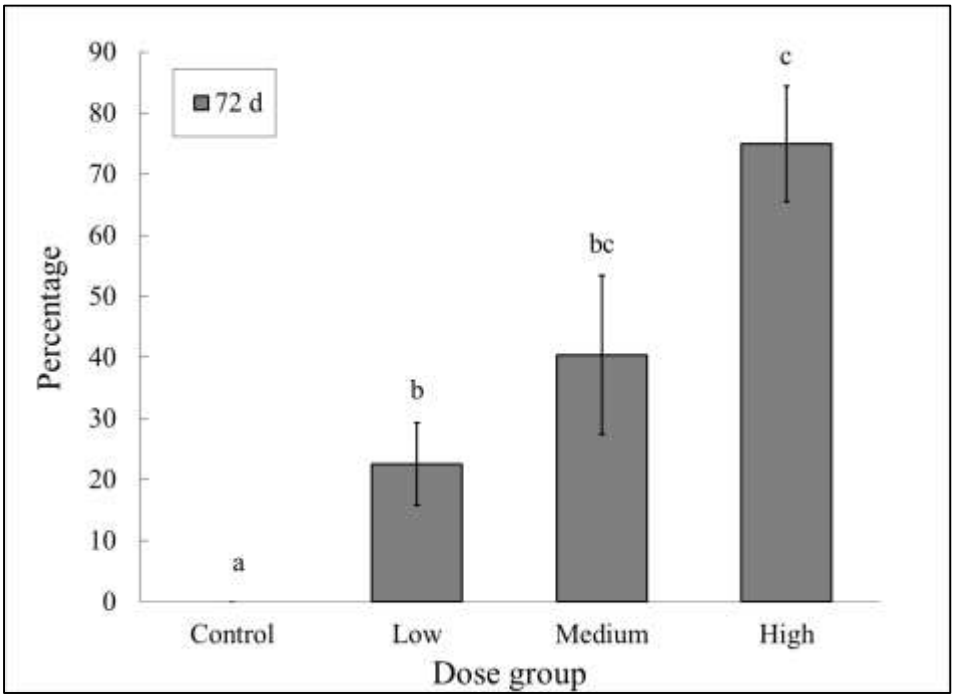


Figure 2.3. Percent of fish with an edema score ranking greater than or equal to 2 at day 72. Bars represent average of edema prevalence from 7 tanks per group. Error bars indicate the SEM. Letters indicate significant differences among dose groups.

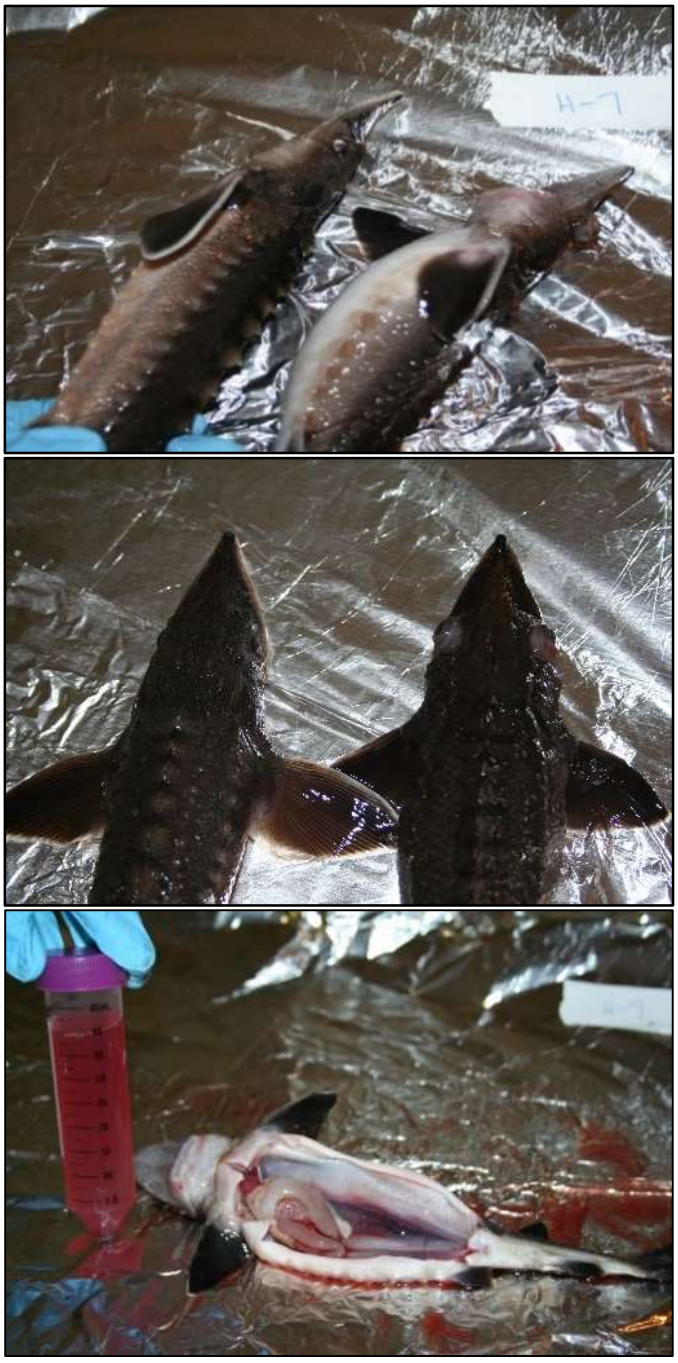


Figure 2.4. Top and Central: A control (left) and moribund high dose (right) fish, day 22. Bottom: shows the fluid collected from the abdomen of the high dose fish as well as the thinness of side muscle.

2.2.4 Growth and Condition Factor

To minimize handling stress fish were not weighed and measured at the start of the present study. Thus, we used the gain in length and mass between the day 10 subsampling and termination of the study as a proxy for growth, assuming similar initial body sizes among treatments. Subsamples collected on day 10 averaged 124 ± 46.5 g and 26.0 ± 2.8 cm fork length (mean ± SD) across all treatment groups, with no significant differences among treatment groups (ANOVA, $p = 0.294$). By day 72 fish in control, low, medium and high doses weighed on average 232 ± 71.8 g, 216 ± 60.1 g, 226 ± 64.2 g, and 129 ± 44.5 g respectively and had fork lengths of 31.5 ± 3.6 cm, 31.2 ± 2.7 cm, 31.2 ± 3.6 cm and 26.4 ± 2.8 cm respectively (Fig. C2.S3). High dose group fish were significantly lighter and shorter than those from all other treatment groups at day 72 (ANOVA, $p < 0.001$) and were not significantly different than high dose fish at day 10 (ANOVA, $p = 1.00$). Fish from the control, low and medium treatment groups were significantly heavier and longer compared to those sampled on day 10 (ANOVA, $p \leq 0.013$). Condition factor (CF) was not different among dose groups at day 10 or day 72, or within dose groups between the two time points (KW, $p = 0.653$).

2.4.5 Histopathology and Hepatosomatic Index

Hepatosomatic indices (HSI) were not different among dose groups at day 10 (ANOVA, $p \geq 0.703$) and were not different among control (2.5 ± 0.8), low (2.8 ± 0.5) and medium (2.6 ± 0.8) (mean ± SD) dose groups at day 72 (ANOVA, $p \geq 0.792$). However, HSIs were significantly lesser in the high dose group (1.3 ± 0.8) (mean ± SD) compared to all other groups at day 72 (ANOVA, $p < 0.001$) and compared to HSI at day 10 in the high dose group (ANOVA, $p = 0.019$) (Fig. 2.5A). High dose fish showed a decrease in liver lipid stores measured as a significant reduction in cell surface area (MU, $p = 0.007$, BC $p = 0.017$) (Fig. 2.5B & Fig. 2.6). Average cell surface area was 601.2 ± 92.0 μm^2, 595.3 ± 87.4 μm^2, 599.9 ± 76.0 μm^2 and 251.5 ± 65.0 μm^2 (mean ± SD) in the control, low, medium and high dose groups respectively.

Visual inspection indicated a trend toward an increase in frequency and size of MMAs (Fig. 2.6). Targeted analysis of the most affected areas using ImageJ showed a significant

increase in surface area covered by MMAs in livers of high dose group fish compared to control fish (ANOVA, Tukey HSD, p = 0.014). Average MMA surface area was 340.0 ± 118.7 μm^2, 420.0 ± 181.3 μm^2, 354.1 ± 99.2 μm^2 and 867.9 ± 214.7 μm^2 (± SD) in control, low, medium and high dose groups respectively. There was no difference among treatment groups in the categories of apoptosis, necrosis and general cell health. Although reported in similar studies, vacuolar degeneration and glycogen depletion were not observed in fish from any of the treatment groups in the present study (Table C2.S4).

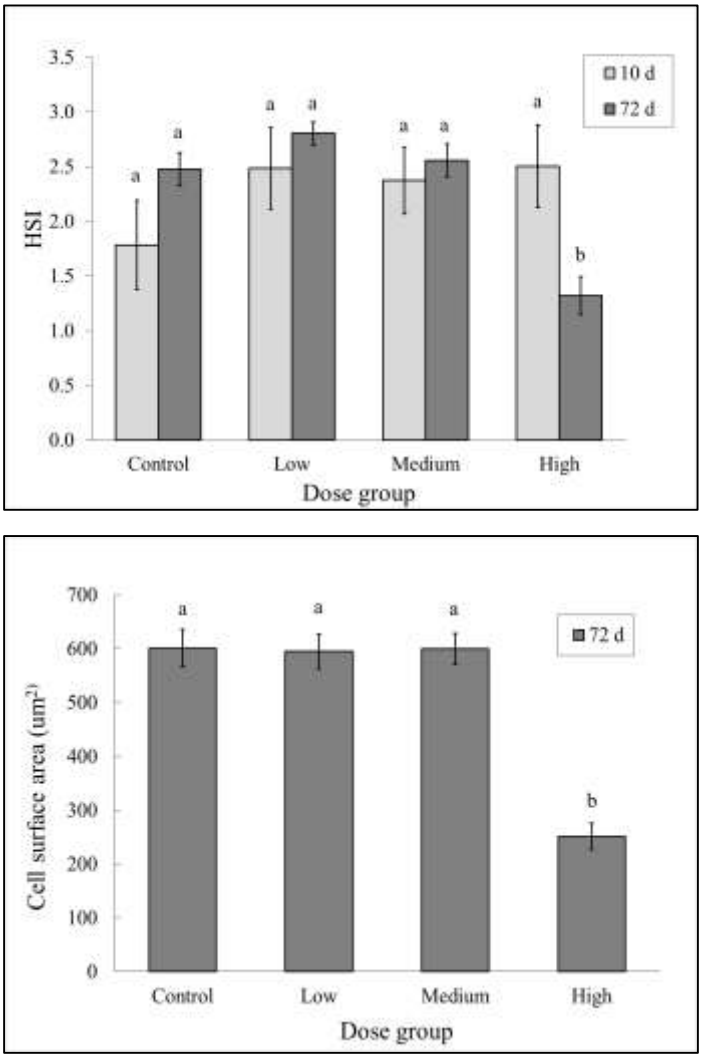


Figure 2.5. Top (A): Average HSI at day 10 and 72, and Bottom (B): Average surface area of liver cells at day 72. Error bars indicate SEM. Letters indicate statistical differences between dose groups.

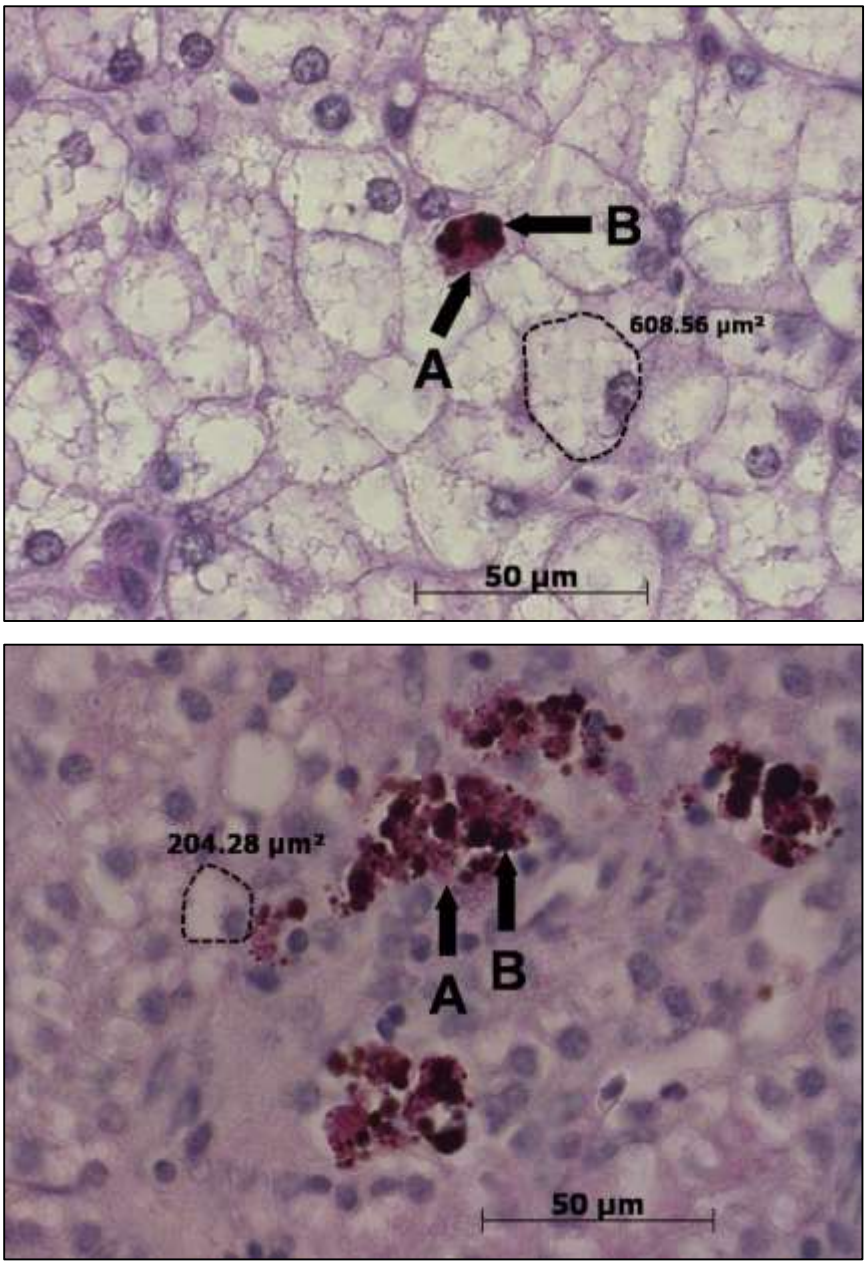


Figure 2.6. PAS stained sections of a control (top) and high (bottom) dose group liver section at 40x magnification (oil immersion). A and B indicate lipofuscin and melanin respectively in melanomacrophage aggregates (MMAs). The dashed line outlines a cell with surface area indicated beside. The high dose parenchymal cells were much smaller and more darkly stained as cytoplasmic lipid droplets had disappeared.

2.5 Discussion

The present study demonstrated that exposure of juvenile white sturgeon to dietary SeMet resulted in a significant and dose dependent increase of Se in liver and muscle tissues and significant adverse health effects, and histological changes. Over 72 days, adverse health effects linked to increasing dietary SeMet included edema (low, medium and high dose group) leading to morbidity (medium and high dose group), mortality (high dose group), cessation of growth (high dose group), and a significant decrease in HSI (high dose group). Histological analysis of the liver revealed a dose dependent increase in frequency and size of MMAs and decrease of lipid energy stores (high dose group). These findings indicate that Se pollution leading to dietary doses ≥ 22 µg/g (dm) (muscle concentrations ≥ 23.5 µg/g; liver concentrations ≥ 9.2 µg/g) has a negative impact on white sturgeon health. Stage 2 edema was observed at a lowest observable adverse effect concentration (LOAEC) of 5.6 ± 0.02 µg/g (dm) (muscle concentration 5.3 ± 0.90 µg/g; liver concentration 2.9 ± 0.45 µg/g) which places white sturgeon among the most sensitive fish species. However, the biological relevance of stage 2 edema is unknown and further studies are needed to assess the potential impact this phenomenon may have on fish health.

The most predominant and surprising finding was the early onset of edema and the high rate of morbidity and/or mortality in the medium and high treatment groups. Edema severity and frequency increased with dietary dose and over time (Table C2.S3 & Fig. C2.S2). Edema may be caused by altered[6] cell permeability resulting in 'leaky' organs (Lemly, 2002a). It has also been suggested that excess Se causes oxidative stress (De Riu et al., 2014; Palace et al., 2004) which could lead to membrane damage and possibly edema. Reports in the literature are inconsistent with regard to this edema endpoint in fish exposed to dietary SeMet. Subchronic dietary exposure studies conducted with white sturgeon over 8 weeks (Tashjian et al., 2006; De Riu et al., 2014) and 23 weeks (Linville, 2006) using comparable doses (0.4 – 191.1 µg/g dm) with Sacramento River white sturgeon reported no signs of edema and essentially no mortalities. In contrast, edema causing popeye and distension of the abdomen have been observed in studies with wild juvenile white crappie (*Pomoxis annularis*) and other fish species in a Se polluted lake

[6] Published version: "Edema may be caused by distortion of selenoproteins in cell membrane structures, which disrupts cell permeability resulting in 'leaky' organs (Lemly, 2002a)". This statement has been edited for greater accuracy.

(Lemly, 2002a), in Se exposed catfish in a laboratory study (Ellis et al., 1937) and in bluegill used in two separate cage and laboratory Se exposure studies (Table 2.1) (Finley, 1985). In the laboratory study, bluegill suffering from edema, abdominal distention and popeye after exposure to dietary Se, showed symptom remission after refusing all food for 4 - 11 days. However despite the initial remission, once feeding resumed symptoms returned. Over the 44 day exposure period, 75% of the Se treated bluegill (given wild mayflies, 54.4 µg Se/g) died, while there were no mortalities among the controls (Finley, 1985). In contrast, no comparable remission of symptoms was observed in white sturgeon that avoided food in the present study.

Food avoidance can be a confounding factor in dietary Se studies and has been observed in other species of fish and mammals (e.g. cattle, National Research Council, 1983; rainbow trout, Hilton et al., 1980 and Hamilton, 2004). Interestingly no food avoidance was observed in other white sturgeon studies (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014), although Tashjian et al. (2006) did observe that swim activity decreased in groups fed Se at concentrations $\geq$ 40 µg/g (Table 2.1). In the present study, food avoidance was notable in some high dose tanks by day 13, and by day 21 all fish in the high dose tanks had stopped eating. This makes it difficult to attribute the observed mortalities, decreases in growth, and reduction of lipid stores in the liver to Se toxicosis, self-enforced starvation or a combination of the two. However, since Se accumulation continued throughout the present study, regardless of food avoidance (Fig. 2.2), observed effects are most likely due to selenosis. Perhaps some residual uptake of feed, that was not visually observed, occurred and was sufficient to maintain exposure levels. Fish accumulated Se in a dose dependent manner with average TTFs of 0.9 and 0.6 in muscle and liver respectively, across doses. This is similar to other selenium exposure studies with white sturgeon that found TTFs of approximately 1.0 in muscle and liver (Table C2.S2) (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). The avoidance of highly seleniferous feed in the laboratory suggests that sturgeon may be able to detect and avoid toxic food stuffs successfully in the wild.

Histological analysis of the liver showed only a low degree of tissue damage, which is in contrast to what was expected based on the observed severe edema and mortalities in the present study and earlier reports of white sturgeon exposures to SeMet (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). Glycogen depletion, vacuolar degeneration, necrosis, apoptosis, changes in vein and canaliculi structures, biliary hyperplasia and biliary stasis have previously

been reported for white sturgeon exposed to ≥ 20 µg Se/g (dm) through the diet but were not observed in the present study (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014).

Table 2.1. Selenium exposures and resultant effects in various fish species, including white sturgeon, expanded from Hamilton, 2004

Species	Selenium Species	Dietary Concentration (ug/g dm)	Tissue Concentration (ug/g dm)	Parameter Affected	Reference
Rainbow trout	SeVI	9		Mortality	Hamilton, 2004 review
		11 - 12	4.0 - 4.5 (WB)	Kidney, weight	
		13	5.2 (WB)	Mortality, growth, food aversion	
Rainbow trout	SeMet	18.16*	8.84 (M)	Growth	Wiseman et al., 2011
Chinook salmon	SeMet	9.6	5.4 (WB)	Mortality	Hamilton, 2004 review
		18.2	10.8 (WB)	Growth	
Bluegill	SeMet	6.5	4.3 (WB)	Mortality	Hamilton, 2004 review
		5.1	5.5 (WB)	Mortality	
		33	19 (WB)	Reproduction	
Bluegill	Wild caught mayflies	54.4*	26* (M), 178.8* (L)	Mortality, edema, food aversion, loss of equilibrium	Finley, 1985
White sturgeon	SeMet	≥ 22.4	23.5 (M), 9.3 (L)	Mortality/morbidity	The present study
		≥ 5.6	5.3 (M), 2.9 (L)	Edema	
		≥ 104.4	64.1 (M), 91.7 (L)	Growth, energy stores, liver histopathologies, food aversion	
White sturgeon	SeMet	≥ 20	21.6 (M), 19.5 (L)	Liver histopathologies	Linville, 2006
White sturgeon	SeMet	≥ 20.5	22.9 (M), 22.0 (L)	Kidney histopathologies	Tashjian et al., 2006
		≥ 41.7	36.8 (M), 37.4 (L)	Growth, liver histopathologies, swim activity	
White sturgeon	SeMet	≥ 40.1	41.3 (M), 30.1 (M)	Growth, energy stores, kidney histopathologies	De Riu et al., 2014
Zebra fish	SeMet	≥ 3.7	7.2 (WB)	Swim performance, increased energy stores, increased growth	Thomas & Janz, 2011
		≥ 26.6	18.6 (WB)	Mortality	
Fathead minnow	SeMet	≥ 9.9	9.4 (WB)	Swim performance	McPhee & Janz, 2014

WB = whole body SeIV = selenite
L = liver SeMeth = selenomethionine
M = muscle * = converted from wet weight assuming 75% moisture (Lemly, 2002)

However, the previous three studies were inconsistent in terms of the type of pathologies described as well as their severity (Table C2.S4). Tashjian et al. (2006) noted statistically significant differences in the categories of vacuolar degeneration, biliary stasis, biliary hyperplasia and glycogen depletion. De Riu et al. (2014) qualitatively observed vacuolar

degeneration and glycogen depletion but made no mention of biliary statis or hyperplasia. Linville (2006) qualitatively found an increase in vacuolar degeneration, biliary stasis and biliary hyperplasia but did not mention glycogen depletion, even though glycogen was pointed out in histological figures. Linville (2006) also found increases in focal necrosis and immune cells while Tashjian et al. (2006) and De Riu et al. (2014) did not investigate these pathologies. The present study did not find any of the liver pathologies listed above. Although the exact reason for the inconsistencies in histological lesions remains unknown, the lack of repeatability may be because little baseline information regarding sturgeon liver histology is available and normal tissue morphology has yet to be established.

A prevalent histopathological change observed in the present study was the increase in size and frequency of melanomacrophage aggregates (MMAs) in exposed fish. Similar observations were reported by Linville (2006) and De Riu et al. (2014) but not Tashjian et al. (2006). MMAs are believed to function as part of the immune response system, in cell debris clean up, in storage for recyclable molecules, and in permanent storage for molecules that cannot be broken down and disposed of further (Passantino et al., 2014; Agius & Roberts, 2003; Wolke, 1992; Fournie et al., 2001). The observed increase in size and frequency of MMAs in the liver suggest that there is increased cellular damage occurring and/or a greater need to protect against oxidative stress and/or direct Se toxicity (Passantino et al., 2014; Agius & Roberts, 2003; Wolke, 1992; Fournie et al., 2001; Blazer et al., 1987). Therefore the absence of other signs of cellular damage such as apoptosis and necrosis was surprising. It has been suggested that MMAs can be used as a general indicator for toxic exposures (Agius & Roberts, 2003; Wolke, 1992; Fournie et al., 2001). However, they should not be used as specific indicators of Se exposure as they also increase in fish due to aging, nutritional status, infectious disease, and various contaminant exposures, and are therefore considered a generalized response (Passantino et al., 2014; Agius & Roberts, 2003; Fournie et al., 2001). MMAs do however add to the body of evidence in the present study supporting the case that SeMet in excess amounts is toxic to white sturgeon (Passantino et al., 2014; Fournie et al., 2001).

White sturgeon liver tissue is naturally more lipid dense than that of other fish species (Linville, 2006), with lipid droplets filling up most of the parenchymal cell cytoplasm. In the present study, significant depletion of lipid energy stores was observed in the high treatment group (Fig. 2.5B & 2.6) and HSI was also significantly lower (Fig. 2.5A), likely driven by lipid

depletion. While this was not specifically discussed by Linville (2006), images included in that dissertation suggest similar lipid depletion may have occurred in fish fed 52.5 µg Se/g. In proximate composition analyses Tashjian et al. (2006) found that lipid content was negatively correlated with Se concentration, and De Riu et al. (2014) found a significant decrease in lipid content of fish fed ≥ 40 µg/g. The depletion of lipid stores suggests that energy was being used to compensate for and repair damage caused by toxic assault.

Toxicity thresholds for dietary Se exposure of the most sensitive species are between 3 µg Se/g (juvenile salmonids) and 6.5 µg Se/g (centrarchids) (Lemly, 2002b). According to Lemly (2002b), the most sensitive fish species have toxicity thresholds of 8 µg Se/g in muscle and 12 µg Se/g in liver tissue when considering reproductive, growth and mortality endpoints (Table 2.1). Following these suggestions for sensitivity thresholds, previous white sturgeon studies have found this species to be relatively tolerant (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014).

Results of the present study place white sturgeon as widely ranging from tolerant to sensitive depending on the endpoint considered. Based on growth and mortality endpoints white sturgeon were found to be moderately sensitive to SeMet exposure. For growth, they were less sensitive than rainbow trout (Hamilton, 2004; Wiseman et al., 2011) and bluegill (Hamilton, 2004), but more sensitive than fathead minnow (*Pimephales promelas*) (McPhee & Janz, 2014) and zebrafish (*Danio rerio*) (Thomas & Janz, 2011). Other white sturgeon studies found decreased growth rates when fish were fed ≥ 40 µg Se/g (dm) (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). Data from the present study can be considered consistent with previous white sturgeon study findings as no change in growth at 22.4 µg/g was observed and a decreased growth rate at 104.4 µg/g was observed with no intermediate dosage tested. Based on rates of mortality, the present study found white sturgeon to be less sensitive than rainbow trout, bluegill and chinook salmon (Hamilton, 2004), but more sensitive than fathead minnow (McPhee & Janz, 2014) and white sturgeon tested in other studies (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014). Sensitivity was approximately the same as that reported for zebrafish (Thomas & Janz, 2011), with significant increases in morbidity in the present study at LOAECs of 22.4 µg Se/g in the diet, 23.5 µg Se/g in muscle tissue, and 9.3 µg Se/g (dm) in liver tissue.

Results of the present study place white sturgeon among the most sensitive species to SeMet exposure when edema is used as the endpoint. By day 72 edema was occurring in the low

dose treatment group (21% of fish exhibited stage 2 edema), which had concentrations of 5.6 µg Se/g in the diet, 5.3 µg Se/g in muscle tissue, and 2.9 µg Se/g (dm) in liver tissue. The sensitivity of white sturgeon to developing edema may be of concern as recent studies have reported that common prey exceed this dietary concentration in some white sturgeon habitats (e.g. an average 15 µg Se/g [dm] in clams in the San Francisco Bay) (Linville, 2006; Linville et al., 2002; Luoma & Presser, 2006). However, the biological relevance of the various stages of edema is still unknown and warrants further research. Of the other fish species tested in the laboratory only bluegill has also been shown to develop edema at comparable dietary Se concentrations (54.4 µg Se/g food [wild mayflies]) (Finley, 1985).

There are a number of possible reasons for the differences between the responses observed in the present study and those in other studies conducted with white sturgeon. Other studies have used juvenile white sturgeon raised from (potentially multigenerational) captive brood stock from farms based in Sacramento (CA, USA) (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014), whereas the present study used wild brood stock from the Upper Columbia River. Potentially differing histories of parental exposures and the number of generations in hatchery conditions may account for differing Se tolerances. Also, the wild Sacramento and Columbia populations have been geographically separated since 1938 by the Bonneville dam (Hildebrand et al., 1999) which may have caused the development of greater or lesser tolerances to Se. Especially as California is known for naturally seleniferous soil, thus likely resulting in higher background Se levels. Alternatively, the size and developmental stages of the fish used may have had an effect on Se tolerance, although this is unlikely as earlier life stages of fish are generally more sensitive to contaminant exposures than older fish (present study, 124g; Linville, 2006, 575g; Tashjian et al., 2006, 30g; De Riu et al., 2014, 30g). Laboratory water quality differences could also have affected fish tolerance. Laboratory water hardness and alkalinity were 220 mg/L $CaCO_3$ and 180 mg/L $CaCO_3$, respectively, in a prior study (Linville, 2006) and 172 mg/L $CaCO_3$ and 140 mg/L $CaCO_3$, respectively, in the present study. Regardless, the exact reasons for the differences in sensitivities observed among white sturgeon studies are unknown and further experiments are required to investigate the specific causes for these differences.

Taken together, results of the present study and those of previous studies indicate that excess dietary SeMet could reduce the fitness of juvenile white sturgeon at environmentally relevant concentrations. The large variation in tolerances according to different endpoints

suggests a high degree of complexity in the mechanism(s) of Se toxicity to this species. As an extension of the present study, ongoing research with samples collected from the same fish is investigating oxidative stress in the liver, changes in blood chemistry (cortisol response, steroidogenesis), and alterations in gene expression patterns across the whole transcriptome to further elucidate the specific mechanism(s) of SeMet toxicity in white sturgeon.

CHAPTER 3: IS HEPATIC OXIDATIVE STRESS A MAIN DRIVER OF DIETARY SELENIUM TOXICITY IN WHITE STURGEON (*ACIPENSER TRANSMONTANUS*)?[2]

[2] This chapter has been submitted to Environmental Toxicology and Safety under joint authorship with Sarah Patterson (University of Saskatchewan), Steve Wiseman (University of Saskatchewan), and Markus Hecker (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the references section of this thesis. Supplementary material submitted to the journal have been included in Appendix A.

3.1 Abstract

Most species of sturgeon have experienced significant population declines and poor recruitment over the past decades, leading many species, including white sturgeon (*Acipenser transmontanus*), to be listed as endangered. While the reasons for these declines are not yet fully understood, benthic lifestyle, longevity, and delayed sexual maturation likely render them particularly susceptible to factors such as habitat alteration and contaminant exposures. Toxicity studies have shown white sturgeon to be among the most sensitive species of fish to pollutants such as metals, dioxin-like chemicals and endocrine disrupters. Selenium (Se) in the aquatic ecosystem is of particular concern, especially in its more bioavailable form selenomethionine (SeMet), because it is known to efficiently bioaccumulate in the food chain. The toxic effects of Se have been observed in the wild and in laboratory settings. Therefore, the aim of the present study was to link physiological effects observed in a previous laboratory study to key molecular events of toxicity. Oxidative stress in liver tissue was focused on as it was hypothesized to be a primary mode of toxicity. Specifically, 4 year old white sturgeon were exposed for 72 days to 1.4, 5.6, 22.4 or 104.4 µg SeMet (dm) per g feed. Doses were chosen to range over a necessary Se intake level, current environmentally relevant intakes and an intake representing possible scenarios of Se release. Lipid hydroperoxides, end products of lipid oxidation, were measured using a standard assay kit. Antioxidant response was measured via changes in gene expression of glutathione peroxidase (GPx), superoxide dismutase, catalase, glutathione S-transferase, apoptosis inducing factor and caspase 3 using real-time PCR. Results of the lipid hydroperoxide assay were highly variable within dose groups and therefore no dose response was observed. GPx expression was significantly increased in the low dose group indicating an induced antioxidant response. No other genes were significantly induced or suppressed. Overall, indicators of oxidative stress were few and therefore oxidative stress is not believed to be a main driver of toxicity in white sturgeon exposed to selenium.

3.2 Introduction

For oviparous vertebrates, selenium (Se) is both an essential micronutrient and a toxicant depending on the concentration (Chapman et al., 2009; Mayland, 1994). In surface waters, Se contamination increases as a result of natural geological processes and/or anthropogenic disturbances such as mining, smelting operations, fossil fuel by-product waste disposal and irrigation runoff (Lemly, 2002b; Chapman et al., 2009; Janz, 2012; Maher et al., 2009). Once in the aquatic ecosystem elemental and inorganic Se are biotransformed to selenomethionine (SeMet), an amino acid analogue, by bacteria, phytoplankton and other low trophic level organisms. These organisms can tolerate relatively high Se body burdens, which are then transferred to higher trophic level consumers that are less tolerant; although sensitivity to Se exposure is species specific (Fan et al., 2002; Chapman et al., 2009; Maher et al., 2009). Among aquatic organisms, fish are particularly sensitive to SeMet exposures, and one species that recently has received much attention with regard to their vulnerability is the white sturgeon (*Acipenser transmontanus*). White sturgeon are believed to be at risk due to their benthic life-style, longevity, and the adult's position at the top of the food chain (Linville, 2006; Moyle, 2002; Doroshov et al., 1997). They are known to be among the most sensitive species of fish to other environmental pollutants such as metal ions and dioxin-like compounds (Vardy et al., 2011; Vardy et al., 2012; Doering et al., 2012; Doering et al., 2014), and previous studies have found elevated levels of Se in wild white sturgeon (Linares-Casenave et al., 2014; Kruse, 2000).

Exposure to elevated doses of SeMet causes adverse health effects in vertebrates including mammals (National Research Council, 1983), aquatic birds (Hoffman, 2002) and a wide range of fish species (*Oncorhynchus mykiss* Wiseman et al., 2011; *Lepomis cyanellus*, *Pomoxis annularis*, *Micropterus salmoides*, *Gamusia affinis*, *Notropis lutrensis*, *Ictalurus punctatus,* Lemly, 2002a; *Lepomis microlophus,* Sorensen, 1988 and Sorensen & Bauer, 1984; *Oncorhynchus tshawytscha*, Hamilton, 2004; *Lepomis macrochirus*, Finley, 1985; *Danio rerio*, Thomas & Janz, 2011; *Pimephales promelas*, McPhee & Janz, 2014; *Acipenser transmontanus,* Tashjian et al., 2006, Linville, 2006, De Riu et al., 2014 and Zee et al., 2015; *Acipenser medirostris,* De Riu et al., 2014). The most common route of Se uptake is through the diet. Fish require 0.1-0.5 µg Se/g feed for proper health; however, toxic effects have been reported at > 3 µg Se/g feed in some fish species (Gaitlin & Wilson, 1984; Poston et al., 1976; Hodson &

Hilton, 1983; Lemly, 1997). Symptoms caused by elevated Se body burdens in fish include changes to concentrations of sex steroid hormones and cortisol in the blood (Wiseman et al., 2011), reproductive failure, teratogenesis (Lemly, 2002a; Sorensen, 1988), pathological lesions in gills, liver, kidney, heart and ovary (Zee et al., 2015; Tashjian et al., 2006, De Riu et al., 2014; Linville, 2006; Lemly, 2002a; Sorensen et al., 1984; Sorensen & Bauer, 1984; Sorensen, 1988), decreased energy reserves (Zee et al., 2015; Tashjian et al., 2006), impaired swim performance (McPhee & Janz, 2014; Tashjian et al., 2006) and growth (Zee et al., 2015; Tashjian et al., 2006), cataracts (Lemly, 2002a), edema leading to popeye (exophthalmos) (Zee et al., 2015; Lemly, 2002a; Finley, 1985; Sorensen et al., 1984; Ellis et al., 1937) and mortality (Zee et al., 2015; Finley, 1985; Lemly, 2002b; Hamilton, 2004). Four studies have exposed juvenile white sturgeon to dietary SeMet in the laboratory and various toxic symptoms were observed including histopathological liver changes (Zee et al., 2015; De Riu et al., 2014; Linville, 2006), reduced energy reserves (Zee et al., 2015; Tashjian et al., 2006), reduced swimming activity (Tashjian et al., 2006), slowed growth rates (Zee et al., 2015; Tashjian et al., 2006), and severe edema and mortality (Zee et al., 2015).

One of the possible explanations for some of the pathologies, such as the severe edema, observed in fishes exposed to Se in previous studies (Zee et al., 2015; Lemly, 2002a; Sorensen et al., 1984; Sorensen & Bauer, 1984; Ellis et al., 1937) could be an increase in oxidative damage, which can alter cell membrane permeability and fluidity causing organs and/or capillaries to become ‘leaky’. This hypothesis is supported by the increases in size and frequency of melanomacrophage aggregates in liver (Zee et al. 2015; Linville, 2006; De Riu et al., 2014), which suggested that there was increased cellular damage occurring and/or a greater need to protect against oxidative stress. The need to combat oxidative stress may also explain depleted energy reserves and slowed growth rates observed in white sturgeon (Tashjian et al., 2006; Zee et al., 2015). Cross-linking of actin filaments due to oxidative damage in muscle was proposed as a reason for decreased swimming activity in white sturgeon given excess dietary SeMet (Tashjian et al., 2006). Studies that exposed Siberian sturgeon (*Acipenser baerii*) to dietary selenocysteine (SeCys) for 60 or 90 days observed signs of antioxidant response in the liver, including increased glutathione peroxidase, superoxide dismutase, glutathione reductase and catalase activity, and a positive correlation between glutathione s-transferase activity and Se concentration (Pacini et al., 2013; Elia et al., 2014). A significant increase in oxidized

glutathione (GSSG) concentration and the ratio of oxidized glutathione to reduced glutathione (GSSG:GSH) was observed in the liver of rainbow trout given 20 µ Se/g dietary SeMet for ≥ 120 days, indicating an increase in oxidative stress (Holm, 2002). Palace et al. (2004) demonstrated that rainbow trout embryos exposed to SeMet had significantly increased production of superoxide radicals, while Misra et al. (2012) showed that rainbow trout hepatocytes develop an antioxidant response as well as signs of increased oxidative stress when cultured in SeMet spiked media. Miller (2006) and Janz et al. (2009) both propose oxidative stress to be involved in Se related teratogenesis in oviparous animals and other pathologies observed in adults. Symptoms of oxidative stress have also been observed in aquatic birds (Spallholz & Hoffman, 2002; Hoffman, 2002; Janz et al., 2009). In mallards (*Anas platyrhynchos*), willets (*Catoptrophorus semipalmatus*), American coots (*Fulica Americana*) and emperor geese (*Chen canagica*) exposed to Se in the wild or fed SeMet in the laboratory, increases in reactive oxygen species (ROS) and antioxidant activity have been observed (Hoffman, 2002). While oxidative stress/antioxidant response is known to be an important mechanism of toxicity/protection in other species only a handful of studies have researched its role in sturgeon species exposed to contaminants (Pacini et al., 2013; Elia et al., 2014; Palace et al., 1996; Martinez-Alverez et al., 2002; Song et al., submitted).

The purpose of the present study was to investigate whether histopathological changes in the liver, reduced energy reserves, edema and mortality, which were previously observed in juvenile white sturgeon exposed to elevated concentrations of dietary SeMet (Zee et al., 2015), can be explained by oxidative stress as a mechanism of toxicity. To this end, markers of oxidative stress were assessed by quantification of concentrations of lipid hydroperoxides (LHPs) and expression of genes that are important for responding to oxidative stress. All tests were conducted with liver samples as this is a main organ of detoxification, and is one of the tissues where greatest Se accumulation occurs during dietary exposures (Linville, 2006; Tashjian et al., 2006; Linares-Casenave et al., 2014).

3.3 Methods

3.3.1 Exposure

Three-year-old juvenile white sturgeon were exposed to various concentrations of dietary SeMet as described in Zee et al. (2015). All procedures involving live animals were approved by the University of Saskatchewan's Animal Research Ethics Board (Animal Use Protocol #20070049). Briefly, five fish were randomly assigned to seven replicate tanks per treatment group (35 fish/treatment) supplied with carbon filtered municipal water under flow-through conditions in accordance with loading densities recommended by the American Society for Testing and Materials (ASTM) guidelines for testing early life-stage of fishes (ASTM, 2007). For 72 days fish were given diets of commercial trout chow (Proform Aquaculture Feed, Aqua-Balance Trout 52:19 Starter #2 Crumble, Viterra Feed Products; Okatoks, AB, Canada) spiked with either 5.6 ± 0.02 µg/g, 22.4 ± 0.37 µg/g or 104.4 ± 4.81 µg/g seleno-L-methionine (Sigma-Aldrich; Oakville, ON, Canada). No SeMet was added to control diets, which contained 1.4 ± 0.06 µg/g (dm) that was added by the manufacturer as a dietary supplement (nominal concentrations, mean ± SD). A subsample of one fish per tank was taken at day ten and used for gene expression analysis. Remaining fish were sampled on or near day 72 and their livers were analysed for concentrations of lipid hydroperoxides. The exception was the fish in the high-dose group that were euthanized and sampled at day 65 due to high mortality rates and to avoid unnecessary suffering of animals.

3.3.2 Quantitative Real-Time Polymerase Chain Reaction

Expression of genes encoding for antioxidant enzymes and enzymes that mediate apoptosis were quantified in liver samples of white sturgeon after 10 days of exposure. Genes associated with response to oxidative stress were glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST). Genes involved with apoptotic signaling were caspase 3 (Cas3) and apoptosis inducing factor (AIF), which are key

mediators of caspase-dependent and -independent apoptosis, respectively. Total RNA was extracted from approximately 100 mg liver tissue from each fish using an RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON, Canada), according to the manufacturer's protocol. Concentrations of RNA were determined by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and samples of RNA were stored at −80°C until analysis. First-strand cDNA was synthesised from 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Samples of cDNA were stored at −20°C until analysis (Doering et al., 2014).

Quantitative real-time PCR (qPCR) was performed in 96-well plates using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 70 µl reaction mixture of 2× concentrated Power SYBR Green master mix (Qiagen), 3.5 µl cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water was prepared for each cDNA sample and primer combination. Reactions were conducted in triplicate with 20 µl reaction volumes per well. The reaction mixture for PCR was denatured at 95°C for 10 min followed by a thermal cycle profile consisting of denaturing at 95°C for 10 s and extension for 1 min at 60°C for a total of 40 PCR cycles. Target gene transcript abundance was quantified by normalizing to β-actin according to the method described by Simon (2003). A dissociation step was added to ensure only a single product was amplified. Primers against β-actin of white sturgeon were from Doering et al. (2012). Primers against GST, GPx, Cas3, CAT, AIF, and SOD of white sturgeon were designed from a database of the transcriptome of the liver of white sturgeon that was generated by *de novo* assembly of paired-end sequencing reads generated on Illumina MiSeq and HiSeq 2000 sequencing platforms (Illumina, San Diego, CA, USA) (unpublished data). Primers (Table 3.1) were designed by use of Primer 3 software (Koressaar and Remm, 2007; Untergrasser et al., 2012) and were from Invitrogen (Burlington, ON, Canada) (Doering et al., 2014).

Table 3.1. Gene primer sequences, annealing temperatures and efficiencies.

Target Transcript	Accession #	Primer Sequence (5'-3')	Annealing Temp. (°C)	Efficiency (%)
βeta Actin	FJ205611	F: CCGAGCACAATGAAAATCAA R: ACATCTGCTGGAAGGTGGAC	60	96
Glutathione Peroxidase 1		F : AGTTGATGTGAACGGGAAGG R: ACTTGGGGTCAGTCATCAGG	60	109
Glutathione S-transferase theta		F: CTCCAGGATGAAAACCTTGG R: ACTCAATCCCATGCAAAAGG	60	107
Catalase		F: GAACGAAGAAGAGCGCCAG R: GATGCGGCTCCCATAGTCT	60	107
Superoxide Dismutase 3		F: GCAGGTCCGTGGTGATTCAT R: TTCCGATGACACAGCAAGCT	60	99
Caspase 3		F: TCACACAGGGACTGGATGAA R: AGTGACAGCTCTCCCCAGAA	60	104
Apoptosis Inducing Factor		F: ATCGTGGGTGGAGGATTTG R: GCCCCTACGTTGTGATGGA	60	104

3.3.3 Lipid Hydroperoxide Assay

Lipid peroxidation of liver tissue from white sturgeon at termination of the exposure experiments was analyzed using a Lipid Hydroperoxide Assay Kit following the manufacturer's protocol (Cat # 705002, Cayman Chemical Company, Ann Arbor, MI, USA). This kit directly measures lipid hydroperoxides (LHP), rather than malondialdehyde (MDA) degradation products. Concentration of hydroperoxides (nmol/g) were quantified in 100mg (wet mass) of liver tissue by use of a standard curve of LHP and normalizing data by the wet mass of tissue in each sample. Absorbance was measured at 500 nm with a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

3.3.4 Statistics

Statistical evaluation of the data was conducted using IBM SPSS Statistics V20 (IBM Corp., Armonk, NY). All data were tested for normality (Shapiro Wilk test) and homogeneity of variance (Levene's test). One-way analysis of variance (ANOVA) followed by a Tukey's post

hoc test was used to determine significant differences in gene expression among treatment groups. Data for expression of CAT, and LHP did not meet assumptions for parametric statistical tests and therefore were tested for significance using a Kruskal-Wallis (KW) one-way ANOVA followed by a Mann Whitney U (MU) post-hoc test. Statistical significance was accepted when $p < 0.05$. The sample size for LHP and qPCR was n=7. For previously reported details on statistics done on Se concentrations in muscle and liver tissue see Zee et al. (2015).

3.4 Results

3.4.1 Gene Expression

After 10 days of exposure, expression of GPx in liver samples was significantly increased by three-fold in the low dose group compared with the control ($p = 0.002$) (Fig. 3.1) but was not significantly increased in the medium-dose or high-dose groups. Expression of SOD and GST, while not significantly different among dose groups, followed similar trends with greatest expression in the low dose group. There was a trend towards higher expression of CAT in the low and medium dose groups, but effects were not statistically significant because of high variability within treatment groups. Expression of Cas3 and AIF were not significantly different among dose groups, nor were there any trends of increased or decreased expression of these genes (Fig. C3.S1).

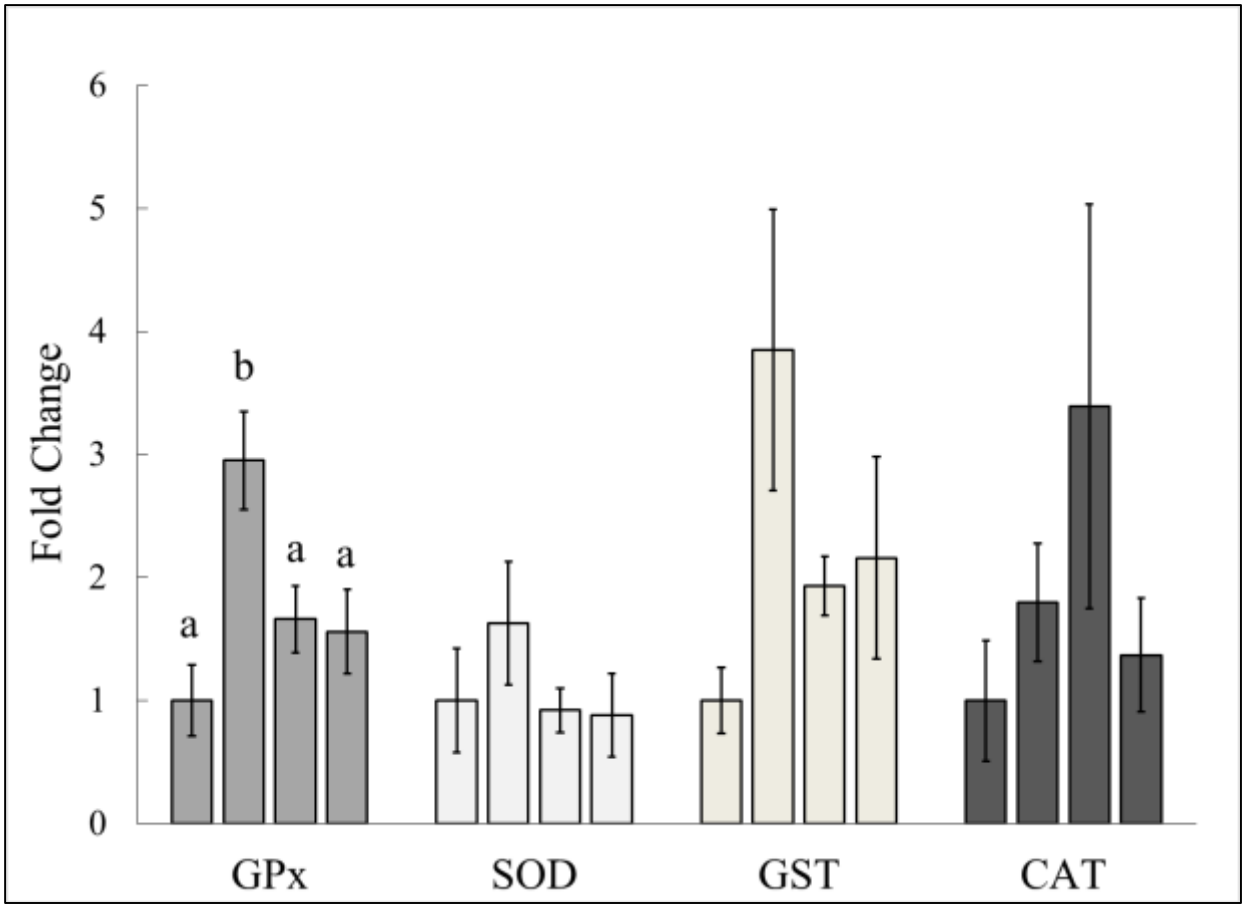


Figure 3.1. Expression of genes responsive to oxidative stress in livers from white sturgeon exposed to dietary SeMet. Bars represent mean ± SD of transcript abundance for each dose group (left to right: control, low, medium, high, n = 7). Different letters indicate significant differences among dose groups.

3.4.2 Lipid Hydroperoxides

LHP concentrations in livers from fish sampled at day 72 and 65 were highly variable within dose groups and there were no statistically significant differences among dose groups (KW, $p = 0.725$) (Fig. 3.2 & Table C3.S1). There was no relationship between Se concentration and LHP concentration in liver tissue at termination of exposure (Fig. 3.3).

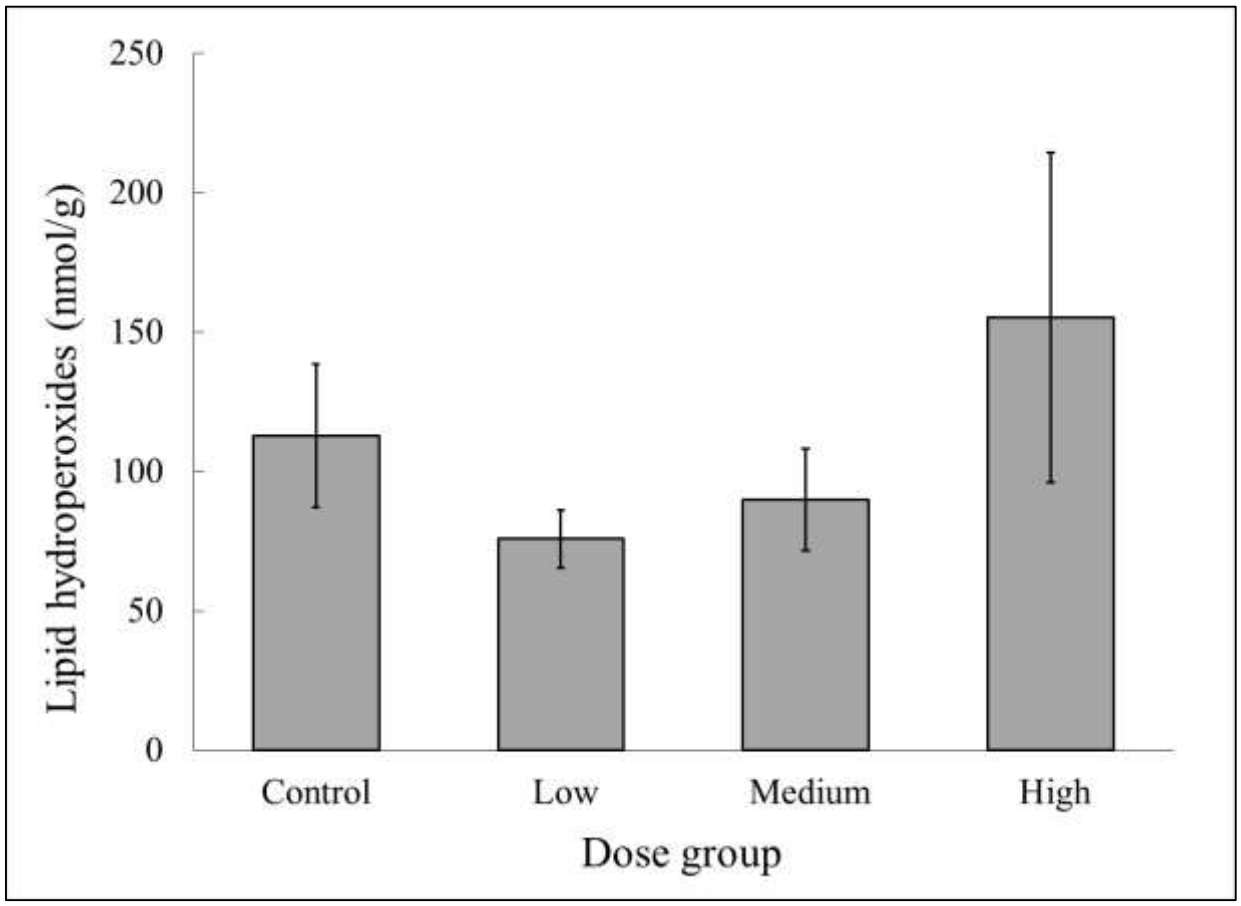


Figure 3.2. Concentration of lipid hydroperoxide in liver tissue. Error bars represent SEM.

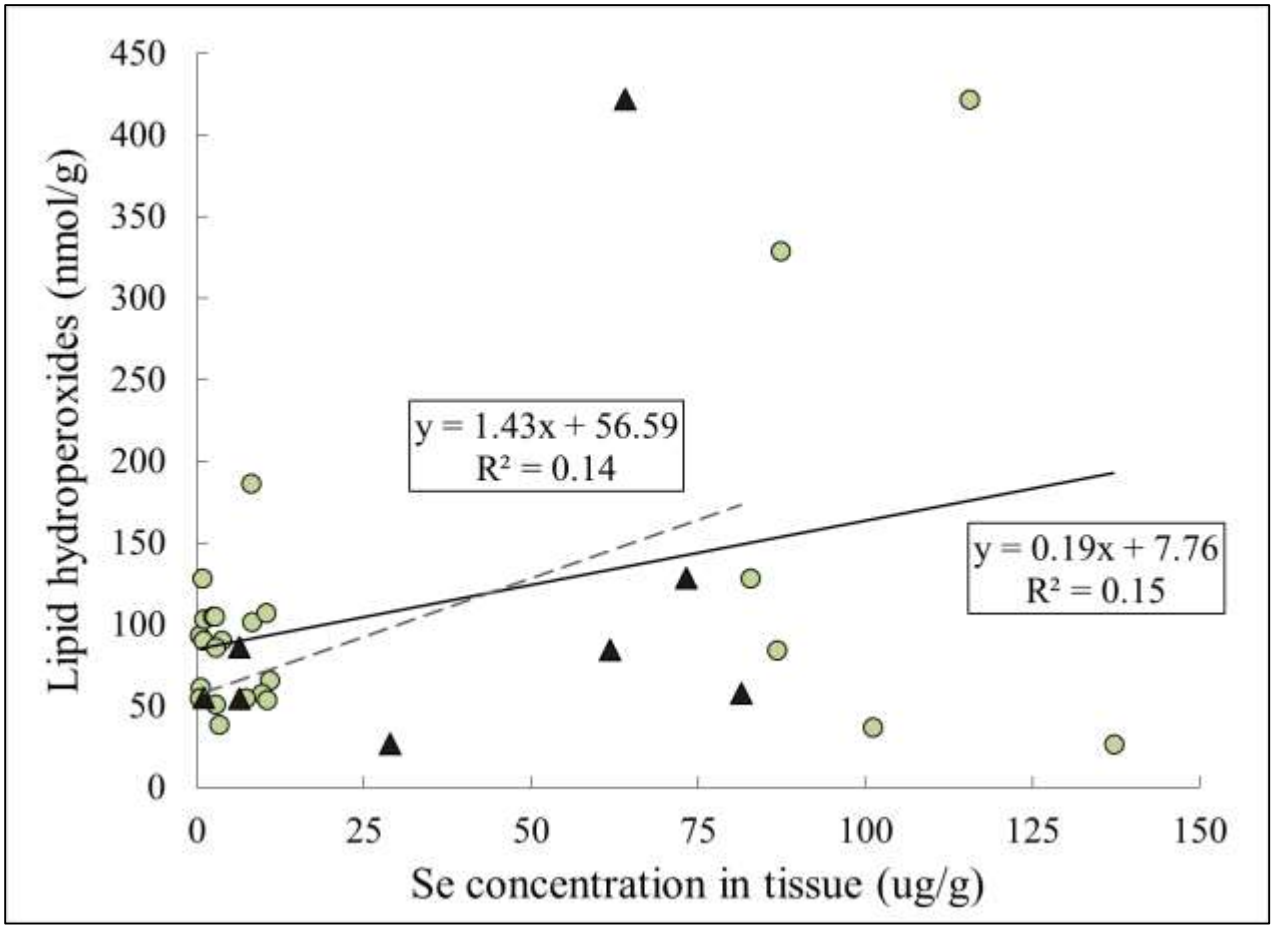


Figure 3.3. Lipid hydroperoxide concentration in liver vs selenium concentration in liver (circle; solid trend line) or muscle (triangle; dashed trend line) tissue after 72 days exposure to dietary selenomethionine. Each point represents a single fish.

3.5 Discussion

Based on a wealth of information from previous studies it was hypothesized that oxidative stress would be a main driver for the toxicosis observed in juvenile white sturgeon exposed to increasing concentrations of dietary SeMet (Zee et al., 2015; Tashjian et al, 2006; De Riu et al., 2014; Linville, 2006). Contrary to this hypothesis overt signs of oxidative stress were not evident in livers, despite the many adverse health effects observed in a companion study (Zee et al., 2015). There was a high degree of variation in the amount of lipid peroxidation in liver within dose groups, which explains the lack of statistically significant differences between dose groups. While there was a trend towards greater expression of GST and CAT in the low and medium dose groups respectively, only GPx had significantly increased expression. Fish given the highest dose of SeMet were already showing signs of severe edema after 10 days of exposure (Zee et al. 2015) which is an indication that normal bodily systems were overwhelmed. An overwhelmed antioxidant system could explain the limited antioxidant gene response in high dose fish while a parallel increase in LHPs was not detected due to a high variability among samples, although there might have been a slight trend towards greater LHP concentration in high dose fish.

A dose dependent relationship between LHP concentration and dietary SeMet had been expected, with ROS hypothesized to be the cause of severe adverse health effects observed during the same study. These effects included mortality, severe edema, decreased hepatic lipid stores and increased hepatic melanomacrophage aggregates (MMAs) (Zee et al, 2015). In a similar study, De Riu et al. (2014) also hypothesized that histopathological changes in livers from white sturgeon given excess dietary SeMet were due to oxidative stress. Other studies conducted with aquatic birds exposed to SeMet found increases in concentrations of a lipid oxidation end product, malondialdehyde (MDA), measured as thiobarbituric acid reactive substances (TBARS) (Hoffman, 2002), as did a study using rainbow trout hepatocyte cells exposed to SeMet (Misra et al., 2012). Contrary to our hypothesis and results from these previous studies, no increase in concentration of LHP was found in white sturgeon subchronically exposed to dietary SeMet.

Studies exposing Siberian sturgeon to excess dietary SeCys also found no differences in concentrations of MDA in liver (Pacini et al., 2013; Elia et al., 2014). These studies found high

variability in MDA levels within dose groups (Control MDA levels: Elia et al., 84.44 ± 24.16; Pacini et al., 80.48 ± 15.62), which is in agreement with the large variability in concentrations of LHP within dose groups of the present study (Control LHP level: Zee et al., 112.74 ± 67.99). This high variability within dose groups could be due to inherent heterogeneity within liver tissue (which is unlikely as liver is a very homogenous tissue), or the instability of these end products, which makes measurement difficult. There is a possibility that this high variability obscured any relationships between average Se concentration and average amount of LHP. However given the general lack of a trend across SeMet treatment groups, with exception of the high dose group that showed a minor increasing trend, it is unlikely that an increase in lipid peroxidation in liver contributed to the toxic effects observed in these fish (Zee et al., 2015).

With the exception of greater expression of GPx, no statistically significant changes in expression of antioxidant genes were identified. Although there was a trend towards greater expression of GST and CAT in the low and medium dose groups respectively, these effects were not statistically significant. Greater GPx enzyme activity has been reported in fish given diets with elevated concentrations of Se. Elia et al (2014) and Pacini et al (2013) found an increase in GPx activity in livers of Siberian sturgeon given 5 and 20 µg SeCys/g feed after 90 and 60 days exposure respectively. Miller (2006) observed that GPx activity in liver tissue increased in brook trout (*Salvelinus fontinalis*) but decreased in rainbow trout with increasing Se concentrations in muscle. Misra et al. (2012) found a significant increase in GPx activity in rainbow trout hetapocytes treated with 1000uM SeMet. Increasing Se concentrations in diet or tissue commonly led to an increase in GPx activity in blood plasma and/or liver tissue of mallards and other aquatic bird species after exposure to SeMet (Hoffman, 2002). Therefore, the increased expression of GPx in livers from white sturgeon exposed to low dose Se is consistent with other studies and indicates a response to oxidative stress. The lack of upregulation of GPx expression in livers from fish given the medium and high dose of SeMet might indicate that the antioxidant systems had been overwhelmed, although if this was the case then a corresponding increase in LHP would have then been expected. It is possible that the increase in expression of CAT, which also acts on hydrogen peroxides, may have compensated for the lack of greater expression of GPx in fish given the medium dose as it has been observed that CAT is more significant than GPx in combating severe oxidative stress (Mates, 2000).

An alternative explanation for the lack of greater expression of genes important for the response to oxidative stress could be that peak responses to SeMet occurred prior to day 10, thus significant changes were not observed. It has been proposed that fish respond to a toxic assault at the transcription level in less than 24 hrs and changes are most pronounced after the first 2 days of exposure (Ankley & Villeneuve, 2015). However the lack of greater concentrations of LHP in the fish given the high dose of SeMet, despite severe edema by day 10 (Zee et al., 2015), coupled with the lack of transcriptional response renders it an unlikely scenario. It is more likely that oxidative stress in not a main driver of SeMet toxicosis in white sturgeon.

There are great intra- and interspecies differences in the response to oxidative stress in fishes exposed to SeMet. In the present study, expression of CAT was only marginally increased in fish given the medium dose. The lack of statistical significance is most likely due to the large variability among samples within this dose. Studies of Siberian sturgeon (*Acipenser baerii*) reported a dose dependent decrease in CAT activity in livers of fish exposed to 5 and 20 µg/g of dietary SeCys at 30 and 60 days of exposure (Pacini et al., 2013) but significantly greater activity at 90 days of exposure (Elia et al., 2014). Misra et al. (2012) found a significant increase in CAT activity in rainbow trout hetapocytes treated with 1000 µM of SeMet for 24 hours. For SOD, we observed no significant induction in livers of white sturgeon after 10 days exposure to SeMet. Hepatic SOD activity in Siberian sturgeon either did not change over 90 days of exposure (Elia et al., 2014) or, in fish exposed to $\geq$ 1.25 µg/g, was significantly greater after 30 days before returning to control levels by 60 days (Pacini et al., 2013). Pacini et al. (2013) found that SOD activity was positively correlated with Se concentration. Misra et al. (2012) observed a significant increase in SOD activity in rainbow trout hetapocytes treated with 1000uM for 24 hours. For GST, gene expression increased slightly but non-significantly in liver tissue of exposed white sturgeon, with the greatest increase observed in the low dose group. Again, lack of significance may be due to the large variability between samples within each dose group. GST activity levels also did not change in Siberian sturgeon liver tissue (Pacini et al., 2013) after dietary SeCys exposure. Taken together, the varying results of this and other studies point to a complicated array of factors involved in the response to oxidative stress and its measurement. Since multiple, interrelated pathways are involved in combating oxidative stress and the contribution of each pathway can vary with tissue type, species, age, gender, season and/or health, nutrition, reproductive and developmental status, cross study comparisons are difficult

(Miller, 2006; Van der Oost et al., 2003). While oxidative stress in response to SeMet exposure has been shown in other species of fish and aquatic birds, it has not previously been tested for in white sturgeon. Perhaps the unique physiology of white sturgeon makes oxidative stress a less relevant mechanism of toxicity than in other species.

Overall it does not appear that oxidative stress in hepatic tissue is a main driver of SeMet mediated toxicity in white sturgeon, despite being detected in a variety of other species. Although significant adverse effects, such as severe edema, morbidity and mortality, were observed throughout the exposure (Zee et al., 2015) only GPx expression was significantly upregulated and no increase in LHP was detected. Furthermore, histological analysis of livers revealed no differences among treatment groups in the categories of apoptosis, necrosis and general cell health (Zee et al., 2015). This absence of tissue damage supports the notion that oxidative stress is not the main driver of Se toxicosis in these fish. Since oxidative stress in liver tissue does not appear to be the main mechanism of SeMet toxicity additional studies are required to identify the mechanism of toxic action of SeMet to white sturgeon.

CHAPTER 4: GENERAL DISCUSSION

4.1 Summary

The purpose of the present study was to characterize adverse health effects in white sturgeon exposed to dietary SeMet, and to link physiological effects to oxidative stress – the hypothesized mechanism of toxicity. Earlier studies have pointed to oxidative stress as a critical mechanism of action (MOA) for dietary Se in various species, but this had not yet been confirmed in sturgeon. Furthermore, the present study aimed to characterize the sensitivity of geographically distinct populations by comparing effects observed in juvenile white sturgeon originating from brood stock of a landlocked, wild population in the transboundary reach of the Columbia River to juveniles from farmed brood stock (Sacramento River basin origins) used in previous studies (Tashjian et al., 2006; Linville, 2006; De Riu et al., 2014).

Over the past 100 years sturgeon populations have been severely depleted due to overharvesting, poor recruitment, habitat alteration and pollution. White sturgeon populations in the Columbia River are considered endangered by most conservation bodies and Se, especially in the organic form of SeMet, has become a contaminant of concern as it is known to bioaccumulate in preferred prey items of white sturgeon. It is important to study white sturgeon directly as Acipenseriformes are quite different physiologically from modern teleosts, having branched off hundreds of millions of years ago. Therefore, common fish models in ecotoxicology, such as rainbow trout, zebrafish and fathead minnow, are likely not appropriate for predicting the vulnerability of ancient fishes to environmental contaminants. Discovering the MOA for SeMet is of interest to the academic community, while protection of endangered white sturgeon is of interest to conservationists, and governments and industries operating in critical habitats.

White sturgeon are benthivores and their food preferences at most life stages are known to be able to bioaccumulate high concentrations of SeMet. This puts them at risk of Se poisoning. Fish in the present study accumulated Se at rates similar to previous studies (Table C2.S2) with a trophic transfer factor (TTF) of around 1. A TTF of 1 indicates they are not likely to biomagnify

Se but will mirror the Se concentrations found in their diets. This suggests that predictions of accumulation of Se in sturgeon can be based on dietary concentrations rather than extensive tissue samples in endangered populations. For this reason it is important to understand and monitor how Se is moving through lower levels of the food web.

Early in the exposure visible signs of edema began to occur. While edema is a known effect of Se toxicosis it was unexpected, as similar white sturgeon studies had not reported any edema. It is unlikely that the edema is an artifact of study design as edema is a known symptom of Se toxicity in other fish species. The presence of edema and popeye in the low dose group in the present study is of concern because this places the genetically distinct transboundary reach white sturgeon among the most sensitive and vulnerable fish species to dietary SeMet exposures. The greater sensitivity of this and potentially other populations in the Columbia and Fraser rivers, is important information for the accurate assessment of risk as human development moves forward in the Columbia and Fraser watersheds.

While edema was a significant finding in the present laboratory study, what its impact on wild populations would be is unknown. Edema in wild white sturgeon has not been reported. In the wild, fish freely move into and out of contaminated areas varying their exposure, however populations restricted by dams may not have this option depending on the extent of contamination. In the present study fish in all high dose tanks exhibited food avoidance by day 21. This suggests that white sturgeon are able to detect toxic levels of SeMet in their feed via chemoreception, which makes sense since the high dose feed had an obvious odour to researchers dispensing it. Perhaps low and medium dose fish could also detect toxic levels of Se but the trade-off between toxicity and starvation was not yet great enough to cause food avoidance.

A significant reduction of hepatic lipid stores was observed in high dose fish and measured as a decrease in average cell surface area. Food avoidance in the high dose group made it difficult to attribute the reduction of lipid stores to Se toxicosis, self-enforced starvation or a combination of the two. However, since Se accumulation continued throughout the present study, regardless of food avoidance (Fig. 2.2), observed effects were most likely due to selenosis. The reduction in lipid stores suggests that energy was needed to fight toxicity and to repair consequent cellular damage. The significantly lesser average HSI in the high dose group at day

72 compared to other dose groups at day 72, and to the high dose subsample at day 10, was likely a reflection of the decreased hepatic lipid stores.

Cessation of growth occurred in the high dose group, which resulted in significantly smaller size (length and weight) compared to all other dose groups, and was likely due to energy resources being allocated to detoxifying the large amounts of Se ingested as well as repairing damage caused by toxicity. Food avoidance would have exacerbated the energy depletion by reducing energy intake. Besides growth rate, reduced energy reserves may affect wild sturgeon's ability to escape predators, find food, migrate, and reproduce. Since the concentration at which growth suppression occurred (high dose) is not yet environmentally relevant the ecological relevance of this end point remains uncertain.

A number of histopathological alterations were expected to occur in white sturgeon due to the high rate of mortality and prevalence of edema observed in the same fish, as well as based on earlier reports of SeMet toxicity in white sturgeon (Table C2.S4) and other fish species (Lemly, 2002a, Sorensen, 1986). However, no significant differences in apoptosis, necrosis, vacuolar degeneration, biliary stasis or general cell health were detected among dose groups as they had been in other white sturgeon studies (Table C2.S4). A trend towards a dose dependent increase in frequency and size (surface area) of MMAs was observed however, with a significant increase in the high dose group compared to the control. An increase in size and frequency of MMAs suggested that chronic liver damage was occurring. The lack of dose dependent damage in the categories of vacuolar degeneration, apoptosis, necrosis, pyknosis, and cell wall degradation was surprising considering the other morphological changes and high rate of mortality. If oxidative stress was a MOA occurring in the liver, more histopathological lesions would have been expected, unless damages were being repaired expediently. One reason for the inconsistencies in histopathological findings between studies may be that, to my knowledge, there are no atlases of sturgeon liver diseases by which to vet results. Overall greater histopathological changes were expected due to the adverse health effects observed during the exposure and the hypothesis of oxidative stress in the liver being a main MOA.

Definitive signs of oxidative stress have been observed in the liver of other species exposed to SeMet (Pacini et al., 2013; Elia et al., 2014; Palace et al., 2004; Misra et al., 2012; Hoffman, 2002) and therefore oxidative stress was expected to be a key MOA in the present study. Liver, as a detoxifying organ known to accumulate comparatively high concentrations of

Se, was hypothesized to sustain high levels of oxidative damage, or to produce high levels of ROS combative antioxidant enzymes. Therefore liver tissue was tested for signs of lipid peroxidation, and changes in expression of genes involved in apoptotic signaling; however, only limited signs of oxidative damage were observed. An assay for lipid hydroperoxides, end products of lipid peroxidation, found no significant differences or trends between dose groups. Similarly, no significant changes in the expression of AIF and Cas3 were observed, which suggested that the apoptotic pathway had not been affected by SeMet accumulation. If extensive oxidative damage had occurred LHP concentrations would have increased in a dose dependent manner as would have expression of genes involved in caspase-independent (AIF) and -dependent (Cas3) apoptotic signaling. When cells are severely damaged they self-destruct in a process called apoptosis. Since oxidative damage was not observed either oxidative stress was not occurring or the antioxidant response system was able to control the damage. Therefore tissues were tested for changes in gene expression of antioxidant enzymes. Of the antioxidant genes tested, only GPx in the low dose group was significantly induced. SOD and GST followed similar trends with greatest expression in the low dose group but this effect was not significant. CAT was not significantly induced but did have greater expression in the low and medium dose groups. The induction of GPx indicated that some level of oxidative stress did occur, however the lack of change in the expression of SOD, CAT and GST suggested that this stress is likely minimal and could be neutralized with only small physiological changes.

The results of the lipid hydroperoxide assay and expression of all six genes were highly variable within each dose group. This variability could have obscured small relationships between Se concentration and the tested endpoints. Alternatively, multiple, interrelated antioxidant pathways may have worked together to combat oxidative stress without obvious large inductions in the gene expression endpoints tested. Since only six genes were tested changes to other pathways or individual genes may have been missed. However considering the lack of histopathological damage, limited presence of LHP and modest changes in expression of genes encoding important antioxidant enzymes it seems most likely that oxidative stress was not a main driver of toxicity. Contrary to our hypothesis, overt signs of oxidative stress were not evident in white sturgeon liver, despite the many adverse health effects observed.

4.2 Conclusions and Future work

The present study showed that white sturgeon from the transboundary region of the Columbia River are susceptible to Se toxicity when faced with elevated concentrations of SeMet in the diet. Adverse health effects included mortality/morbidity, edema, decreased growth, and food avoidance. Surprisingly, the only significant histopathological changes in the liver were a dose dependent increase in melanomacrophage aggregates and a decrease in lipid stores. Although oxidative stress was hypothesized by other studies to be a main mechanism of Se toxicity, the work presented here demonstrated a lack of a clear antioxidant response or oxidative damage. Only GPx gene expression was significantly upregulated in response to SeMet accumulation and LHP concentrations were not different among dose groups. If oxidative stress had been a main driver of toxicity then we would have expected either an increase in expression of antioxidant enzymes or, an increase in LHP concentrations and expression of caspase-dependent and -independent apoptotic genes. Since neither of these responses occurred it can be surmised that SeMet accumulation did not greatly increase oxidative stress in juvenile white sturgeon liver. Further work needs to be conducted in order to elucidate the specific mechanism(s) driving Se toxicity in this species, especially with respect to the severe edema and mortality observed in the present study.

One recently developed approach that may be helpful in discovering MOAs not previously considered is the use of novel sequence-by-synthesis technology such as whole transcriptome sequencing (RNAseq). Comparing the entire transcriptome of treated and control fish would allow for unbiased and thorough investigation of effects of SeMet on the expression of all genes in an organism. It could also help narrow down the search by highlighting or eliminating pathways of concern. Since such severe adverse health effects were observed it is likely that there were also histopathological damages in organs other than the liver. Looking into histopathologies of kidney and gill tissue might help explain problems with osmotic regulation, which could have been involved in formation of the edema reported here. Investigating kidney and intestinal tissue could give clues to the uptake/excretion routes of Se, and therefore, possibly the MOA. A better overall understanding of sturgeon physiology as compared to teleost

physiology would be helpful in understanding how Se and other contaminants act on sturgeon body systems.

Future research should also include a refined dose range by including dietary doses between 20 and 40 µg Se/g as a number of pathologies observed in the present and other studies first occurred in this range. Understanding toxicities in the low dose range (5 µg/g) will help conservationists set dietary limits. There were some significant differences between the present study and previous studies conducted with white sturgeon. Firstly the present study observed severe edema, morbidity and high mortality rates, which had not been observed previously. There were also differences in the presence/absence of various hepatic lesions between studies. The discrepancies between studies could have been due to differences in genetics, or parental exposure. Fish in the present study came from wild brood stocks from the transboundary reach of the Columbia River while previous studies used farmed fish from the Sacramento watershed. For risk assessment purposes it will be important to come to grips with the differences between the Upper Columbia and Sacramento populations as it appears that they have differing sensitivities and should not be used as representative of one another. In general industries and conservationists should be cautious about the amount of Se that enters the food web and work to understand its movements at the low trophic levels. For what happens at these lower trophic levels has the greatest impact on white sturgeon.

In order to recreate and maintain sustainable white sturgeon populations north of the Sacramento river basin some of the past damage caused by overharvesting and habitat alteration needs to be reversed. Attention should be given to the contaminants that enter surface waters, and anticipation of their effects are necessary. Studying the effects of SeMet pollution on white sturgeon is a small piece of the conservation puzzle.

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APPENDICES

Appendix A: Supplementary Materials

Supplementary materials submitted with manuscripts are included here. The figure or table number is presented as Cx.Sy format, where 'Cx' indicates chapter number and 'Sy' indicates figure or table number.

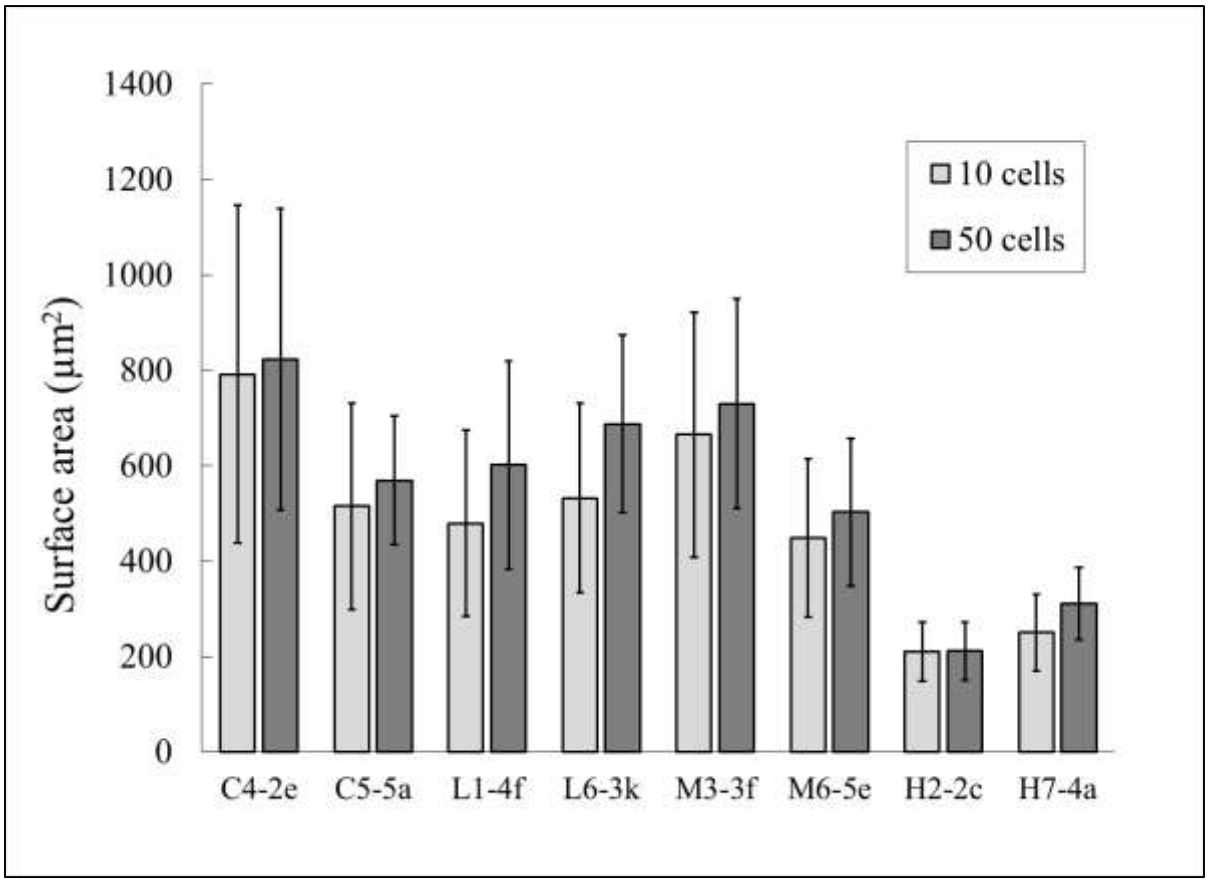


Figure C2.S1. Validation of cell surface area measurements. The same slides were used for both the 10 cell and 50 cell analyses. Slides were coded as follows: dose group, tank number – fish number, slide identification.

Table C2.S1. Validation of cell surface area measurements. Slide (A) 10 cell surface areas were measured. Slide (B) 50 cell surface areas were measured. Slides were coded as follows: dose group, tank number – fish number, slide identification.

Slide	Average Surface Area (μm^2)	SD
C4-2e (A)	792.20	354.76
C4-2e (B)	823.08	316.14
C5-5a (A)	515.11	216.41
C5-5a (B)	569.61	135.37
L1-4f (A)	479.12	195.29
L1-4f (B)	602.21	217.99
L6-3k (A)	532.61	197.86
L6-3k (B)	687.98	185.48
M3-3f (A)	665.82	256.97
M3-3f (B)	730.34	219.51
M6-5e (A)	448.78	165.36
M6-5e (B)	502.75	155.06
H2-2c (A)	210.88	62.28
H2-2c (B)	211.72	60.59
H7-4a (A)	250.32	80.89
H7-4a (B)	311.42	75.81

Tissue Permeating Procedure (Paraffin)

45 min 70% alcohol room temperature, no vacuum

45 min 70% alcohol room temperature, under vacuum

45 min 70% alcohol room temperature, under vacuum

45 min 80% alcohol room temperature, under vacuum

45 min 95% alcohol room temperature, under vacuum

45 min 100% alcohol room temperature, under vacuum

45 min 100% alcohol room temperature, under vacuum

45 min 100% alcohol room temperature, under vacuum

45 min clearant room temperature, under vacuum

45 min clearant room temperature, under vacuum

1 hr paraffin 60°C, under vacuum

1 hr paraffin 60°C, under vacuum

Table C2.S2. Comparison of trophic transfer factors from various studies exposing white sturgeon to dietary SeMet.

	Se Concentration (ug/g)			Trophic Transfer Factor	
	Diet	Muscle	Liver	Muscle	Liver
Linville, 2006 Median	1.0	2.1	2.9	2.0	2.7
	20.1	21.6	19.5	1.1	1.0
	35.6	39.7	40.2	1.1	1.1
	52.5	53.0	69.8	1.0	1.3
Tashjian et al., 2006 Mean ± SEM	0.4	8.2 ± 0.6	15.7 ± 0.7	20.5	39.3
	9.6	17.2 ± 0.7	18.8 ± 1.2	1.8	2.0
	20.5	22.9 ± 1.5	22.0 ± 1.2	1.1	1.1
	41.7	36.8 ± 1.8	37.4 ± 1.7	0.9	0.9
	89.8	52.9 ± 3.2	53.1 ± 8.3	0.6	0.6
	191.1	54.8 ± 2.8	82.7 ± 12.7	0.3	0.4
De Riu et al., 2014 Mean ± SEM	2.2	9.2 ± 0.7	4.2 ± 0.1	4.2	1.9
	19.7	27.0 ± 1.1	28.0 ± 10.4	1.4	1.4
	40.1	41.3 ± 0.6	30.1 ± 1.0	1.0	0.8
	77.7	57.9 ± 1.2	56.3 ± 2.6	0.7	0.7
Zee et al. Mean ± SEM	1.4	1.1 ± 0.1	0.7 ± 0.1	0.80	0.5
	5.6	5.3 ± 0.4	2.9 ± 0.2	1.00	0.5
	22.4	23.5 ± 1.7	9.2 ± 0.5	1.10	0.4
	104.4	64.1 ± 6.4	91.7 ± 12.4	0.60	0.9

Table C2.S3. Fate and fluid volume in all high dose group fish. Fish were labeled with a letter denoting the treatment group followed by the tank number – fish number. Fish were numbered in order sampled. No fish tags were employed while in the tanks. Where fluid volume was not measured notes were included in as written in the lab book. "Little fluid" indicates that the fluid volume present was too small to collect in a vial. Blank spaces indicate no notes were written and it can be assumed there was no fluid present.

Fish	Fate	Fluid volume (mL) or observational notes	Weight (g)	Weight - Fluid (g)
H2-1	10 day subsample	50	120	70
H7-1	10 day subsample	Full of fluid	152.5	152.5
H8-1	10 day subsample	Full of fluid	126	126
H12-1	10 day subsample		217.5	217.5
H15-1	10 day subsample		62.5	62.5
H24-1	10 day subsample		204	204
H25-1	10 day subsample		141.5	141.5
H25-2	Euthanize day 22	Full of fluid	-	264.5
H7-2	Euthanize day 22	40	149	109
H8-2	Mortality day 25	20	174	154
H24-2	Mortality day 25	20	189.5	169.5
H24-3	Euthanize day 28	35	161.5	126.5
H15-2	Mortality day 35	Extremely bloated	144	103.5
H12-2	Euthanize day 43	27	139.5	102.5
H24-4	Euthanize day 43	9	178	154
H8-3	Euthanize day 43		223	200
H12-3	Euthanize day 57	Extremely bloated	219.5	-
H2-2	65 day take down	No fluid	112	112
H2-3	65 day take down	0.85	160.5	159.65
H2-4	65 day take down	Little fluid	100	100
H2-5	65 day take down		112	112
H7-3	65 day take down	No fluid	156	156
H7-4	65 day take down	20	187	167
H7-5	65 day take down	Little fluid	61.5	61.5
H8-4	65 day take down	Little fluid	58.5	58.5
H8-5	65 day take down		174	174
H12-4	65 day take down		58	58
H12-5	65 day take down		101.5	101.5
H15-3	65 day take down		113.5	113.5
H15-4	65 day take down		204	204
H15-5	65 day take down	25.5	198	172.5
H24-5	65 day take down		151.5	151.5
H25-3	65 day take down		101.5	101.5
H25-4	65 day take down		192	192
H25-5	65 day take down		152.5	152.5
H25-6	65 day take down	7	119.5	112.5

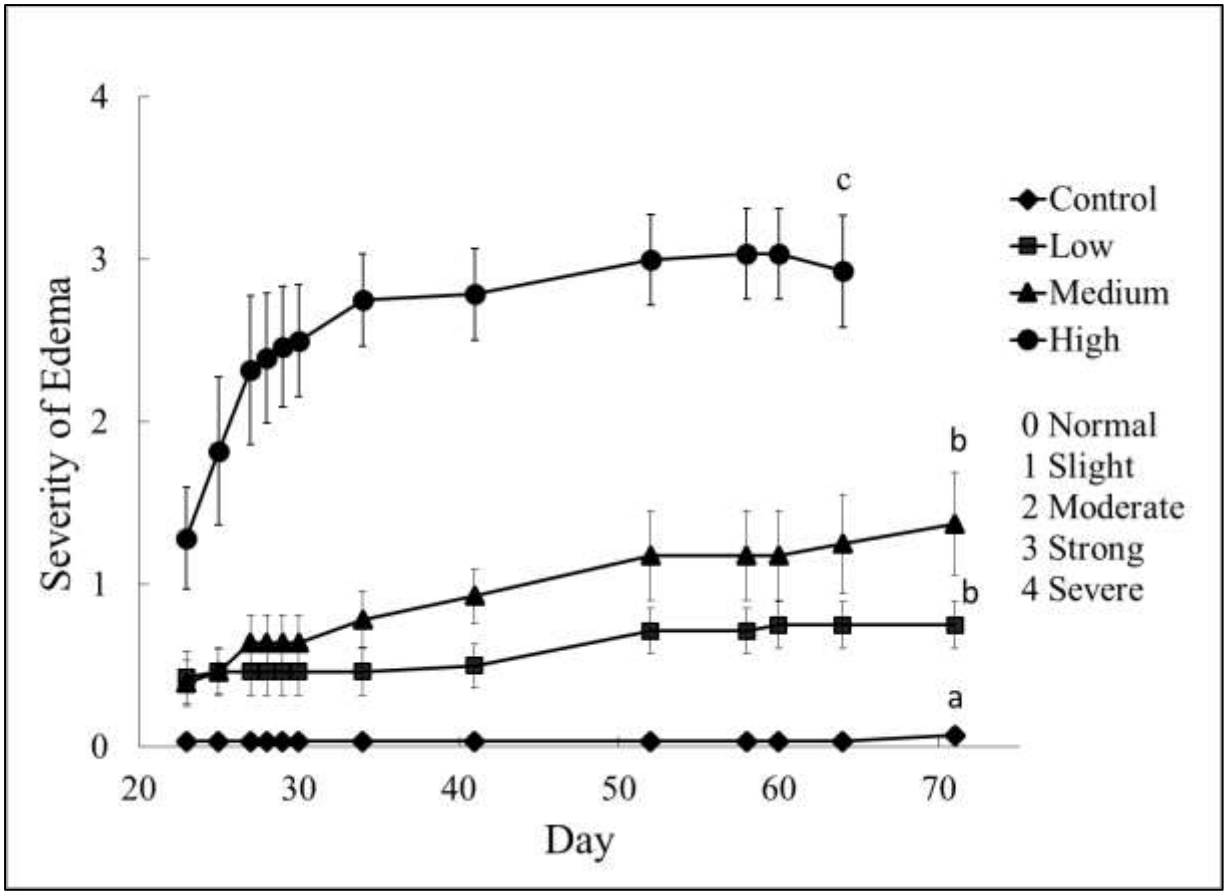
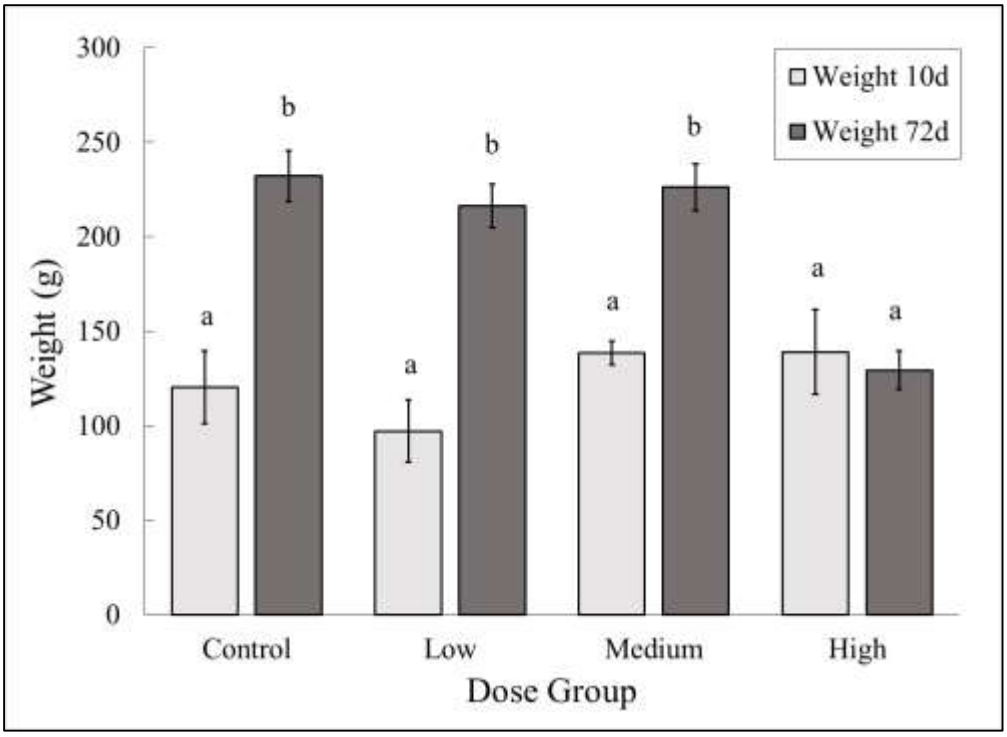


Figure C2.S2. Average edema severity rankings over time. Data points represent averages of edema scores at a given time point. The high dose group was taken down on day 65 due to the high rate of mortalities. Error bars indicate the SEM.



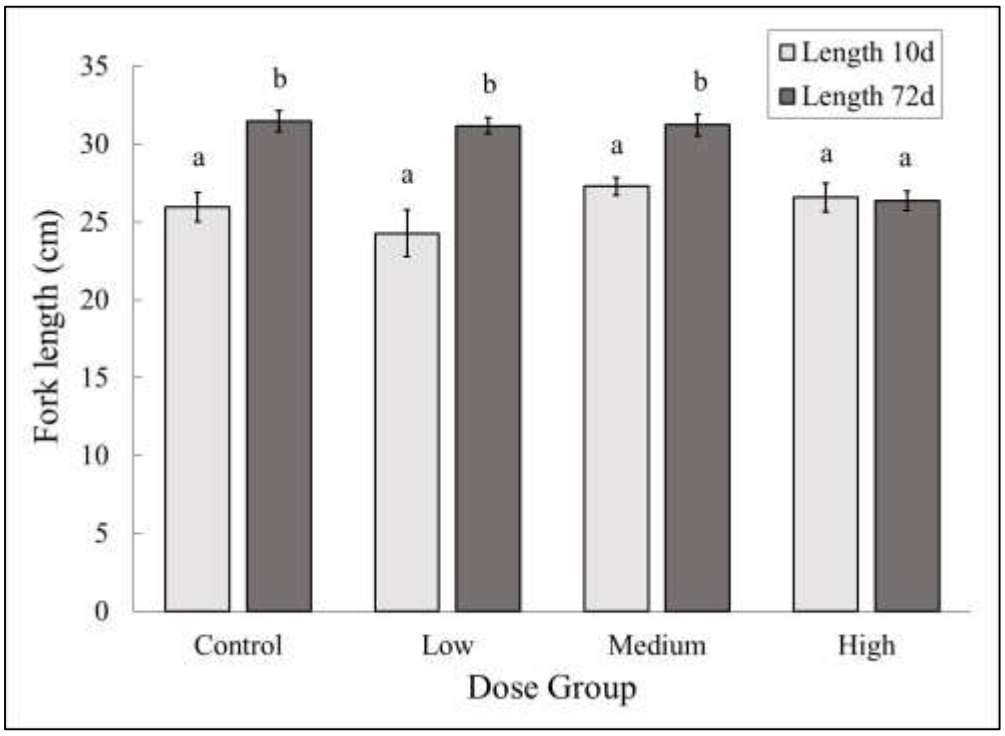


Figure C2.S3. Average weight (top) and length (bottom) of fish at day 10 and day 72. Error bars indicate SEM and letters indicate significant differences.

Table C2.S4. Comparison of histopathological changes in white sturgeon liver observed in various studies.

	Dietary Selenium Concentrations (dm)			
Observed Effect	**Linville, 2006**	**Tashjian et al., 2006**	**De Riu et al., 2014**	**Zee et al.**
Glycogen depletion		≥ 42 ug/g	78 ug/g	
Hepatocellular vacuolar degeneration		≥ 42 ug/g	78 ug/g	
Deformed cell nuclei				
Necrosis or cell swelling	≥ 20 ug/g	≥ 42 ug/g		
Swollen central veins	≥ 20 ug/g			
Changes to bile ducts and canaliculi	≥ 20 ug/g	≥ 42 ug/g		
Biliary stasis	≥ 20 ug/g			
Increased immune cells	36 ug/g			
Increase in Melanomacrophage aggregates	≥ 36 ug/g		78 ug/g	104 ug/g
Decrease in perisinusoidal lipid droplets				104 ug/g

Table C2.S5. Comparison of various dietary SeMet studies with white sturgeon. When there was no mention of a certain parameter in the paper the square was left blank.

Parameters	**Linville, 2006**	**Tashjian et al., 2006**	**De Riu et al., 2014**	**The present study**
Selenium source	Selenized yeast -SeMet predominates	L-selenomethionine	L-selenomethionine	L-selenomethionine
Age	47 weeks post hatch			3 yrs
Weight at Initiation	575 g	30 g	30 g	124 g
Length of Study	23 weeks	8 weeks	8 weeks	10 weeks
Doses	1.0, 20.1, 35.6, 52.5 ug/g	0.4, 9.6, 20.5, 41.7, 89.8, 191.1 ug/g	2.2, 19.7, 40.1, 77.7 ug/g	1.4, 5.6, 22.4, 104.4 ug/g
Lethal Effect	none[1]	none[2]	none	54% at 104.4 ug/g 22% at 22.4 ug/g
Growth Rate	Decreasing trend in high dose	Significant decline in the 41.4-191.1 ug/g treatments (BWI)	Significant decrease in 40.1 -77.7 ug/g treatments (%BWI/d)	Significant decrease in 104.4 ug/g treatment
CF		Significantly lower in 191.1 ug/g treatment		No change
HSI		Significantly lower in 191.1 ug/g treatment		Significantly lower in 104.4 ug/g treatment
Edema	none	none	none	104.4 ug/g by 10 days

[1] One death in the high and low group

[2] 99 ± 0.43% survival

[3] Lethal effects include morality and morbidity (severe edema, loss of equilibrium)

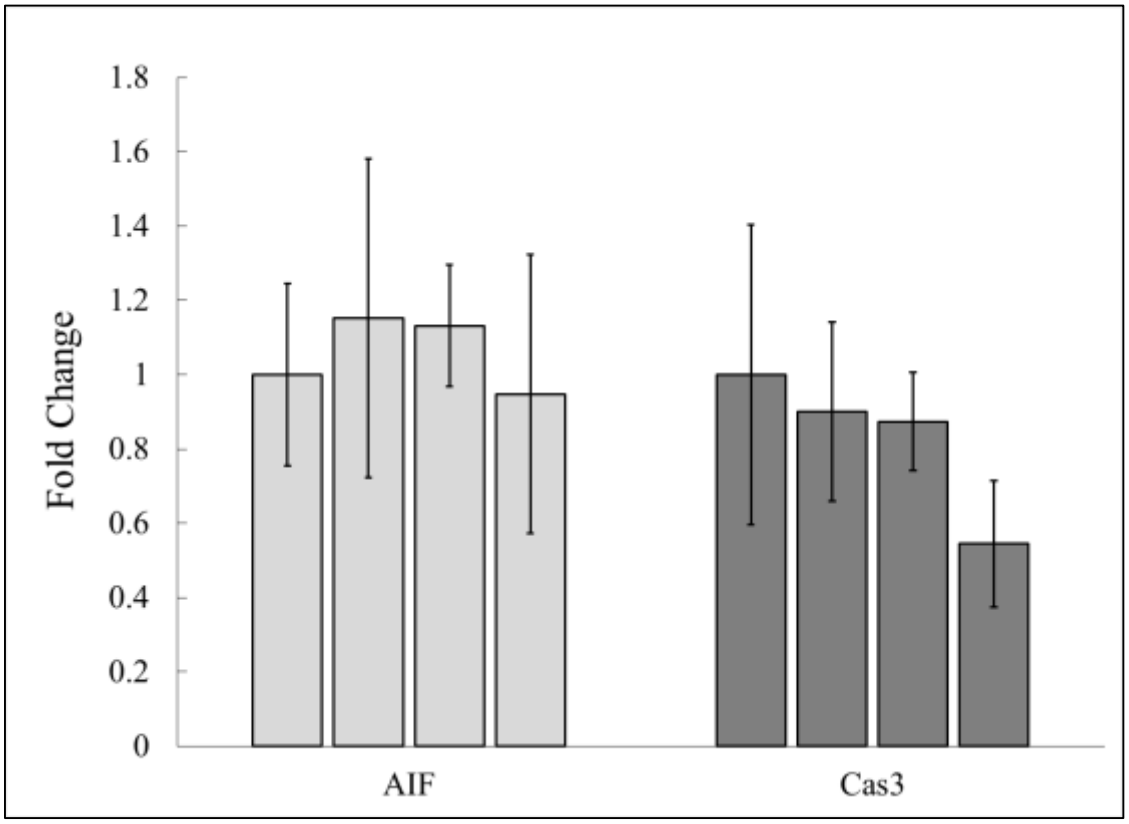


Figure C3.S1. Fold change in gene expression of apoptosis inducing factor (AIF) and caspase 3 (Cas3). Error bars represent SD.

Table C3.S1. Lipid hydroperoxides and selenium concentrations in individual fish. n/a indicates that this data was not available. Fish were labeled as follows: dose, tank – fish.

	LHP	Liver [Se]		LHP	Liver [Se]		LHP	Liver [Se]		LHP	Liver [Se]
Control	nmol/g	ug/g	Low	nmol/g	ug/g	Medium	nmol/g	ug/g	High	nmol/g	ug/g
C4-2	93.92	0.41	L1-4	104.88	2.39	M3-3	66.08	10.84	H2-2	329.04	87.30
C5-5	128.64	0.81	L6-3	90.64	3.63	M9-3	102.08	8.14	H7-4	422.16	115.50
C10-5	103.36	1.05	L11-3	105.12	2.66	M13-5	57.76	9.63	H8-5	37.36	101.03
C17-3	61.76	0.54	L14-4	38.64	3.23	M16-5	186.80	8.11	H12-5	84.56	86.72
C18-5	90.48	0.91	L19-5	51.04	2.77	M21-4	54.16	10.43	H15-5	26.88	137.16
C20-5	256.24	n/a	L23-2	86.32	2.74	M22-3	55.04	7.20	H24-5	128.32	82.74
C26-2	54.80	0.39	L27-2	54.32	n/a	M28-3	107.04	10.36	H25-5	57.92	n/a
Averages	**112.74**	**0.69**		**75.85**	**2.90**		**89.85**	**9.24**		**155.18**	**101.74**
SD	**67.99**	**0.28**		**27.36**	**0.45**		**48.17**	**1.42**		**156.53**	**21.16**
Median	**93.92**			**86.32**			**66.08**			**84.56**	

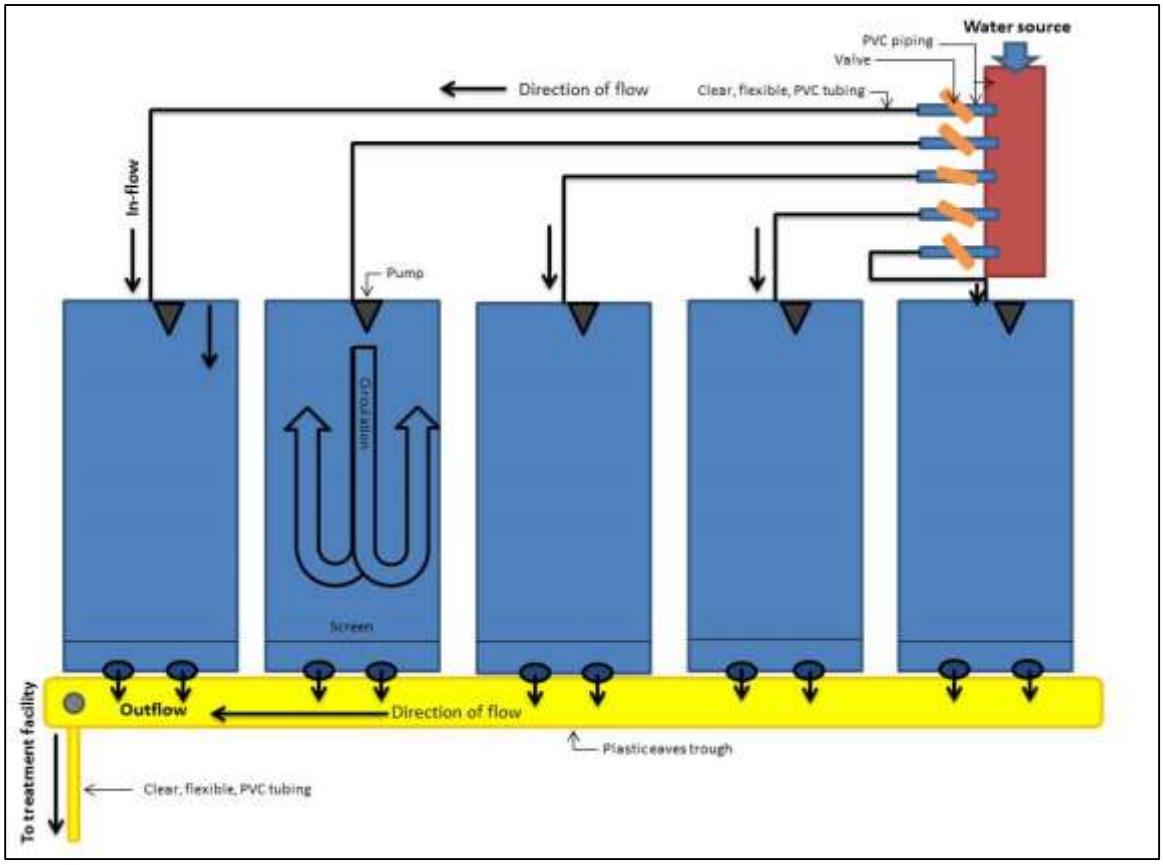
Table C3.S2. Mean fold change, standard deviation and stand error of the mean for various antioxidant genes.

	GPx			**SOD**		
	Mean fold change	SD	SEM	Mean fold change	SD	SEM
Control	1.00	0.29	0.11	1.00	0.42	0.16
Low	2.95	0.40	0.15	1.63	0.50	0.19
Medium	1.66	0.27	0.10	0.92	0.18	0.07
High	1.56	0.34	0.13	0.88	0.34	0.13
	GST			**CAT**		
	Mean fold change	SD	SEM	Mean fold change	SD	SEM
Control	1.00	0.27	0.10	1.00	0.49	0.19
Low	3.85	1.14	0.43	1.80	0.48	0.18
Medium	1.93	0.24	0.09	3.39	1.64	0.62
High	2.16	0.82	0.31	1.37	0.46	0.17
	Cas3			**AIF**		
	Mean fold change	SD	SEM	Mean fold change	SD	SEM
Control	1.00	0.40	0.15	1.00	0.25	0.09
Low	0.90	0.24	0.09	1.15	0.43	0.16
Medium	0.87	0.13	0.05	1.13	0.16	0.06
High	0.54	0.17	0.06	0.95	0.38	0.14

Appendix B: Tank Set – up

The tanks used were the same ones used by Vardy et al. (2015), constructed from high density polyethylene (HDPE) and screens were fabricated from plexi-glass with fiberglass mesh. The tanks were developed specifically to minimize "dead spaces" and create uniform flow. The baffles used by Vardy et al. not used in this experiment. Tanks were arranged in 6 rows of 5 with 1 unused tank in both the first and last row. Each row of tanks was arranged as shown (see following schematic). Water came from a single head tank chilled to 12^0C and delivered to each tank via manifolds. Circulating pumps and air bubblers were installed utilized.

Vardy DW, Doering JA, Santore R, Ryan A, Geisy JP, Hecker M. 2015. Assessment of Columbia River Sediment Toxicity to White Sturgeon: Concentrations of Metals in Sediment, Pore water and Overlying water. Supplementary Material. *Journal of Environmental and Analytical Toxicology* 5:2.



Appendix C: Histological Staining

Permeating with Parffin

Permeated with paraffin in an automated MVPI Modular Vacuum Processor.

45 min 70% alcohol no temperature, no vacuum

45 min 70% alcohol no temperature, vacuum

45 min 70% alcohol no temperature, vacuum

45 min 80% alcohol no temperature, vacuum

45 min 95% alcohol no temperature, vacuum

45 min 100% alcohol no temperature, vacuum

45 min 100% alcohol no temperature, vacuum

45 min 100% alcohol no temperature, vacuum

45 min clearant no temperature, vacuum

45 min clearant no temperature, vacuum

1 hr paraffin 60C, vacuum

1 hr paraffin 60C, vacuum

Haematoxylin and Eosin

Solutions and procedure provided by histology technicians of the Western College of Veterinary Medicine, University of Saskatchewan.

2 min xylene 1

2 min xylene 2

3 min absolute (100%) alcohol 1

2 min absolute (100%) alcohol 2

2 min absolute (100%) alcohol 3

2 min 95% alcohol

2 min 70% alcohol

Dip into running tap water (few seconds)

Dip into distilled water (few seconds)

5 min haematoxylin

Wash off with running tap water (remove excess stain)

Dip into acid alcohol (2 seconds)
10 min running tap water
Dip distilled water (few seconds)
3 min eosin
Dip in running water (remove excess stain)
Dip in each alcohol once 70%, 95%, 100% alcohol 3, 100% alcohol 2
1 min 100% alcohol 1
2 min xylene

Results
Nuclei – blue
Background - pink

1% acid alcohol solution
1000 mL 70% alcohol
10 mL concentrated hydrochloric acid

Best Carmine

Luna LG, ed. 1968. Best's Carmine Method for Glycogen. Manual of Histological Staining Methods of the Armed Forces Institute of Pathology 3rd Edition. American Registry of Pathology. Toronto, ON, Canada: McGraw-Hill Inc.

1) Deparaffinize and hydrate to distilled water
2 min xylene 1
2 min xylene 2
3 min absolute (100%) alcohol 1
3 min absolute (100%) alcohol 2
2 min 95% alcohol
2 min 70% alcohol
Dip into running tap water (few seconds)
Dip into distilled water (few seconds)

2) Staining

Harrison's hematoxylin solution (1 hematoxylin: 1 distilled water) for 15 min.

Wash in running water for 15 min.

Working carmine solution for 30 min.

Differentiating solution for a few seconds

Rinse quickly in 70% alcohol

3) Dehydrate

Dip in each alcohol once 70%, 95%, 100% alcohol 3, 100% alcohol 2

1 min 100% alcohol 1

2 min xylene

Results

Glycogen – pink to red

Nuclei – blue

Carmine stock solution

2 g carmine

1 g potassium carbonate

5 g potassium chloride

60 mL distilled water

Boil in an evaporating dish gently and cautiously for several minutes. When cool (room temperature) add 20 mL of 28% ammonium hydroxide. Store in refrigerator.

Carmine working solution

10 mL carmine stock solution

15 mL ammonium hydroxide, 28%

15 mL methanol

Differentiating solution

20 mL alcohol, 100%

10 mL methanol

25 mL distilled water

Prussian Blue Method for Hemosiderin

Clark G, ed. 1981. Staining Procedures 4th Edition. Published for the Biological Stain Commission. Baltimore, MD, USA: Williams & Wilkins, Waverly Press Inc.

1) Deparaffinize and hydrate to distilled water

2 min xylene 1

2 min xylene 2

2 min absolute (100%) alcohol 1

2 min absolute (100%) alcohol 2

2 min absolute (100%) alcohol 3

2 min 95% alcohol

2 min 70% alcohol

Dip into running tap water (few seconds)

Dip into distilled water (few seconds)

2) Stain

Prussian blue staining solution for 1 hr at room temperature

Counterstain for 2 min in 0.2% safranin O in 1% acetic acid

Wash in 1% acetic acid

3) Dehydrate

Dehydrate with 95% and 100% alcohol

Clear in xylene

Results

Hemosiderin – blue or green

Nuclei – red
Background – pink

Prussian blue solution freshly made (light sensitive)
1 g potassium ferrocyanide
50 mL distilled water
50 mL 2% hydrochloric acid, C.P. (or 5% acetic acid)

Safranin O (0.2%)
0.2 g safranin O in 100 mL 1% acetic acid

Periodic Acid Schiff's

Schiff's reagent was provided by technician Jim Gibbons of the Western College of Veterinary Medicine, University of Saskatchewan.

Method was modified from IHCWorld. PAS (Periodic Acid Schiff) Staining Protocol. http://www.ihcworld.com/_protocols/special_stains/pas.htm

1) Deparaffinize and hydrate to distilled water
2 min xylene 1
2 min xylene 2
2 min absolute (100%) alcohol 1
2 min absolute (100%) alcohol 2
2 min absolute (100%) alcohol 3
2 min 95% alcohol
2 min 70% alcohol
Dip into running tap water (5 second dip)
Dip into distilled water (5 second dip)

2) Staining
5 min 0.5% periodic acid

Rinse in distilled water

30 min Schiff's reagent at room temperature

5 min lukewarm running tap water

1 min Harrison's hematoxylin counterstain (1 hematoxylin:1 distilled water)

5 min wash in tap water

Rinse in distilled water

3) Dehydrate

Dip in each alcohol once 70%, 95%, 100% alcohol 3, 100% alcohol 2

1 min 100% alcohol 1

2 min xylene

Results

Lipofuscin – pink

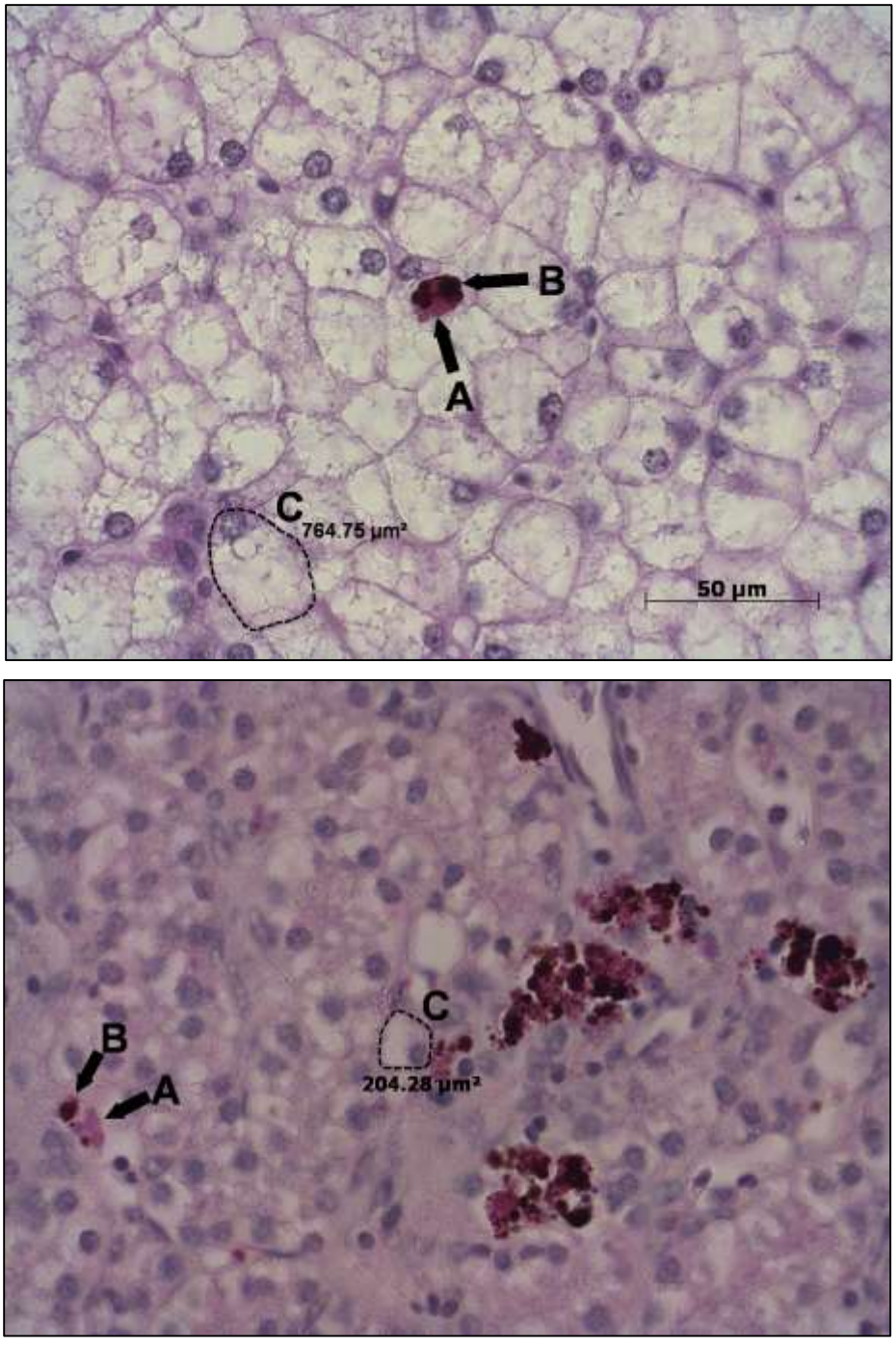
Nuclei – blue

Periodic Acid

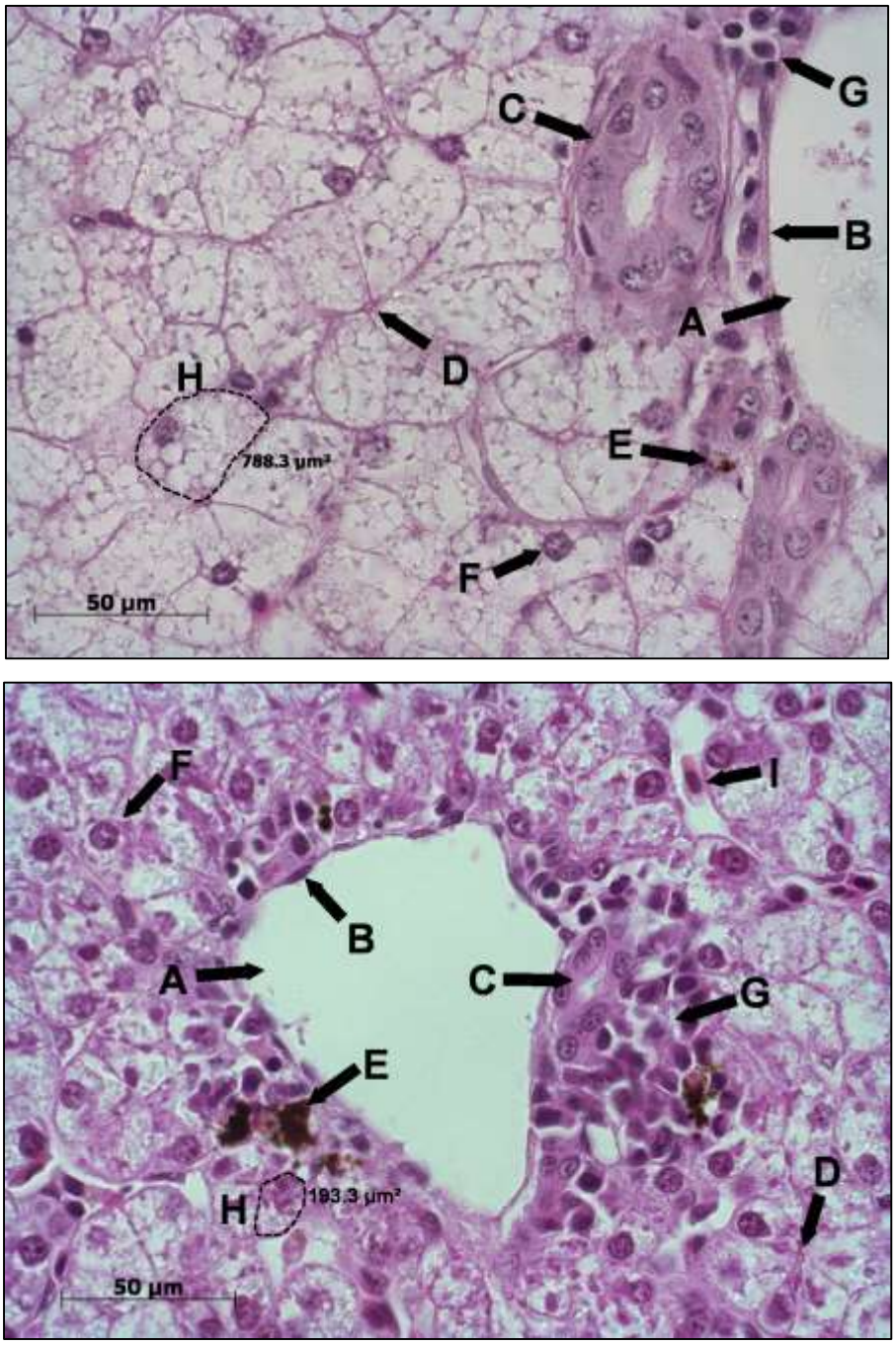
5 g periodic acid

100 mL distilled water

Appendix D: Histological Images



Control (C20-5l) (top) and High dose (H15-5q) (bottom) liver sections stained with PAS at 40x magnification. A indicates lipofuscin and B indicates melanin within a melanomacrophage aggregate. C indicates a single cell with a measured surface area (Zeiss software). Clear spaces within the cells are from lipid stores that dissolved in slide processing.



Control (C4-2b) (top) and High dose (H24-5e) (bottom) liver sections stained with H&E at 40x magnification. A - vein; B - endothelial cell; C - bile duct; D – canaliculus; E – melanomacrophage aggregate; F – parenchymal nucleus; G - hematopoeitic cells; H - a single cell with a measured surface area (Zeiss software); I - nucleated red blood cell.

Appendix E: Lipid Hydroperoxide (LPO) Assay Kit protocol (Cayman Chemical)

Procedure

Tried to keep everything on ice as much as possible when extracting

Liver tissues were snap frozen and stored at -80^{0}C.

Small tubes containing liver tissue were taken from the freezer and put on ice

~100 mg sections were cut off with a scalpel

Tissue was homogenized in HPLC-grade water as per instruction manual (used nanopure water)

100 mg tissue + 2mL water -> homogenize

Preparation:

Aliquot 500 µL sample into a glass tube

Add 500 µL Extract R (from kit) saturated methanol

Add 1 mL cold, deoxygenated chloroform (bubbled with nitrogen to remove oxygen)

Vortex 10 sec

Centrifuge at 1,500 x g for 5 min at 0^{0}C

Collect the bottom chloroform layer and store on ice

There was a thick lipid layer on the top and it was difficult to avoid at least a little water. To avoid this the collected chloroform was centrifuged again and 500 µL chloroform was put in a new tube.

Standard curve (chloroform: methanol blank) and water blank.

Assay:

500 µL chloroform

Add 450 µL chloroform: methanol solvent (2:1) (deoxygenated the chloroform and methanol first)

Add 50 µL fresh chromogen

Vortex

Let stand at room temperature for 5 min

Transfer 300 µL from each tube into the 96 well plate (3 wells per sample)

Read the absorbance at 500 nm using a plate reader

Colour is stable for 2 hours. Beware evaporation

Lipid Hydroperoxide nmol/g Calculation

1) 0.1 g tissue / 2.0 mL nanopure = 0.05 g/mL

2) 0.05 g/mL * 0.5 mL homogenate used = 0.025 g tissue in tube

Add 0.5 mL extract R and 1 mL chloroform to 0.5 mL homogenate

3) 0.025 g/ 2 mL total volume = 0.0125 g tissue/mL

There is now 0.0125 g of "liver tissue" (aka hydroperoxides = HPO) in the 1 mL chloroform layer

Take 500 µL of the chloroform layer

4) 0.0125 g/mL * 0.5 mL = 0.00625 g

There is 0.00625 g "liver" (HPO) in each assay tube

5) X nmol / 0.00625 g = ??? nmol HPO/g liver

Appendix F: Edema Data

Fish were subjectively categorized based on edema severity by a single observer based on the following system: normal (0) – normal appearance with eyes flush to the skull; slight (1) – eyes appeared slightly raised from the skull - a minor variation from the classified normal state; moderate (2) – definite protruding of eyes from skull; strong (3) – greater protruding of eyes from skull and often noticeable bloating of the abdomen; and severe (4) – severe protruding of eyes and severe abdominal bloating. Fish were not tagged, therefore the data is representative of the tank only, not of specific individual fish within the tank. Fish were observed daily during feeding and edema stages were compared to the previous day. Data was recorded when changes were observed. For example when a tank which previously contained four stage 1 fish was found to contain one stage 2 fish and three category 1 fish. Tanks were labeled by letter denoting dose group and number. "Jumper" indicates that the fish was found on the floor having jumped out of the tank. Mesh covers were put in place after this occurred.

Tank	13-Jun	15-Jun	17-Jun	18-Jun	19-Jun	20-Jun	24-Jun	1-Jul	12-Jul	18-Jul	20-Jul	24-Jul	1-Aug
L1	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	2	2	2	2	2
	2	2	2	2	2	2	2	2	2	2	2	2	2
H2	0	0	2	2	2	2	2	2	2	2	2	2	
	1	1	2	2	2	2	2	2	2	2	2	2	
	1	2	2	2	2	2	3	3	3	3	3	3	
	2	2	3	3	3	3	3	3	3	3	3	3	
M3	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	2	2	2	2	2	2	2	2	2	2	3
	2	2	2	2	2	2	2	2	2	2	2	2	3
C4	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
C5	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
L6	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	1	1	1	1	1
	2	2	2	2	2	2	2	2	2	2	2	2	2

Tank	13-Jun	15-Jun	17-Jun	18-Jun	19-Jun	20-Jun	24-Jun	1-Jul	12-Jul	18-Jul	20-Jul	24-Jul	1-Aug
H7	1	1	2	2	2	2	3	3	3	3	3	3	
	1	1	3	3	3	3	3	3	3	3	3	3	
	3	3	3	3	3	3	3	3	3	3	3	3	
	5	5	5	5	5	5	5	5	5	5	5	5	
H8	0	0	2	2	2	2	2	1	1	1	1	1	
	1	2	2	2	2	2	3	2	2	2	2	2	
	1	5	4	4	4	4	4	4	5	5	5	5	
	3	3	5	5	5	5	5	5	5	5	5	5	
M9	0	0	0	0	0	0	0	0	2	2	2	2	2
	0	0	0	0	0	0	0	1	2	2	2	2	2
	1	1	1	1	1	1	2	2	3	3	3	3	3
	2	2	2	2	2	2	3	3	3	3	3	4	4
C10	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
L11	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	1	1	1	1	1
	0	1	1	1	1	1	1	1	2	2	2	2	2
H12	0	0	1	1	1	1	1	1	1	1	1	1	
	0	0	2	2	2	2	2	2	2	2	2	2	
	1	1	2	2	2	2	2	2	4	5	5	5	
	2	2	2	2	2	2	3	4	5	5	5	5	
M13	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
L14	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	1	1	1
	0	0	0	0	0	0	0	0	1	1	2	2	2
	1	1	1	1	1	1	1	1	2	2	Jumper 5	Jumper 5	Jumper 5
H15	0	0	0	0	0	0	0	0	0	0	0	0	
	0	0	0	0	0	0	0	0	0	0	0	0	
	0	0	0	0	0	0	3	3	4	4	4	1	
	0	0	0	2	3	4	4	5	5	5	5	5	
M16	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	1	1	1	1	1	1	1	1	1	1	1
	0	0	1	1	1	1	1	1	1	1	1	1	1
C17	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0

Tank	13-Jun	15-Jun	17-Jun	18-Jun	19-Jun	20-Jun	24-Jun	1-Jul	12-Jul	18-Jul	20-Jul	24-Jul	1-Aug
C18	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
L19	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	1	1	1	1	2	2	2	2	2
C20	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
M21	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	2	2	2	2	2	2	2	2	2	2	2
	1	1	2	2	2	2	2	2	3	3	3	3	3
M22	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	1	1	1	1	1	1
	0	0	0	0	0	0	1	1	1	2	2	2	2
	0	1	1	1	1	1	2	2	2	Jumper 5	Jumper 5	Jumper 5	Jumper 5
L23	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1
H24	2	2	2	2	2	2	2	2	2	2	2	2	
	2	2	2	2	2	2	2	4	5	5	5	5	
	2	5	5	5	5	5	5	5	5	5	5	5	
	2	5	5	5	5	5	5	5	5	5	5	5	
H25	0	0	0	0	0	0	0	0	0	0	0	0	
	0	2	2	2	2	2	2	2	2	2	2	2	
	1	2	2	2	3	3	3	2	2	2	2	2	
	5	5	5	5	5	5	5	5	5	5	5	5	
C26	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	1
L27	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	1	1	1	1	1	1
M28	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	1	1	1	1	1	1
	0	0	0	0	0	0	0	1	2	2	2	2	2
	1	1	1	1	1	1	1	1	2	2	2	2	3