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

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ABSTRACT

Sturgeon are an ancient family of fish comprised of 26 known species, most of which are threatened or endangered. Populations of white sturgeon (WS; *Acipenser transmontanus*), which are endemic to western North America, have been declining since the 1800's due to overharvesting, habitat alteration and increased contaminant loads from industrial and agricultural activities. The longevity, benthic lifestyle and late sexual maturation might make WS particularly susceptible to bioaccumulative toxicants, such as selenium (Se). Although Se is an essential micronutrient, it can be toxic to aquatic organisms at low concentrations and it is prevalent in surface waters due to various sources, including natural weathering of rocks and soil, agricultural runoff, discharge from mining and milling operations, and fossil fuel combustion. One organic form of Se, selenomethionine (SeMet), is a particular environmental concern due to its ability to persist and bioaccumulate through the food chain. Selenomethionine is known to persist in some WS habitats and it has been detected in their tissues and prey at concentrations above dietary and tissue toxicity thresholds. Selenomethionine can adversely affect a range of physiological processes in fish, including the physiological stress response; however, studies assessing the sensitivity of WS to SeMet are limited and the effects of SeMet on their physiological stress response are unknown. While oxidative stress has been hypothesized as one of the primary mechanisms of SeMet toxicity in teleosts, the mechanisms leading to adverse effects in WS are poorly understood. Therefore, the goal of this research was to expand the understanding of WS sensitivity to SeMet by assessing sublethal effects of dietary SeMet exposure, including effects on their ability to mount a stress response. As well as to provide insight into potential pathways of SeMet toxicity in WS, by linking molecular responses to adverse whole organism effects observed in a parallel study. Juvenile WS were fed either a control diet containing 1.4 µg Se/g dry mass (dm), or a spiked diet containing 5.6, 22.4 and 104.4 µg Se/g dm, for 72 d. Effects of SeMet on the cortisol stress response were evaluated by subjecting fish to a 2 min handling stressor on the final exposure day, followed by quantifying concentrations of cortisol, glucose and lactate in blood plasma, and glycogen levels in muscle and liver tissue. Furthermore, abundances of transcripts of genes along the hypothalamus-pituitary-interrenal (HPI) axis were analyzed using qPCR. RNA-seq analysis was conducted on liver samples from control and medium dosed fish, collected on day 10 of the exposure, to assess transcriptional responses and identify physiological processes

that might be adversely impacted by SeMet. Exposure to dietary SeMet for 72 d did not impact the ability of WS to mount a cortisol response to an acute handling stressor; however, this response could only be assessed in the low and medium dose fish due to high mortalities in the high dose group. There was some indication that the medium dose of SeMet altered the ability of WS to utilize muscle glycogen for energy, following exposure to the acute handling stressor. Basal levels of plasma cortisol, glucose, lactate, and liver glycogen were significantly altered in the high dose fish. RNA-seq analysis identified 178 and 147 transcripts that were significantly down- and up-regulated, respectively. Biological pathways associated with the differentially expressed transcripts were analyzed through ClueGO, a Cytoscape plug-in. Selenomethionine altered several physiological processes, but the majority were involved with the regulation of energy, cholesterol/lipid and protein metabolism. Alterations in these pathways appeared to be associated with reduced hepatic energy stores and growth, as well as increased edema and mortality observed in WS by the end of the exposure. Overall, the results of this research indicate that WS are sensitive to dietary SeMet at concentrations ≥ 22.4 μg/g dm based on the biochemical and molecular endpoints assessed in this research. Effects on energy, cholesterol/lipid and protein metabolism likely play an important role in the development of SeMet toxicity in WS and should be investigated in future studies. WS also appear to respond differently to SeMet from other fishes, as seen by the lack of effects on their stress response at lower concentrations, the lower magnitude cortisol response, and the development of edema at low concentrations. These differences highlight that cross-species extrapolations in regard to the toxicity of SeMet in fishes, cannot always be made, and this should be considered in future environmental risk assessments for Se.

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LIST OF ABBREVIATIONS

abcb11b – ATP binding cassette, sub family B member 11

abcd3a – ATP-binding cassette, sub family D member 3

acaa2 – acetyl-coenzyme A acyltransferase 2

acsbg2 - long chain fatty acid ligase

ACTH – adrenocorticotropic hormone

adh5 - alcohol dehydrogenase class 3

aif – apoptosis inducing factor

ANOVA – analysis of variance

apoa1a – apolipoprotein a-1a

atp6v0a1b – ATPase h+transporting lysosomal v0 subunit a lb

atpaf2 - ATP synthase mitochondrial F1 complex assembly factor 2

ATRF – Aquatic Toxicology Research Facility

β-actin – beta actin

CCME – Canadian Council of Ministers of the Environment

cDNA – complementary deoxyribonucleic acid

CPM – counts per million

cpt2 - carnitine palmitoyltransferase

CRH – corticotropin releasing hormone

ctrl – chymotrypsin-like

ctsc - cathepsin C

cyp1a - cytochrome p450 1a

cyp7a1a – cholesterol 7-alpha-monooxygenase

dgat2 - diacylglycerol O-acyltransferase 2

dm – dry mass

dpp1 - dipeptidyl peptidase 1

ela2l - elastase 2 like

ELISA – enzyme linked immunosorbent assay

fabp7b – fatty acid binding protein 7, brain

fabp – fatty acid binding protein

fbp1a - fructose- 1,6-bisphosphatase

FDR – false discovery rate

gc - vitamin D-binding protein

GCR – glucocorticoid receptor (protein)

gcr – glucocorticoid receptor (gene)

GO – gene ontology

g6pca – glucose-6-phosphatase

gst – glutathione-s-transferase

hbaa1 - hemoglobin, alpha adult 1

HPI – hypothalamic-pituitary-interrenal

HSI – hepatic somatic index

HSD11B2 – corticosteroid 11 beta dehydrogenase 2 (protein)

hsd11b2 – corticosteroid 11-beta dehydrogenase 2 (gene)

idh1 - isocitrate dehydrogenase 1

k – Fulton condition factor

KEGG – Kyoto Encyclopedia of Genes and Genomes

lipc - hepatic lipase

LOEC – lowest observed effect concentration

MATC – maximum acceptable toxicant concentration

MC2R – melanocortin 2 receptor (protein)

mc2r – melanocortin 2 receptor (gene)

mRNA – messenger ribonucleic acid

mvp – major vault protein

NOEC – no observed effect concentration

PCR – polymerase chain reaction

pcsk5 - proprotein convertase subtilisin kexin type 5

PEPCK – phosphoenolpyruvate carboxykinase (protein)

pepck – phosphoenolpyruvate carboxykinase (gene)

P450scc – cytochrome P450 side chain cleavage (protein)

p450scc – cytochrome P450 side chain cleavage (gene)

qPCR – quantitative real-time polymerase chain reaction

RNA – ribonucleic acid

RNA-seq – RNA sequencing

ROS – reactive oxygen species

Se – selenium

S.E.M. – standard error of the mean

SeMet – selenomethionine

SD – standard deviation

STAR – steroidogenic acute regulatory protein

s*tar* – steroidogenic acute regulatory protein (gene)

tpp1 – tripeptidyl peptidase 1

try - trypsin-like

USEPA – United States Environmental Protection Agency

WS – white sturgeon

ww – wet weight

PREFACE

This thesis is presented in manuscript-style according to the requirements set by the College of Graduate Studies and Research. Chapter 1 is a general introduction, Chapters 2 and 3 are organized as manuscripts for publication in scientific journals, and Chapter 4 is a general discussion and conclusion. Thus, there is some repetition between the introduction and materials and methods sections in each chapter. Chapter 2 has been published in *Aquatic Toxicology* (2017) Volume 186, Pages 77-86. The tables, figures, supporting information, and references cited in each chapter have been reformatted here to meet requirements for a consistent thesis style. References cited in each chapter are combined and listed in the References section of the thesis.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Sturgeon

Sturgeons are ancient freshwater fish that have existed for over 100 million years (LeBreton et al., 2004). Sturgeons have shark-like bodies covered with five separate rows of bony scutes for protection and an almost completely cartilaginous skeleton (Hochleithner and Gessner, 2001). They are among the largest freshwater fish species and are incredibly long-lived. Sturgeons live exclusively in regions of the Northern Hemisphere, including Northern Europe, Asia and North America where they inhabit many large river and lake systems, as well as coastal marine waters (Hochleithner and Gessner, 2001).

Sturgeons are among the most commercially valuable species of fish, due to their importance in fisheries and aquaculture, as well as the value of their caviar (Jaric and Gessner, 2012). In North America, they are also culturally important to Aboriginal Peoples. Sturgeon were an integral part of Aboriginal culture prior to European colonization as they depended on them for their meat, roe, oil, skin and a glue-like product, isinglass (LeBreton et al., 2004). European settlers quickly discovered the economic value of sturgeon meat and roe, resulting in overfishing and habitat destruction, which almost completely extinguished populations in North America (LeBreton et al., 2004). Sturgeons remain at risk to this date, with global populations declining steadily. These declines are a result of several anthropogenic factors including overharvesting, pollution from agriculture and mining activities, and construction of dams which block migration to spawning grounds and alter water flow (Hochleithner and Gessner, 2001; Little et al., 2012). Ultimately, sturgeon populations are facing habitat fragmentation and degradation.

Most of the 26 known species of sturgeon are classified as endangered or threatened by the International Union for Conservation of Nature (IUCN) (Birstein, 1993; LeBreton et al., 2004; IUCN, 2017). Many populations of sturgeon occurring in Canada (Atlantic, green, lake, shortnose and white sturgeon) are considered threatened, endangered, or a species of special concern, by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC). White sturgeon (WS; *Acipenser transmontanus*) populations in the Kootenay, Nechako, Upper Columbia and Upper Fraser Rivers are officially listed as endangered by the Canadian Government under Schedule 1 of the *Species at Risk Act* (SARA) (COSEWIC, 2012; SARA, 2018).

White sturgeon are semi-anadramous and found in major rivers and estuaries along the Pacific Coast of North America, mainly in the Fraser, Columbia and Sacramento-San Joaquin Rivers, as well as the San Francisco Bay-Delta (Hochleithner and Gessner, 2001). White sturgeon are the largest sturgeon species in North America, growing to over 6 m in length and weighing over 800 kg (Hochleithner and Gessner, 2001). They are also long-lived, reaching upwards of 100 years of age, and slow to reach sexual maturation, with males and females not reaching sexual maturity until the ages of 12 and 16-35 years, respectively (Billard and Lecointre, 2001). White sturgeon are bottom feeders, spending most of their time in close proximity to the sediment searching for prey. They feed primarily on benthic invertebrates, crustaceans, shrimp, mollusks, amphipods and small fish (Billard and Lecointre, 2001).

Poor annual recruitment due to loss of early life stage fish from spawning grounds is of concern in many Canadian WS habitats. The COSEWIC predicts that WS populations in the Upper Columbia and Upper Kootenay Rivers could be extinct within the next 30 years, if successful remediation of natural recruitment is not achieved (UCWSRI, 2002; DFO, 2007; Paragamian and Hansen, 2008). In hopes of saving this unique species from extinction, conservation and recovery planning for WS populations in the Columbia and Kootenay Rivers has become a priority. One of the factors thought to be driving declines in WS populations is contamination of habitats from surrounding mining and industrial activities (current and historical), and therefore, part of the recovery planning involves evaluating the presence and effects of contaminants in their habitats (UCWSRI, 2002; Little et al., 2012).

White sturgeon are more vulnerable to bioaccumulation of contaminants than other species due to their longevity and benthic lifestyle. Therefore, given the need for conservation of this endangered species, further understanding regarding their sensitivity to contaminants is required (Vardy et al., 2011; 2013; Little et al., 2012). One emerging contaminant of concern for WS is selenium (Se), due to its release from surrounding industrial and agricultural activities into WS habitats, such as the Kootenay River and San Francisco (S.F.) Bay-Delta regions (Luoma and Pressser, 2000; Dessouki and Ryan, 2010).

1.2 Selenium

Selenium is an elemental nonmetal naturally found in soil, that can exist as an inorganic (selenite, selenate) or organic (e.g. seleno-amino acids and seleno-proteins) compound (Fan et al., 2002; reviewed in Janz, 2012. Selenium is prevalent in surface waters as a result of various natural and anthropogenic sources, such as natural weathering of Se rich rocks and soil, agricultural runoff, fly ash from coal fired power plants, discharge from mining and milling operations, and fossil fuel combustion (Lemly and Smith 1987; Sappington, 2002). Natural concentrations of Se in aquatic ecosystems are usually low (0.01 - 0.1 μg/L) but they can be as high as 5 - 50 μg/L near some shale deposits (Maher et al., 2010).

Once Se enters aquatic ecosystems it can be taken up by aquatic organisms, bind to particulate matter, or remain free in solution (Lemly and Smith, 1987). Over time Se tends to accumulate in sediment. However, through several different physical, chemical and biological processes, Se can be mobilized and cycled back into food chains, causing long term dietary exposure to aquatic organisms, even years after Se inputs stop (Lemly and Smith, 1987; Lemly, 1993, 1997a). Selenium becomes biologically available via oxidation of sediments, as well as by direct uptake of Se by rooted plants, benthic invertebrates and fish (Lemly and Smith, 1987). Selenite and selenate are the dominant Se species found in surface waters, followed by organoselenides. Inorganic Se species are taken up by primary producers (bacteria, algae, and plants) and are converted to organoselenides, such as selenomethionine (SeMet), and then transferred through the food chain to consumers, including invertebrates and fish (Fan et al., 2002; Maher et al., 2010; Stewart et al., 2010; Janz, 2012). Selenomethionine is a major dietary source of Se for aquatic organisms and it has become a particular environmental concern as it persists and bioaccumulates through the food chain (Lemly and Smith, 1987; Fan et al., 2002). Dietary intake of SeMet, rather than waterborne exposure, is the main source leading to bioaccumulation of Se to toxic levels in fish (Lemly and Smith, 1987).

The Canadian Council of Ministers of the Environment (CCME) has set a water quality guideline for the protection of aquatic life for Se at 1 μg/L in freshwater (CCREM, 1987) (Table 1.1). Certain provinces within Canada have also set their own guidelines including British Columbia (BC), which has set the guideline at 2 μg Se/L. The water quality criteria set by the U.S. Environmental Protection Agency (USEPA) to protect aquatic organisms from chronic exposure

to Se was 5 μg Se/L in freshwater (USEPA, 1999), until recently. The only other countries with water quality guidelines for Se are Australia, New Zealand and South Africa (reviewed in Janz, 2012). North American water quality guidelines for Se have been under review because of the complex biogeochemical cycling of Se in aquatic environments and the ability of low concentrations of Se in the water column to bioconcentrate and bioaccumulate to potentially toxic levels, which renders it difficult to accurately predict toxicological risks from aqueous concentrations. Se concentrations can increase up to several thousand-fold between uptake of Se from water to primary producers, leading to transfer of toxic levels to more sensitive organisms such as fish (Lemly and Smith, 1987; Skorupa, 1998; Stewart et al., 2010; Janz, 2012). Therefore, many agencies are converting to the use of tissue concentration-based and site-specific criteria, considering that Se in the water at concentrations below the guidelines (1 - 5 μg Se/L) can be concentrated to toxic levels in prey of more sensitive organisms, such as fish (Lemly and Smith 1987; Skorupa, 1998; Muscatello et al., 2008; Janz et al., 2010; Stewart et al., 2010). One study established dietary and tissue concentration-based thresholds for Se in fish species in the San Diego Creek, using larval rainbow trout (*Oncorhynchus mykiss*) chronically exposed to SeMet through the diet. They estimated a dietary Se lowest observed effect concentration (LOEC) of 4.6 μg/g and a whole-body burden LOEC of 1.2 μg/g wet weight (ww) (Vidal et al., 2005).

After the research presented in this thesis was conducted, the USEPA (2016) published the finalized freshwater selenium ambient water quality criterion, which provides recommendations for fish tissue and water-based Se concentrations to protect aquatic life, replacing the previous water quality criterion of 5 μg/L (USEPA, 1999) (Table 1.1). The BC Ministry of Environment also approved new ambient water quality guidelines for Se to protect aquatic life, including an interim chronic dietary guideline based on invertebrate tissue and chronic guidelines for fish tissues (whole-body, muscle/muscle plug and egg/ovary), water column and sediment (Beatty and Russo, 2014; BC Ministry of Environment, 2014) (Table 1.1).

Table 1.1. Chronic Aquatic Life Ambient Water Quality Criterion/Guidelines recommended by the USEPA (2016), BC Ministry of Environment (2014) and CCREM (1987)[a].

Environmental Media	USEPA Aquatic Life Ambient Water Quality Criterion (2016)	BC Ministry of Environment Ambient Water Quality Guidelines (WQGs) for Se–Aquatic Life (2014)	CCREM (1987)
Dietary Invertebrate tissue	N/A	4 μg/g dw - interim	N/A
Fish Tissue Whole-body Muscle Egg/ovary	 8.5 μg/g dw 11.3 μg/g dw 15.1 μg/g dw	 4 μg/g dw 4 μg/g dw - interim 11 μg/g dw	N/A
Water Column	Freshwater 1.5 μg/L - lentic systems 3.1 μg/L - lotic systems	Freshwater and marine 1μg/L - alert concentration 2 μg/L - WQG	Freshwater 1 μg/L
Sediment	N/A	2μg/g - alert concentration	N/A

[a] Adapted from USEPA (2016), BC Ministry of Environment (2014) and CCREM (1987).

1.3 Toxicity of selenium in fish

Selenium is an essential micronutrient required by all vertebrates, including fish, for incorporation into selenoproteins (reviewed in Janz, 2012). Selenoprotein families that have been characterized in fish include glutathione peroxidases and thioredoxin reductases, which are involved in redox metabolism and defense against oxidative stress, as well as iodothyronine deodinases, which are important for regulating thyroid hormone metabolism (reviewed in Janz, 2012). Fish require between 0.1 and 0.5 μg Se/g dm in their diet to maintain optimal nutrition and normal physiological function, however, there is a fine line between Se essentiality and toxicity (Hodson and Hilton, 1983; Lemly, 1997a, 1997b; Hamilton, 2004). Selenium can be toxic to aquatic organisms when concentrations exceed 3.0 μg Se/g dm (Lemly, 1997a; Hamilton, 2004). Toxic effects include altered behavior, developmental malformations, impaired reproduction and mortality (Lemly, 1997a, 2002; reviewed in Janz, 2012).

1.3.1 Mechanisms of toxicity

Studies have shown that Se can be bioaccumulated through food chains and cause toxicity in fish when concentrations exceed 3 μg/L in water or 3 μg/g in the diet (Lemly and Smith, 1987; Lemly 1997a). The mechanism(s) leading to deformities in early life stages of fish, have been the main focus of studies. Selenomethionine accumulates in tissues by substituting for the essential amino acid methionine, during protein synthesis. Due to their similar molecular structure, cellular enzymes cannot distinguish between the two (Spallholz and Hoffman, 2002; Janz, 2012). Selenomethionine is primarily accumulated in the liver, where the yolk protein, vitellogenin, is also produced. Increased accumulation of SeMet could lead to increased substitution of methionine in vitellogenin, which is then transferred to oocytes. Developing embryos incorporate the maternally transferred organic Se during reabsorption of the yolk, which could lead to developmental deformities (Fan et al., 2002; Janz 2011). Substitution of SeMet into proteins was previously thought to cause deformities by altering disulfide linkages leading to improper protein folding and function, but this theory is no longer supported as methionine does not play a significant role in forming disulfide bonds (Lemly, 2002; reviewed in Janz, 2012). Recently, oxidative stress has been hypothesized as the primary cause of SeMet toxicity in fish embryos (Palace et al., 2004). Selenomethionine is biotransformed to selenoxides that are capable of producing reactive oxygen species (ROS); therefore, excessive accumulation of SeMet could lead to overproduction of ROS, causing oxidative stress and damage to proteins and tissues (Palace et al., 2004; Janz, 2012; Lavado et al., 2012). Isolated rainbow trout hepatocytes exposed to increasing concentrations of SeMet had decreased glutathione concentrations and increased generation of ROS, lipid peroxidation and activation of caspase enzymes which supports the theory that oxidative stress and apoptosis play a role in SeMet toxicity in fish (Misra et al., 2010, 2012a).

1.3.2 Effect on development, reproduction and morphology

Research has shown SeMet to be chronically toxic to fish, causing several deleterious effects. One of the most well documented effects of SeMet exposure (>10 μg/g dm) in fish is teratogenesis leading to poor annual recruitment and population decline (Lemly, 2002).

Malformations include spinal curvatures, craniofacial deformities, fin deformities and edema (cranial, yolk sac, pericardial), which result from maternal transfer of SeMet during vitellogenesis (Lemly, 1993, 2002; Fan et al., 2002; Janz et al., 2010). Other effects include decreased respiratory capacity, myocarditis, pericarditis, cataracts and pathological changes in the kidney, liver, heart and ovaries (Lemly, 1993, 2002; Fan et al., 2002). One of the worst cases of Se contamination in an aquatic environment occurred in Belews Lake, NC, USA. Fishes in Belews Lake were chronically exposed to Se wastewater from a coal fired power plant and exhibited several pathological changes including swollen gill lamellae, dilated blood sinusoids, reduced hematocrit (sign of anemia), corneal cataracts, exopthalmus (protruding eyeballs) due to edema in the body cavity and head, developmental malformations (including deformities of the spine, head, mouth and fins) and damaged egg follicles in the ovaries (Lemly, 1993, 2002). Reproductive failure and teratogenicity in these fishes led to the eradication of most of the species from Belews Lake (Lemly, 1993, 2002). Other studies have investigated SeMet's effects on behavior and physiological processes such as energy homeostasis, the physiological stress response and steroidogenesis (Thomas and Janz, 2011; Wiseman et al., 2011a, 2011b; Thomas et al., 2013).

1.3.3 Effect on the physiological stress response, energetics and steroidogenesis

Organisms, including fish, have adapted to cope with natural and anthropogenic stressors by activating the physiological stress response to maintain homeostasis (Wendelaar Bonga, 1997; Barton, 2002). The stress response can be divided into primary, secondary and tertiary phases. The primary phase of the stress response, or neuroendocrine response, involves release of catecholamines from chromaffin cells and stimulation of the hypothalamus-pituitary interrenal (HPI) axis, followed by the release of corticosteroids (Wendelaar Bonga, 1997). The secondary phase is often dependent on neuroendocrine responses in the primary phase and involves changes in concentrations of blood plasma constituents such as glucose and lactate (Mommsen et al., 1999; Barton, 2002). The tertiary phase involves responses at the whole-organism level such as growth, condition, disease resistance, behaviour and overall survival (Wedemeyer and McLeay, 1981; Barton, 2002). Cortisol is the primary corticosteroid released from inter-renal tissue in response to a stressor (Mommsen et al., 1999). The release of cortisol is regulated by the HPI axis and initiated by the release of the corticotropin releasing hormone (CRH) from the hypothalamus, which

stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary (Wendelaar Bonga, 1997; Mommsen et al., 1999; Barton, 2002). Adrenocorticotropic hormone stimulates interrenal cells in the head kidney by binding to the melanocortin 2 receptor (MC2R), activating corticosteroidogensis (Alura and Vijayan, 2009). Chronic exposure to contaminants such as SeMet can exhaust or impair the physiological stress response and alter cortisol production (Wendelaar Bonga, 1997; Barton, 2002; Vijayan et al., 2005; Wiseman et al., 2011a). Changes in cortisol secretion can negatively affect energy metabolism and oxygen consumption in fish leading to depleted energy stores (Wedemeyer and McLeay, 1981). Energy put towards maintaining homeostasis during chronic exposure to contaminants can deplete energy available for immune function, growth and reproduction, which can be adverse for the health and survival of fish over time (Wedemeyer and McLeay, 1981).

Studies have shown zebrafish (*Danio rerio*) exposed to dietary SeMet had altered swim performance (decreased critical swimming speed) and significant increases in energy stores (triglycerides and glycogen) when fed > 3.7 μg Se/g. Zebrafish fed > 26.6 μg Se/g also had elevated cortisol levels (Thomas and Janz, 2011). Rainbow trout chronically exposed to dietary SeMet (8.47 mg Se/kg dm) were found to have elevated levels of cortisol in the plasma but were unable to mount a cortisol response when subjected to an acute handling stressor (Wiseman et al., 2011a). The fish unable to mount a stress response were found to have elevated concentrations of cortisone, which is an inactive cortisol metabolite, suggesting attenuation of the cortisol response to stress was due to inactivation of cortisol (Wiseman et al., 2011a). The exposed trout also had significantly greater hepatic somatic index (HSI), body mass and condition factor, as well as increased concentrations of glycogen and triglycerides in the muscle. However, glycogen and triglyceride concentrations decreased 24 h after the handling stressor (Wiseman et al., 2011a). Selenomethionine has also been shown to have effects on other aspects of the endocrine system, including stimulation of gonadal steroidogenesis. Female rainbow trout chronically fed SeMet (4.54 mg/kg ww) had significantly increased plasma concentrations of androstenedione, estrone and estradiol (39.5-, 3.8- and 12.7-fold greater, respectively) compared to control fish. The treated fish were also found to have testosterone in their plasma, while the control fish did not. Exposed trout were also found to have significantly greater transcript abundance of vitellogenin and zona-radiata protein compared to control fish, probably due to the increased estradiol concentrations (Wiseman et al., 2011b).

1.4 Toxicity of selenium in sturgeon

Sturgeons have been shown to be among the most sensitive species of fish to several environmental contaminants including selected endocrine disruptors and metals (Feist et al., 2005; Little et al., 2012; Vardy et al., 2011, 2013). Several persistent contaminants have been detected at concentrations of concern in sturgeons such as methylmercury, polychlorinated biphenyls, and chlorinated pesticides, and have been negatively correlated with parameters such as condition factor, plasma triglyceride concentrations, gonad size and circulating androgen concentrations (Foster et al., 2001; Feist et al., 2005; Webb et al., 2005). Metals have become a particular concern for WS populations in larger river systems as a result of surrounding metal mines, pulp and paper mills, oil refineries, smelters and runoff from other industrial and urban areas (Foster et al., 2001; Vardy et al., 2011, 2013). Highly contaminated sediments have been detected in WS spawning and nursing areas within the Columbia River, which is concerning for early life stage WS (Bortleson et al., 2001). Early life stages of WS, as well as juvenile WS, inhabit benthic environments and consume benthic organisms, making them more susceptible to exposure to pollutants that accumulate in sediments, such as metals, compared to other species of fish (Little et al., 2012; Vardy et al., 2011, 2013). The longevity of sturgeons such as WS also makes them particularly susceptible to accumulation of persistent lipophilic contaminants, with studies showing positive correlations between age and increasing levels of contaminants (Webb et al., 2005).

Selenium contamination could become an increasing issue for some WS populations including populations in the Kootenay River and San Francisco (S.F.) Bay-Delta (Luoma and Pressser, 2000; DFO, 2007; Dessouki and Ryan, 2010). Selenium concentrations have increased in the Kootenay River, which contains critical habitat for endangered WS, because of weathering of accumulated waste rock from upstream coal mines. Based on water quality monitoring, Se concentrations in the Kootenay River are increasing by 0.012 μg/L per year and if this rate continues aquatic life guidelines for Se will be exceeded within the next 15 years (Dessouki and Ryan, 2010). Recruitment in the Kootenay population has been negligible over the last 40 years and with an aging population, extinction will be inevitable if natural recruitment cannot be restored (DFO, 2007). There have not been any studies done on the effects of Se on WS in this region, but

increased Se contamination would likely exacerbate the recruitment issues and increase the likelihood of extinction.

The main causes for increased Se concentrations in the S.F. Bay-Delta are petroleum wastewaters and irrigation runoff from agriculture activities in the San Joaquin Valley (Luoma and Pressser, 2000). In this region, WS prey on species that can readily accumulate Se, such as the Asian clam (*Potamocorbula amurensis*), which has been found to contain up to 20 μg/g dm (Linville et al., 2002). This non-native bivalve species has dominated over native species and become a major food source for WS and other benthic-consuming organisms in the S.F. Bay-Delta. Presser and Luoma (2006) have predicted that increasing Se inputs from agricultural drainage in the S.F. Bay-Delta estuary will increase Se concentrations in bivalves, such as the Asian clam, to levels several times higher than the dietary threshold level for Se (> 3 μg/g) previously reported to cause toxicity in fishes (Lemly 1997a; Hamilton, 2004). This could be detrimental to WS as a study by Tashjian et al. (2006), using dietary SeMet concentrations which were predicted to be present in the S.F. Bay-Delta (Luoma and Presser, 2000), showed that exposure to > 40 μg Se/g caused adverse effects on growth rate, activity and behavior in juvenile WS. Exposure to concentrations > 20 μg Se/g caused histopathological changes in kidney, liver and gill tissue (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014; Zee et al., 2016a). While a significant increase in the prevalence of mild to moderate edema was observed at dietary concentrations as low as 5.6 μg Se/g (Zee et al., 2016a).

White sturgeon sampled from the S.F. Bay-Delta have been found to have up to 15 and 30 μg Se/g in their muscle and liver tissue, respectively, and between 7 and 20 μg Se/g in their ovaries (White et al., 1988; Urquhart and Rigalado, 1991; Linville et al., 2002; Linville, 2006; Linares-Casenave et al., 2014). Ovarian tissue from Kootenai River WS contained up to 12 μg Se/g (Kruse and Scarnecchia, 2002). Se accumulated in WS can be transferred to offspring, leading to developmental malformations and mortality of larvae, which are considered to be the most sensitive life stage in fishes due to the teratogenic properties of SeMet. Se concentrations > 11 to 15 μg/g in WS larvae, have been found to cause significant increases in spinal deformities, edema and mortality (Linville, 2006).

The dietary Se threshold level for fishes previously reported in the literature was 3 μg Se/g, and the tissue thresholds were 4 μg/g for whole-body, 8 μg/g for muscle, 12 μg/g for liver, and 10 μg/g for ovary and eggs (Lemly, 1997a; Hamilton, 2004). Currently, the BC Ministry of

Environment (2014) recommends a chronic dietary guideline of 4 μg Se/g dw (invertebrate tissue), and fish tissue guidelines of 4 μg Se/g dw in whole-body and muscle tissue, and 11 μg Se/g dw in egg/ovary; while the USEPA (2016) recommends 8.5 μg/g dw for fish whole-body, 11.3 μg/g dw for muscle tissue and 15.1 μg/g dw for egg/ovary (Table 1.1). The dietary threshold concentration for WS has been estimated to be between 10 and 20 μg/g (Tashjian et al., 2006). As discussed above, the Se concentrations detected in WS tissues and prey exceed toxicity threshold concentrations previously suggested for other fish species (Lemly, 1997a; Hamilton, 2004) and the current fish tissue criteria/guidelines recommended by the USEPA (2016) and BC Ministry of Environment (2014).

One explanation for the decrease in the recruitment of WS in regions such as the S.F. Bay-Delta or the Kootenay River, could be due to toxicity from the accumulation of Se. If WS in the wild are exposed to high enough concentrations of Se to alter behavior, decrease activity and deplete energy stores, this could reduce their ability to forage, avoid predators, swim upstream for spawning and cope with other secondary stressors, which would be detrimental for survival of already endangered populations (Tashjian et al., 2006). With the potential for increasing Se inputs into WS habitats, and therefore increasing SeMet concentrations in food sources, it is important that the sensitivity of WS to SeMet and mechanisms of toxicity be investigated further to ensure protection of this endangered species. Knowledge of SeMet's effect on the physiological stress response in WS is also important. The key to maintaining homeostasis in times of stress is being able to mount a cortisol response to stress and without this ability, fish health could be adversely affected.

To date, studies assessing molecular mechanisms of Se toxicity have primarily been conducted in teleost species such as rainbow trout and have utilized quantitative real-time polymerase chain reaction (qPCR) or microarray analysis (Palace et al., 2004; Misra et al., 2010, 2012a; Knight et al., 2016). Although oxidative stress appears to be the primary mechanism leading to toxicity in teleosts (Palace et al., 2004; Misra et al., 2010, 2012a; Lavado et al., 2012), the specific drivers of SeMet toxicity in WS are unclear. Some evidence suggests that oxidative stress is not associated with toxicity in WS (Zee et al., 2016b), and therefore there is a need to investigate other potential drivers of toxicity. Next-generation sequencing technology, such as RNA-sequencing (RNA-seq), has emerged as an ideal tool for assessing the toxicity of contaminants in non-model species with little available genetic information, such as WS. These

technologies enable performing unbiased whole transcriptome analysis, and unlike microarrays or qPCR, RNA-seq does not require pre-selection of transcripts for analysis, making it ideal for identifying novel molecular responses to contaminant exposure (Mehinto et al., 2012; Reviewed in Qian et al., 2014; Illumina, 2017).

This research aimed to provide further insight into the sensitivity of WS to SeMet by assessing sublethal effects of dietary SeMet exposure, including effects on the physiological stress response and the whole transcriptome. Other endpoints were assessed in the same fish by Zee et al. (2016a; 2016b), including gross pathologies, liver histopathology, growth, and oxidative stress, and these findings will be discussed in this thesis in order to try to establish links between adverse effects observed at the end of the study and initial molecular responses to SeMet exposure. This research will assist in obtaining more accurate estimates of toxic thresholds for regulatory purposes and provide knowledge about potential mechanisms leading to toxicity. Since WS are potentially more susceptible and sensitive to environmental contaminants than teleosts, regulatory decisions based on data from teleost research may not be protective of WS and therefore, this research could provide information regarding species differences that will be beneficial in future risk assessments of Se contaminated regions.

1.5 Objectives and Hypotheses

The overall objective of this study was to characterize the sensitivity and develop a better understanding of how dietary SeMet affects the physiology of WS by linking specific pathological endpoints with molecular pathways of toxicity.

Objective 1) To determine the effect of chronic dietary exposure to SeMet on the ability of WS to mount a stress response. To address this objective, plasma concentrations of cortisol, lactate and glucose were analyzed, as well as concentrations of glycogen in liver and muscle tissue. Transcript abundance of key regulatory genes in corticosteroidogenesis were also analyzed to determine the effects of SeMet on the stress response.

Null-Hypothesis 1 ($H_0 1$): There are no statistically significant differences in the cortisol, glucose, lactate, and glycogen response to stress, between control fish and fish exposed to increasing concentrations of dietary SeMet.

Objective 2) To identify novel toxicity pathways of SeMet in juvenile white sturgeon by use of sequence-by-synthesis technology to determine changes in gene expression across the whole transcriptome. Determining which physiological processes may be adversely affected by dietary exposure to SeMet aims to identify specific endpoints that will inform future studies.

Null-Hypothesis 2 ($H_0$2): There are no statistically significant changes in gene expression across the whole transcriptome between control fish and fish exposed to dietary SeMet.

Null-Hypothesis 3 ($H_0$3): Chronic exposure to SeMet does not affect specific molecular pathways and functions in WS that are indicative of an altered physiological state, and that can be used to develop specific toxicity endpoints for SeMet.

CHAPTER 2: EFFECTS OF CHRONIC EXPOSURE TO DIETARY SELENOMETHIONINE ON THE PHYSIOLOGICAL STRESS RESPONSE IN JUVENILE WHITE STURGEON (*ACIPENSER TRANSMONTANUS*)

2.0 Preface

This Chapter discusses the impact of dietary selenomethionine (SeMet) exposure on the ability of WS to mount a cortisol response to an acute handling stressor, as well as other endpoints related to the physiological stress response and energetics, as a means to assess the sensitivity of WS and further understand the potential drivers of SeMet toxicity. The results from this Chapter were later discussed along with the pathway analysis results in Chapter 3, to assess whether any alterations in the physiological stress response could be associated with the development of SeMet toxicity in WS.

This chapter has been published in *Aquatic Toxicology* (2017) Volume 186, Pages 77-86, under joint authorship with Jenna Zee (University of Saskatchewan), Steve Wiseman (University of Saskatchewan, University of Lethbridge) and Markus Hecker (University of Saskatchewan). The tables, figures and references cited have been reformatted here to the thesis style. References cited in this chapter are listed in the references section of this thesis. Some of the references differ from how they appeared in the published version, specifically, the references for Lemly, 1997 and Wiseman et al., 2011 were changed to Lemly, 1997a and Wiseman et al., 2011a, to differentiate from other references included in this thesis. Section 2.0 Preface has been added to provide a brief explanation of Chapter 2.

The author contributions to Chapter 2 were as follows:

- Sarah Patterson (University of Saskatchewan) conducted the SeMet exposure, collected the samples, performed all cortisol, glucose, lactate, glycogen and qPCR analysis, and wrote the manuscript.
- Jenna Zee (University of Saskatchewan) co-managed the SeMet exposure, assisted with sample collection and extracting mRNA and cDNA, and reviewed the manuscript.
- Steve Wiseman (University of Saskatchewan, University of Lethbridge) provided guidance

and assistance in conducting the stress test and collecting samples, as well as, provided guidance in the cortisol and molecular analysis, and revised the manuscript.

- Markus Hecker (University of Saskatchewan) provided funding to conduct this research and provided guidance and assistance with the experimental design, collection of samples, and data analysis, and reviewed and revised the manuscript.

2.1 Abstract

Selenium (Se) is an essential micronutrient, but at low concentrations it can be toxic to aquatic organisms. Selenomethionine (SeMet) is the primary dietary form of Se aquatic organisms are exposed to and is an environmental concern because it persists and bioaccumulates. White sturgeon (WS; *Acipenser transmontanus*) might be particularly susceptible to bioaccumulative toxicants, such as SeMet, due to their longevity and benthic lifestyle. Se exposure is known to have adverse effects on the physiological stress response in teleosts, but these effects are unknown in WS. Therefore, the goal of this study was to determine effects of dietary SeMet on the ability of WS to mount a stress response. Juvenile WS were administered diets containing 1.4 (control), 5.6, 22.4 and 104.4 µg Se/g dry mass (dm) for 72 days. Lower doses were chosen to represent environmentally relevant concentrations, while the high dose represented a worst case scenario exposure. On day 72, fish were subjected to a 2 min handling stressor, and they were sampled at 0, 2 and 24 h post-stressor. Cortisol, glucose and lactate concentrations were quantified in blood plasma and glycogen concentrations were quantified in muscle and liver. Transcript abundance of genes involved in corticosteroidogenesis and energy metabolism were determined using qPCR. Under basal conditions, WS fed 104.4 µg Se/g dm had significantly greater concentrations of plasma cortisol and lactate, and significantly lower concentrations of plasma glucose and liver glycogen, compared to controls. Corticosteroid 11-beta dehydrogenase 2 (*hsd11b2*) abundance was lower in WS fed 22.4 and 104.4 µg Se/g dm, indicating less conversion of cortisol to cortisone. Abundance of the glucocorticoid receptor (*gcr*) was significantly lower in high dose WS, suggesting lower tissue sensitivity to glucocorticoids. The increasing trend in phosphoenolpyruvate carboxykinase (*pepck*) abundance, with increasing SeMet exposure, was consistent with greater cortisol and glucose concentrations in high dose WS. Exposure to an acute handling stressor elicited a typical cortisol response, but the magnitude of the response appeared to be significantly lower than those typically observed in teleosts. Selenomethionine also did not appear to modulate the cortisol response to a secondary stressor. However, WS exposed to 22.4 µg Se/g dm and sampled 2 h post-stressor, had significantly higher concentrations of muscle glycogen compared to controls, indicating effects on their ability to utilize muscle glycogen for energy. Overall, the results indicate that chronic exposure to dietary SeMet concentrations > 22.4 µg/g can affect cortisol dynamics and mobilization of energy substrates in juvenile WS.

2.2 Introduction

Selenium (Se) is an essential micronutrient required by all vertebrate species for incorporation into selenoproteins. However, there is a fine line between Se essentiality and toxicity (Janz, 2012; Lemly, 1997a). Dietary requirements for maintaining normal physiological function range between 0.1 and 0.5 μg Se/g dry mass (dm) in fish, but studies have shown concentrations > 3 μg Se/g dm in the diet can result in toxicity (Hamilton, 2004; Lemly, 1997a). Inorganic Se is the most prevalent form found in surface waters, where it is rapidly bioaccumulated and biotransformed by microorganisms into organoselenides, such as selenomethionine (SeMet). These organoselenides are transferred up the food chain to more sensitive, higher trophic level organisms (Janz, 2012; Stewart et al., 2010). Selenomethionine is the major dietary form of Se that aquatic organisms are exposed to and is of particular concern because it is known to persist and bioaccumulate through the food chain (Lemly and Smith, 1987).

There is significant concern about the effects of Se contamination on aquatic vertebrates, including fish. Among these, white sturgeon (WS; *Acipenser transmontanus*) are of great interest because their longevity and benthic lifestyle make them more susceptible to exposure and bioaccumulation than other fish species (Little et al., 2012; Vardy et al., 2011, 2013). In particular, populations of WS in the Kootenay River and San Francisco (S.F.) Bay-Delta are at risk of exposure to Se because of increasing Se inputs into these ecosystems (Presser and Luoma, 2006; Dessouki and Ryan, 2010). Selenium concentrations up to 12 μg/g, which exceed the toxic effect threshold recommended by Lemly (1997a) and approach the US Environmental Protection Agency's (EPA) criterion of 15.1 mg/kg (USEPA, 2016), were detected in ovarian tissue from Kootenai River WS (Kruse and Scarnecchia, 2002). Water quality monitoring has also shown total Se concentrations in the Kootenay River are increasing at a rate of 0.012 μg/L per year, and these increases are potentially associated with upstream inputs from open-pit coal mining operations. If these rates continue, Se concentrations are expected to exceed the current CCME water quality guideline (1 μg/L) within 15-32 years (Dessouki and Ryan, 2010). Concerns about concentrations of Se in the S.F. Bay-Delta originate from effluent discharge from oil refineries in the Bay area and agriculture drainage from the San Joaquin Valley. Although total dissolved Se concentrations in the S.F. Bay-Delta are below the water quality criterion (1 μg/L), WS have been found to contain concentrations up to 15 and 30 μg Se/g dm in their muscle and liver tissue, respectively (White et

al., 1988; Urquhart and Rigalado, 1991; Linville et al., 2002; Linares-Casenave et al., 2014), and these concentrations are greater than the fish tissue criteria set by the US EPA (USEPA, 2016). Sturgeon in the S.F. Bay-Delta feed primarily on filter feeding bivalves (eg. *Potamocorbula amurensis*), which readily accumulate Se. *Potamocorbula amurensis* have been found to contain up to 20 µg Se/g dm (Linville et al., 2002), which is greater than the interim chronic dietary guideline suggested for fish (4 µg Se/g dm) (Beatty and Russo, 2014) and Presser and Luoma (2006) predicted Se concentrations in bivalves could reach >100 µg/g dm under certain Se loading scenarios. Without remediation and management of Se inputs, there is an increased risk of dietary intake, bioaccumulation and toxicity in local sturgeon populations (Presser and Luoma, 2006).

Recently, studies have examined the sensitivity of juvenile WS chronically exposed to SeMet through the diet (Linville, 2006; Tashjian et al., 2006; De Riu, 2014; Zee et al., 2016a). Adverse effects on growth rate, activity and feeding behavior were observed in juvenile WS exposed to concentrations of SeMet > 40 µg/g (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014; Zee et al., 2016a). Exposure to concentrations > 20 µg Se/g caused histopathological lesions in the kidneys, livers and gills (Linville, 2006; Tashjian et al., 2006; De Riu et al., 2014; Zee et al., 2016a), and a significant increase in the prevalence of mild to moderate edema was observed at dietary concentrations as low as 5.6 µg Se/g (Zee et al., 2016a). Increased whole body moisture content and decreased lipid and energy content also were observed in WS fed concentrations > 40 µg Se/g (Tashjian et al., 2006; De Riu et al., 2014). These findings suggest that energy homeostasis in juvenile WS is impacted by dietary SeMet exposure, and further investigation is warranted. Altered energy homeostasis could also indicate an effect on the physiological stress response.

Selenium exposure has been shown to affect the physiological stress response and energy homeostasis in several species of teleost fish (Miller et al., 2007; Miller et al., 2009; Miller and Hontela, 2011; Thomas and Janz, 2011; Wiseman et al., 2011a; McPhee and Janz, 2014). Rainbow trout (*Oncorhynchus mykiss*) chronically exposed to dietary SeMet (8.47 mg/kg dm) were found to have elevated levels of plasma cortisol and were unable to mount a cortisol response when subjected to an acute handling stressor (Wiseman et al., 2011a). The exposed trout also had a significantly greater hepatic somatic index (HSI), body mass and condition factor, as well as increased concentrations of glycogen and triglycerides in the muscle. However, glycogen and triglyceride concentrations decreased 24 h after the handling stressor (Wiseman et al., 2011a). Zebrafish (*Danio rerio*) fed > 26.6 µg Se/g had elevated cortisol levels, as well as altered swim

performance and significant increases in energy stores (triglycerides and glycogen) when fed > 3.7 μg Se/g (Thomas and Janz, 2011). Fathead minnows (*Pimephales promelas*) fed > 5.4 μg Se/g had significantly elevated whole body triglyceride concentrations and decreased whole body glycogen concentrations compared to controls (McPhee and Janz, 2014).

Although previous studies showed effects of SeMet on the physiological stress response and energy homeostasis in teleost species (Thomas and Janz, 2011; Wiseman et al., 2011a; McPhee and Janz, 2014), these effects are not known in WS. Therefore, the objective of this study was to determine the potential effects of chronic dietary SeMet exposure on the physiological stress response in juvenile WS. Sturgeon with an altered stress response and impaired energy homeostasis could have a reduced ability to feed, avoid predators, swim upstream for spawning and cope with other secondary stressors, which would be detrimental to the survival of an already endangered species (Tashjian et al., 2006).

This study was informed by Zee et al. (2016a) and aimed to characterize impacts on the stress response and energy homeostasis as an explanation for the severe pathologies reported in the previous study, while oxidative stress was discussed as a potential driver of Se toxicity in Zee et al (2016b). Together these studies will provide further insight into the sensitivity of juvenile white sturgeon to dietary SeMet and the mechanism(s) leading to toxicity.

2.3 Materials and methods

2.3.2 Test chemical and species

Seleno-L-methionine (purity > 98%) was purchased from Sigma-Aldrich (Oakville, ON, Canada). WS were reared in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan, from eggs donated by the Kootenay Trout Hatchery (Fort Steele, BC, Canada), until they were approximately 3 yr old (124 ± 8.6 g wet mass [mean ± S.E.M]). Sturgeon cultures were maintained at approximately 12°C, in 712 L flow-through tanks, that were continuously supplied with dechlorinated City of Saskatoon Municipal water. Fish were fed a diet of trout chow to satiety daily (Martin, Profishent Aquaculture Nutrition 6PT; Elmira, ON, Canada).

2.3.3 Diet preparation

Food pellets made from #2 crumble trout chow (Proform Aquaculture Feed, Aqua-Balance Trout 52:19 Starter #2 crumble, Viterra Feed Products; Okatoks, AB, Canada) were spiked with nominal concentrations of 0, 5, 25 or 125 µg Se/g dm in the form of SeMet as described by Zee et al. (2016a). In brief, SeMet was dissolved in 100 mL of nanopure water, then added to the trout chow and mixed to form a paste, which was processed through a meat grinder and broken into 3mm diameter pellets and dried at 55°C for 18 h. Analysis by inductively coupled plasma-mass spectrometry (ICP-MS), following the procedure described by Zee et al. (2016b), revealed an average Se concentration of 1.4, 5.6, 22.4 and 104.4 µg Se/g (7.14, 28.6, 114.2 and 532.4 mmol/kg) dm in the control, low, medium and high dose feed, respectively (Zee et al., 2016a).

Concentrations for this study were chosen based on concentrations of Se detected and predicted to be in the S.F. Bay-Delta and major food sources of WS in this region, as well as concentrations detected in WS muscle and liver samples (White et al., 1988; Urquhart and Rigalado, 1991; Linville et al., 2002; Presser and Luoma, 2006). The lower concentrations, 5 and 25 µg Se/g, represent environmentally relevant amounts that sturgeon could be exposed to through their diet, while the highest concentration, 125 µg Se/g, represents a worst case scenario for environmental exposure based on a study by Presser and Luoma (2006), which predicts that without remediation, Se concentrations in S.F. Bay-Delta clams could reach > 100 µg/g.

2.3.4 Experimental design

Protocols for culturing and exposure of WS were approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (UCACS-AREB; # 20090108). Exposures were conducted in the ATRF at the University of Saskatchewan and were performed in 120 L tanks that were continuously supplied with dechlorinated City of Saskatoon Municipal water. Tanks were assigned using a randomized design and there were 7 replicate tanks per dose. Five juvenile WS were randomly assigned to each exposure tank and given at least 1 week to acclimate prior to initiation of the exposure. Water temperature was maintained at 13.4 ± 0.4°C (mean ± SD) with a minimum flow rate of 50 L/h to each tank and a 16 h light: 8 h dark photoperiod. During the acclimation

period, sturgeons were given a mix of their normal diet and the re-pelleted control feed before being switched completely to control feed. Once all individuals adjusted to the control feed, they were switched to the diet spiked with SeMet, which marked day 1 of the exposure. White sturgeon were fed 1.5% of their body mass of control or spiked feed, once daily, 6 times a week, for 65 (high dose group) or 72 (control, low and medium dose groups) days. Final take-downs of all groups will be referred to as 72 d throughout the rest of the paper. The fish were given 1 h to eat and any food not consumed during this period was siphoned from the tanks to ensure Se would not leach into the water.

On day 10 of the exposure, a sub-sample of 7 fish from each treatment group (1 from each tank) were euthanized, total length and mass was determined, and liver and head-kidney tissues were dissected, weighed, and flash frozen in liquid N_2 prior to being stored at -80°C, until required for quantification of gene expression by use of quantitative real-time PCR (qPCR). Gene expression analysis was conducted on day 10 samples in an attempt to capture the initial transcription level response to selenium exposure. This time point was selected as it was assumed that significant Se bioaccumulation would have occurred and molecular responses would still be reflective of an initial toxicity response; while at later time points, changes in gene expression pattern would be more reflective of compensatory responses. Key genes encoding proteins involved in corticosteroidogenesis (*star, p450scc, hsd11b2, gcr*) and energy metabolism (*pepck*) were analyzed. STAR mediates the transport of cholesterol from the outer to inner mitochondrial membrane, initiating cortisol synthesis in the head kidney. P450scc converts cholesterol to pregnenolone, which is a rate-limiting step in cortisol synthesis, and GCR mediates the action of glucocorticoids. HSD11B2 catalyzes the conversion of cortisol to the inactive metabolite cortisone. PEPCK converts oxaloacetate to phosphoenolpyruvate, a rate- limiting step in gluconeogenesis (Mommsen et al., 1999). Furthermore, subsamples of liver and muscle tissue were collected and stored at -80°C for future analysis of total Se concentrations. The feeding ratio was adjusted based on the average weight of fish on day 10. Further details on the experimental design can be found in Zee et al. (2016a).

2.3.5 Stress test

At 72 d, WS given the control feed and feed spiked with the low and medium Se concentrations, were subjected to a standardized stress test, which consisted of chasing, netting and holding the fish above water for 2 min (Wiseman et al., 2011a). WS given the diet containing the highest concentration of Se were euthanized on day 65 because of high mortality rates. These fish were not subjected to the handling stressor because the sample size was too small, but they were used to assess effects of Se on basal concentrations of plasma cortisol, glucose and lactate, and muscle and liver glycogen. Fish were sampled at 3 different time points: prior to the handling stressor (0 h), 2 h post-stressor (2 h) and 24 h post-stressor (24 h). Prior to initiation of the stress test, 1 fish from each 2 h and 24 h tank, as well as all fish from the designated 0 h tanks were euthanized with a quick blow to the head and used as controls for the stress response. All other fish were euthanized at 2 h or 24 h after the handling stressor was applied. Once euthanized, fish mass and fork length was recorded to calculate Fulton condition factor (*k*). Blood was collected from the caudal vein and/or heart using heparinized syringes then centrifuged at 5000 rpm for 15 min to separate the plasma, which was stored at -80°C until needed for quantification of cortisol, glucose and lactate. Liver, head-kidney and muscle tissues were excised and immediately frozen at -80°C for further analysis. Livers were weighed to determine the hepatosomatic index (HSI). Growth, *k* and HSI data was reported in Zee et al. (2016a).

2.3.6 Quantification of cortisol in blood plasma

Concentrations of cortisol in blood plasma were determined by use of an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (Oxford Biomedical Research, Oxford, MI, USA). Cortisol was extracted by adding 1 mL of diethyl ether to 100 uL of plasma. Ether was then evaporated under a nitrogen stream and cortisol was reconstituted in buffer provided by the manufacturer. The absorbance value of samples was measured at 650 nm using a microplate reader and compared to a standard curve to quantify cortisol (ng/ml).

2.3.7 Quantification of glucose and lactate in blood plasma

Glucose concentrations in blood plasma were analyzed following methods described by Goertzen et al. (2011). Assay reagents were purchased from Sigma-Aldrich. Absorbance of each sample was measured at 440 nm in a microplate reader and compared to a standard curve to quantify glucose (mg/ml).

Concentrations of lactate were determined by use of an L-Lactate assay kit following the manufacturer's protocol (Eton Bioscience Inc., San Diego, CA, USA). Absorbance of each sample was measured at 490 nm and compared against a standard curve to quantify lactate (mM).

2.3.8 Quantification of glycogen in liver and muscle

Concentrations of glycogen in liver and muscle tissue were determined by use of a modified version of the method presented in Gomez-Lechon et al. (1996), which was validated by Weber et al. (2008) and Goertzen et al. (2011). Reagents used in the assay were purchased from Sigma-Aldrich. Absorbance of each sample was read at 440 nm then compared to a standard curve, created from Type IX bovine liver glycogen (Sigma-Aldrich), to determine glycogen content (mg/g).

2.3.9 Quantitative real-time PCR

Total RNA was isolated from liver and head kidney samples following the manufacturer's protocol using a Qiagen RNeasy lipid mini kit (Qiagen, Mississauga, ON, Canada) and quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol.

qPCR was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 70 μL reaction mixture, consisting of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen), 3.5 μL of cDNA, 3.5 μL of gene-specific PCR primers (Table 2.1) and nuclease free water, was prepared for each cDNA sample and primer

combination. Primers were developed from either publicly available sequences (*β-actin, star*) or a WS reference transcriptome (*p450scc, gcr, pepck, hsd11b2*), by use of BlastN and Primer3 software, and were synthesized by Invitrogen (Burlington, ON, Canada) (Table 2.1). The reference transcriptome was constructed by performing 3 rounds of *de novo* assembly on single-end and paired-end reads of RNA isolated from sturgeon livers from this study and other studies described previously (Doering et al., 2014; 2016). All qPCR reactions were performed in triplicate with 20 µL reaction volumes per well. Before the first PCR cycle, the reaction mixture was denatured at 95°C for 10 min. The thermal cycle profile consisted of denaturing at 95°C for 10 s and extension for 1 min at 60°C for a total of 40 PCR cycles. Dissociation curves were generated for all samples to ensure amplification of a single PCR product. Transcript abundance of target genes was normalized to *β-actin* according to methods previously described by Simon (2003).

Table 2.1. Sequences, efficiency, annealing temperatures and GenBank accession numbers of oligonucleotide primers used in quantitative real-time PCR.

Gene Identity	Accession #	Sequence (5'-3')	Efficiency (%)	Annealing temp. (°C)
β-actin	FJ205611	F: CCGAGCACAATGAAAATCAA R: ACATCTGCTGGAAGGTGGAC	96	60
star	FJ205610	F: CCCGAGCAAAAAGGCTTCA R:TTGGGCCGAAGAACAATACAG	97	60
p450scc	NA	F: TCCACAGATTTTCCCCAGAC R: TTTGGAGGAGGTGAATGAGG	97	60
gcr	NA	F: CTACCAGATGAGCGGTCACA R: GTCTTCCCCCTAGCCTGTTC	97	60
pepck	NA	F: TCAATCCAGAGAACGGCTTT R: TGGTTCTTCCAGGAGGTCAC	100	60
hsd11b2	NA	F: ACAGCCCTTTCCTTGTCTGT R: TTCCTCTCCGTACTCCTCCA	96	60

2.3.10 Statistical analysis

All data was analyzed using SPSS version 22.0 (IBM SPSS Statistics, Armonk, NY, USA). The Shapiro-Wilk test was used to ensure normality of the data and the Levene's test to ensure homogeneity of variance. When necessary, the data was $\log_{10}$ transformed to achieve normality

and homogeneity of variance. Non-transformed data are shown in figures. Two-way analysis of variance (ANOVA) was used to test the effect of SeMet treatment and time post-handling stressor on cortisol, glucose, lactate and glycogen concentrations. If there were significant interactions between SeMet treatments and time post-handling stressor, a one-way ANOVA, followed by Tukey's post-hoc test, was used to determine the effect of different SeMet concentrations within each sampling time point. qPCR data was analyzed using a one-way ANOVA, followed by Tukey's post-hoc test. Differences were considered statistically significant at $p < 0.05$. Data is reported as mean ± standard error of the mean (S.E.M.). Data that did not meet parametric assumptions after log transformation was analyzed using Kruskal-Wallis test followed by Dunn's post-hoc test.

2.4 Results

2.4.1 Selenium analysis, edema, and mortality

Total selenium concentrations in control feed and feed spiked with the low, medium and high amounts of SeMet were 1.4 ± 0.02, 5.6 ± 0.01, 22.4 ± 0.26 and 104.4 ± 2.78 µg Se/g dm (mean ± S.E.M.), respectively, as described in Zee et al. (2016a). Concentrations of Se in the spiked feed were significantly greater than those in control feed ($p < 0.05$).

Tissue concentrations of Se increased with increasing dietary concentrations of Se. Specifically, on day 72, total Se concentrations in the muscle tissue of control, low, medium and high dose groups were 1.1 ± 0.06, 5.3 ± 0.37, 23.5 ± 1.69 and 64.1 ± 6.43 µg Se/g dm (mean ± S.E.M.), respectively, while concentrations in the liver tissue were 0.7 ± 0.11, 2.9 ± 0.18, 9.3 ± 0.54 and 91.7 ± 12.44 µg Se/g dm (mean ± S.E.M.) in the control, low, medium and high dose groups, respectively. WS fed low, medium and high diets had significantly greater ($p < 0.05$) concentrations of Se in their muscle and liver tissue on day 72, compared to controls (Zee et al., 2016a).

A detailed description of edema observations and mortality are provided in Zee et al. (2016a). In brief, edema causing exophthalmos developed within 15 d and 23 d, and lead to mortality by 72 d in 54% and 22% of WS fed 104.4 and 22.4 µg Se/g dm, respectively. Mild edema

occurred in 23% of WS fed 5.6 µg Se/g dm as well, but the biological relevance of this remains unknown.

2.4.2 Concentrations of cortisol in blood plasma

Basal (0 h) plasma cortisol concentrations differed significantly among treatment groups (Fig. 2.1A). Specifically, basal plasma cortisol concentrations were significantly greater ($p < 0.05$) in the highest SeMet exposure group compared to the control, low and medium exposure groups. However, basal cortisol concentrations in blood plasma were not significantly different among WS fed control, low or medium SeMet diets.

Plasma cortisol concentrations were significantly greater ($p < 0.05$) in WS sampled 2 h post-stressor compared to concentrations in fish sampled prior to the handling stressor and 24 h post-stressor, regardless of treatment group (Fig. 2.1B). There was no significant difference between cortisol concentrations in WS sampled prior to the handling stressor and 24 h post-stressor exposure. There was no significant interaction between the time post-stressor exposure and diet on cortisol concentrations in blood plasma.

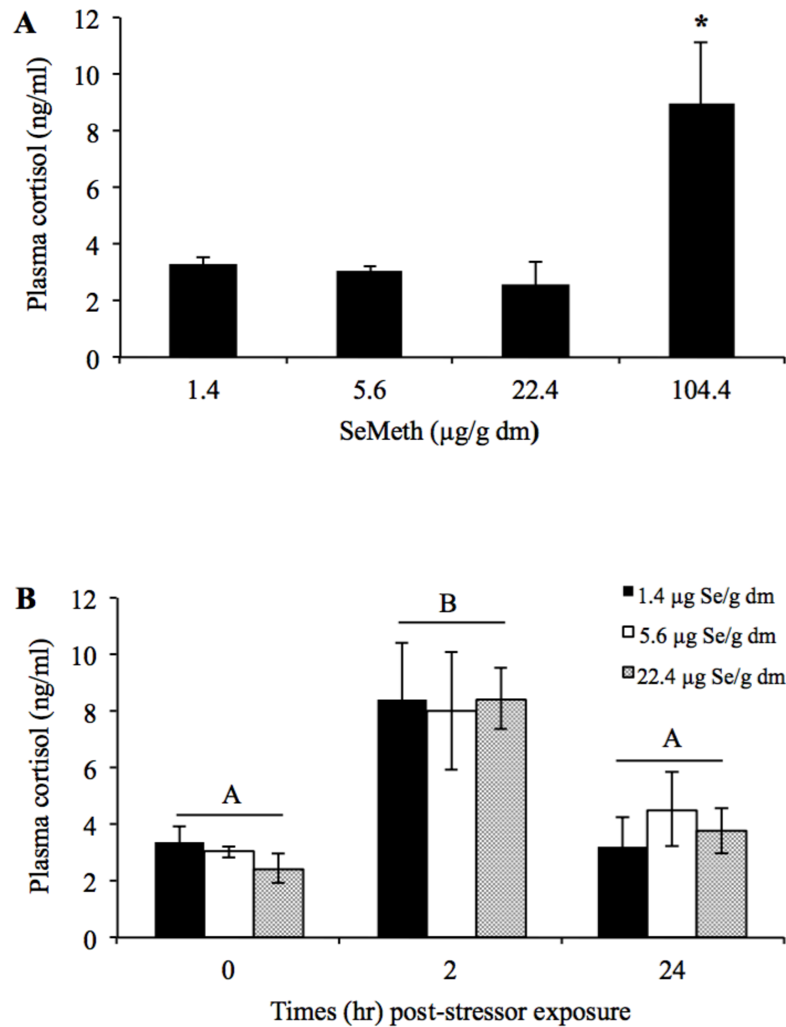

Figure 2.1. (A) Effect of SeMet on basal concentrations of cortisol in blood plasma from juvenile WS sampled prior to being subjected to an acute handling stressor (0 h). Bars represent mean (±S.E.M.) of n = 3-7 sturgeon. An asterisk indicates a significant difference between control and dosed groups (one-way ANOVA with Tukey's post-hoc test, $p < 0.05$). Data was log-transformed prior to statistical analysis. (B) Effect of handling stressor on cortisol concentrations in blood plasma from juvenile WS. Individuals were sampled at 0 h, 2 h or 24 h post-stressor. Bars represent mean (±S.E.M.) of n = 3-8 individuals. Different letters indicate a significant effect of time (two-way ANOVA with Tukey's post-hoc test, $p < 0.05$). Data was log-transformed prior to statistical analysis.

2.4.3 Concentrations of glucose and lactate in blood plasma

There were statistically significant differences in basal glucose and lactate concentrations among treatment groups (Fig. 2.2). Basal glucose concentrations in blood plasma were significantly ($p < 0.05$) lower in the highest SeMet exposure group compared to the control, low and medium exposure groups (Fig. 2.2A). Basal glucose concentrations in blood plasma of WS fed either the control, low or medium SeMet diets were not significantly different. Concentrations of glucose in blood plasma collected 2 h post-stressor from WS exposed to control, low and medium SeMet diets, were significantly ($p < 0.05$) greater than those sampled prior to the handling stressor and at 24 h post-stressor exposure (Fig. 2.2B). Concentrations of glucose in blood plasma from WS sampled prior to the handling stressor and 24 h post-stressor were not significantly different. There was no significant interaction between the time post-stressor exposure and diet on glucose concentrations in blood plasma.

Basal lactate concentrations in blood plasma were significantly ($p < 0.05$) greater in WS fed the highest SeMet diet than in WS fed the control, low or medium SeMet diets (Fig. 2.2C). There was no significant difference in basal lactate concentrations between WS exposed to control, low and medium dose groups. Concentrations of lactate in blood plasma sampled at 2 h post-stressor were significantly ($p < 0.05$) greater than concentrations of lactate in blood plasma sampled prior to the handling stressor and 24 h post-stressor (Fig. 2.2D). Lactate concentrations in blood plasma sampled prior to the handling stressor were not significantly different than lactate concentrations in blood plasma sampled 24 h post-stressor. There was no significant interaction between SeMet diet and time post-stressor exposure on concentrations of lactate in blood plasma.

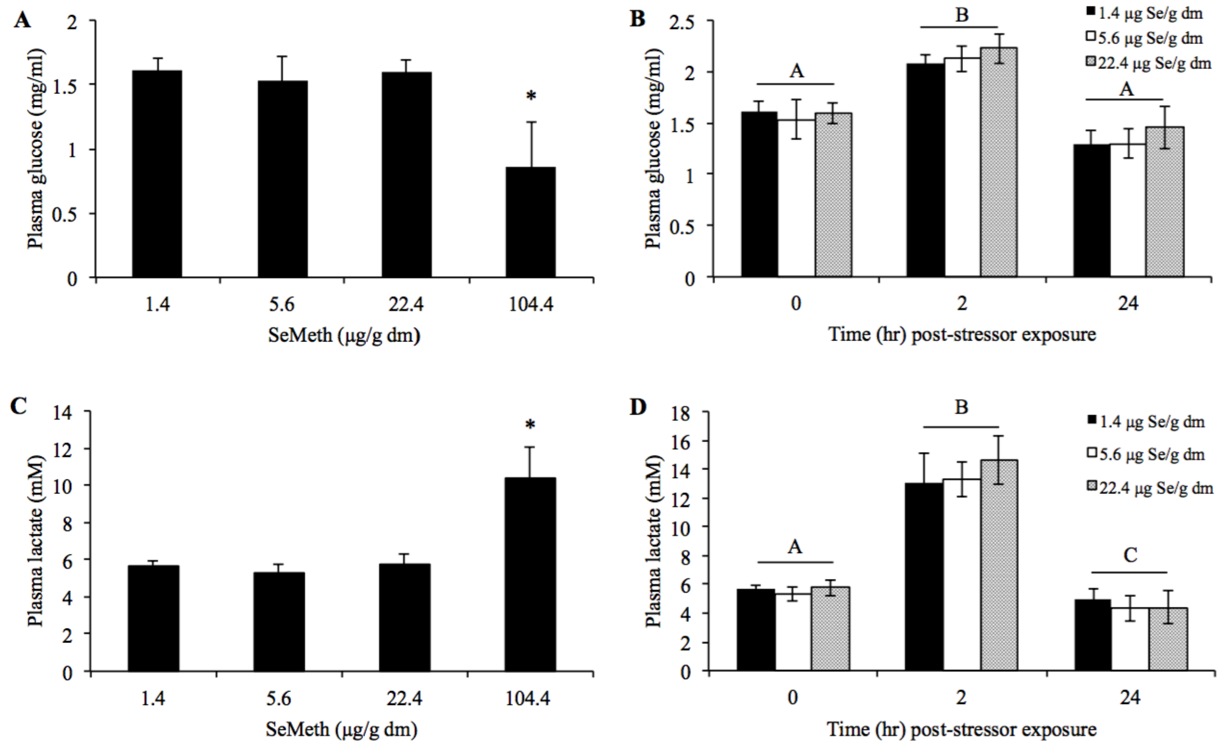

Figure 2.2. (A) Effect of SeMet on glucose concentrations in blood plasma from juvenile WS sampled at 0 h. Bars represent mean (±S.E.M.) of n = 4-7 individuals. An asterisk indicates a significant difference between control and dosed groups (one-way ANOVA with Tukey's post-hoc test, $p < 0.05$). (B) Effect of handling stressor on glucose concentrations in blood plasma from juvenile WS sampled at 0 h, 2 h, or 24 h post-stressor. Bars represent mean (±S.E.M.) of n = 4-7 individuals. Different letters indicate a significant effect of time (two-way ANOVA with Tukey's post-hoc test, $p < 0.05$). (C) Effect of SeMet on lactate concentrations in blood plasma from juvenile WS sampled at 0 h. Bars represent mean (±S.E.M.) of n = 4-9 sturgeon. An asterisk indicates a significant difference between control and dosed groups (one-way ANOVA with Tukey's post-hoc test, $p < 0.05$). Data was log-transformed prior to statistical analysis. (D) Effect of handling stressor on lactate concentrations in blood plasma from juvenile WS sampled at 0 h, 2 h, or 24 h post-stressor. Bars represent mean (±S.E.M.) of n = 4-9 sturgeon. Different letters indicate a significant effect of time (two-way ANOVA with Tukey's post-hoc test, $p < 0.05$). Data was log-transformed prior to statistical analysis.

2.4.4 Concentrations of glycogen in liver and muscle

Effects of SeMet and the handling stressor on muscle and liver glycogen concentrations were variable. Basal concentrations of glycogen in muscle tissue were not significantly affected by SeMet exposure (Fig. 2.3A). However, two-way ANOVA showed a significant effect of SeMet and time post-stressor exposure on muscle glycogen concentrations and a significant interaction between time post-stressor exposure and SeMet concentration (Fig. 2.3B). At 2 h post-stressor, muscle glycogen concentrations in control fish were significantly ($p < 0.05$) lower than those in fish given the medium concentration of SeMet. Muscle glycogen concentrations in control fish sampled at 2 h post-stressor also were significantly ($p < 0.05$) less than concentrations in control fish at 0 h, but not different than concentrations in fish at 24 h post-stressor. There was no significant difference in muscle glycogen concentrations between each sampling time point in WS given the low and medium concentrations of SeMet.

Basal concentrations of liver glycogen were significantly altered by SeMet. WS fed the diet with the highest concentration of SeMet had significantly ($p < 0.05$) lower liver glycogen concentrations compared to the controls at 0 h (Fig. 2.3C). Liver glycogen concentrations in fish sampled at 0 h post-stressor were not significantly different from concentrations in livers from fish sampled at 2 h post-stressor (Fig. 2.3D). However, concentrations of glycogen in livers sampled at 24 h post-stressor were significantly ($p < 0.05$) lower than in livers from fish sampled at 0 h. SeMet did not significantly affect liver glycogen concentrations in WS subjected to the handling stressor.

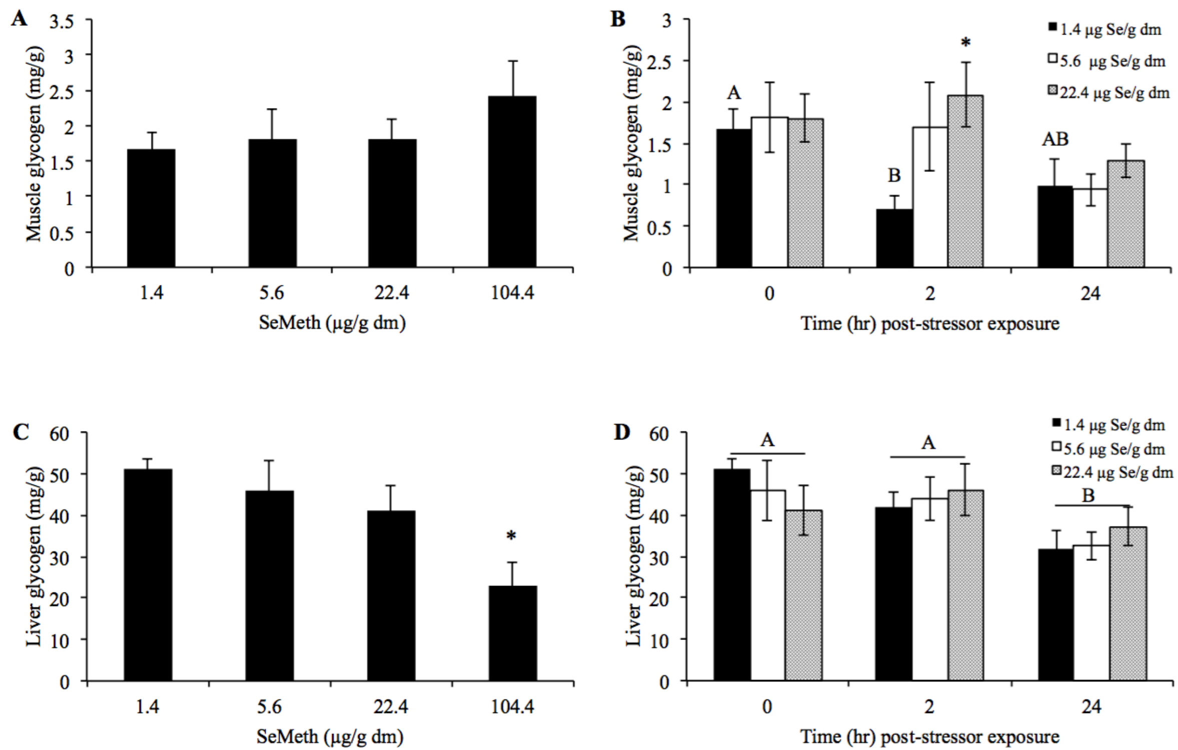

Figure 2.3. Effect of SeMet on glycogen concentrations in muscle (A) and liver (C) tissue from juvenile WS sampled at 0 h. Bars represent mean (±S.E.M.) of n = 6-7 individuals. Different lower case letters indicate a significant difference between doses (one-way ANOVA with Tukey's post-hoc test, $p < 0.05$). Effect of handling stressor on glycogen concentrations in muscle (B) and liver (D) from juvenile WS sampled at 0 h, 2 h, or 24 h post-stressor. Bars represent mean (±S.E.M.) of n = 6-7 sturgeon. Different upper case letters indicate a significant effect of time (two-way ANOVA with Tukey's post-hoc test, $p < 0.05$). An asterisk indicates a significant difference between control and SeMet dosed groups at the same sampling time point.

2.4.5 Abundance of transcripts

Exposure to dietary SeMet for 10 days significantly affected transcript abundance of genes of interest in WS liver and head kidney tissue (Fig. 2.4). Relative to fish given the control diet, transcript abundance of *gcr* was significantly ($p < 0.05$) lower by 4-fold in livers from WS fed the highest SeMet diet (Fig. 2.4A). There were no statistically significant differences in transcript

abundance of *pepck* in the liver; however, there was a trend of increasing transcript abundance with increased concentrations of dietary SeMet (Fig. 2.4B).

In head kidney tissue, transcript abundance of *hsd11b2* was significantly ($p < 0.05$) lower in fish given diets containing the medium and high concentrations of SeMet compared to controls, by 3- and 2.9-fold, respectively (Fig. 2.4C). There were no statistically significant differences in abundance of *star* or *p450scc* transcripts in head kidney tissue of WS fed the control diet or diets spiked with SeMet (data not shown).

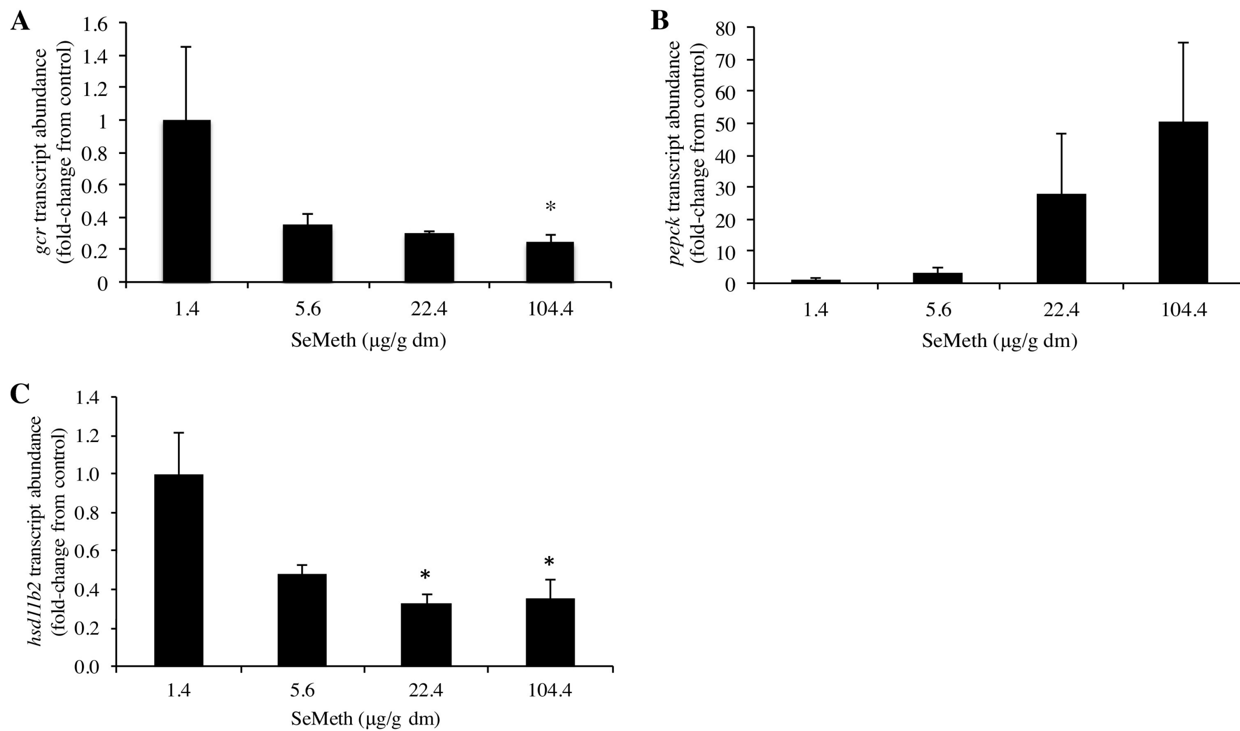

Figure 2.4. Transcript abundance of (A) glucocorticoid receptor (gcr) and (B) phosphopenolpyruvate carboxykinase (pepck) in liver tissue, and (C) 11-β-hydroxysteroid dehydrogenase 2 (hsd11b2) in head kidney tissue, from juvenile WS fed a control diet (1.4 µg Se/g dm) or diet spiked with 5.6, 22.4, 104.4 µg Se/g dm for 10 d. Bars represent mean transcript abundance (±S.E.M) of n = 4-7 individuals, determined by quantitative real-time PCR. An asterisk indicates a significant difference from the control group using Kruskal-Wallis test followed by Dunn's pot hoc test ($p < 0.05$) for (A), and one-way ANOVA of log-transformed data, followed by Tukey's post-hoc test for (B) and (C).

2.5 Discussion

Results from this study suggest that dietary exposure to > 22.4 μg Se/g dm can affect cortisol dynamics and mobilization of energy substrates in juvenile WS. Changes in cortisol dynamics can negatively affect energy metabolism in fish and lead to a depletion of energy available for normal physiological function, and ultimately affect fish health and survival (Wedemeyer and McLeay, 1981). Chronic exposure to 104.4 μg Se/g dm, under basal conditions, altered plasma levels of cortisol, glucose and lactate, as well as liver glycogen content, in WS. Differences in mobilization of muscle glycogen were observed after exposure to an acute handling stressor, in WS fed 22.4 μg Se/g dm, but no effects were observed on other primary (plasma cortisol) or secondary (plasma glucose, plasma lactate and liver glycogen) responses to the acute stressor. Unfortunately, these responses could not be assessed in the highest dose group due to significant mortality.

Prior to the acute handling stressor, plasma cortisol concentrations were significantly elevated in WS exposed to 104.4 μg/g dm of dietary SeMet compared to controls, which is consistent with other studies that showed exposure to dietary SeMet increased concentrations of cortisol in blood plasma of rainbow trout and whole bodies of zebrafish (Thomas and Janz, 2011; Wiseman et al., 2011a). However, much lower concentrations of dietary SeMet (8.47 mg/kg and 26.6 μg/g dm, respectively) caused cortisol levels to increase in rainbow trout and zebrafish compared to WS, indicating potential differences in sensitivity to SeMet exposure between these species. Mechanisms leading to increased plasma cortisol concentrations in response to SeMet exposure are unknown, but previous studies suggested greater concentrations of circulating adrenocorticotropic hormone (ACTH) could lead to greater activation of the melanocortin 2 receptor (MC2R) and a greater availability of cholesterol for cortisol synthesis (Mommsen et al., 1999; Aluru and Vijayan, 2008; Wiseman et al., 2011a). SeMet could also directly activate the MC2R (Wiseman et al., 2011a). Since ACTH levels and *mc2r* transcript abundance or protein expression were not measured in this study, these mechanisms cannot be confirmed. On day 10, the transcript abundance of *hsd11b2* was significantly lower in WS fed 22.4 and 104.4 μg Se/g dm, suggesting the amount of this protein might have been less than in control fish and that conversion of cortisol to its inactive form, cortisone, was less than in controls, which could explain the increase in plasma cortisol (Mommsen et al., 1999).

SeMet exposure also affected *gcr* expression in the liver of juvenile WS. Transcript abundance of *gcr* was down-regulated in the highest exposure group, and albeit not significant, a similar trend was observed at the low and medium concentrations, suggesting lower protein content and lower tissue responsiveness to glucocorticoids, as a result of glucocorticoid stimulation (Mommsen et al., 1999). This down-regulation in response to increased cortisol concentrations is thought to prevent excessive activation of the GCR. However, studies in primary cultures of hepatocytes from rainbow trout found that *gcr* mRNA abundance is regulated through a negative feedback loop by GCR protein content (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). If this is the case in WS, down-regulation of *gcr* transcript abundance might be due to increased GCR protein content in the liver; however, antibodies against WS GCR were not available to test this hypothesis. Reduced GCR protein content in the liver of fish could disrupt cortisol signaling in target tissues and reduce the ability to adjust metabolic pathways necessary to maintain homeostasis, while greater protein expression would allow for increased cortisol signaling (Aluru and Vijayan, 2007; Sandhu et al., 2014). Since changes in energy mobilization occurred along with greater plasma cortisol concentrations, it is unlikely that the cortisol-signaling pathway was inhibited in fish exposed during this study.

Concentrations of cortisol in blood plasma increased in response to the acute handling stressor, but this effect did not appear to be modulated by exposure to SeMet at the concentrations tested. The rapid spike in plasma cortisol levels shortly after stressor exposure and recovery by 24 h is consistent with cortisol responses in rainbow trout exposed to similar acute handling stressors (Aluru and Vijayan, 2006; Gravel and Vijayan, 2007; Wiseman et al., 2007; Wiseman et al., 2011a; Wiseman and Vijayan, 2011; Sandhu et al., 2014). However, the magnitude of the cortisol response appears to be significantly lower in WS compared to teleosts studied to date (Barton and Iwama, 1991; Barton 2002; Pankhurst 2011). In similar studies, plasma cortisol levels in unstressed rainbow trout ranged from ~5-8 ng/ml and increased to ~30-150 ng/ml shortly after exposure to an acute handing disturbance (Aluru and Vijayan, 2006; Gravel and Vijayan, 2007; Wiseman et al., 2007; Wiseman et al., 2011a; Wiseman and Vijayan, 2011), compared to the current study where plasma cortisol in control fed WS increased from 3.4 ± 0.5 ng/ml to 8.4 ± 2.0 ng/ml at 2 h. Lower basal levels of cortisol and low responsiveness in WS compared to teleosts could be due to a lower capacity for cortisol biosynthesis. Differences in the structure of steroidogenic interrenal

cells between chondrostean species and teleosts might also contribute to the difference in cortisol responses (Reviewed in Barton et al., 2000).

Other studies also reported significantly lower cortisol responses to acute handling stressors in pallid sturgeon (*Scaphirhynchus albus)*, hybrid sturgeon (*S. albus x platorynchus*) and paddlefish (*Polydon spathula*), compared to teleosts (Barton et al., 1998; 2000; Barton 2002; Webb et al., 2007). Use of an acute 30 second aerial emersion as a stressor had no significant effect on plasma cortisol over a 24 h period in hybrid and pallid sturgeon; while hybrid and pallid sturgeon exposed to a severe 6 h confinement and handling stressor, had elevated plasma cortisol levels after 1 h of confinement and they remained elevated for the duration of the stressor (Barton et al., 2000). Hybrid and pallid sturgeon had resting plasma cortisol concentrations of ~2 ng/ml and peak concentrations of ~13 ng/ml, but the time to reach peak cortisol concentrations in hybrid sturgeon was 1 h after severe confinement, while pallid sturgeon cortisol levels did not peak until after 6 h of confinement (Barton et al., 2000). However, Belanger et al. (2001) found a large cortisol response (increase from 8.6 ng/ml to ~40 ng/ml) in mature WS, with a peak in cortisol concentrations occurring 30 min after the onset of a water reduction and air emersion stressor. Juvenile WS from the Kootenai River, also had a larger increase in plasma cortisol concentrations (5.6 ng/ml [15.4 nM] to ~32.6 ng/ml [90 nM]) 60 min following a 30 min air exposure (Zuccarelli et al., 2008). Results of these studies and the current study suggest the cortisol response to stress is quite variable in sturgeons and likely dependent on various factors such as genetics, age, size and species of fish, duration and severity of stressor exposure and sampling time point (Reviewed in: Wendelaar Bonga 1997; Barton 2002; Martinez-Porchas et al., 2009; Pankhurst 2011). Small changes in response could also be difficult to detect given the small sample size.

Alternatively, the magnitude of the cortisol response to the acute handling stressor might not have been different from teleosts. Rather, the sampling time point of 2 h post-stressor might have resulted in the observed lower magnitude cortisol response. Peak cortisol levels in fish have been observed within 0.5 – 4 h of exposure to acute stressors (Reviewed in Barton 2002), therefore sampling at a different time point might have been more appropriate to capture peak cortisol concentrations in WS. However, despite differences in study design, lower magnitude cortisol responses seem to be a consistent finding amongst studies in sturgeon compared to teleost species.

The absence of any effect of SeMet on the cortisol response to stress is a contrast to another study where the cortisol response to a handling stressor similar to the one used here, was attenuated

in rainbow trout exposed to 8.47 mg Se/kg dm (Wiseman et al., 2011a). These findings also differ from studies assessing effects of other contaminants on the cortisol response to stress in rainbow trout. Long term exposure to cadmium (Sandhu et al., 2014) and short term exposure to salicylate (Gravel and Vijayan, 2007) and β-naphthoflavone (Aluru and Vijayan, 2006) significantly attenuated the cortisol response to an acute handling stressor in rainbow trout. Although the mechanism(s) of this attenuation is not known, disruption of expression or activity of rate-limiting steroidogenic enzymes, *star* and *p450scc*, and altered ACTH binding to the MC2R, have been proposed as mechanisms of impaired cortisol synthesis in adrenocortical cells of trout exposed to selenite (Miller and Hontela, 2011). Unfortunately, in the current study, the cortisol response could not be assessed in WS fed the highest SeMet diet because of a high mortality rate. Regardless, elevation of cortisol levels and attenuation of a cortisol response to stress occurred at much lower dietary SeMet concentrations in other species of fish (Thomas and Janz, 2011; Wiseman et al., 2011a) compared to WS in the present study, suggesting the cortisol response in WS is less sensitive to SeMet. Effects on the cortisol response were also likely not the main driver of SeMet toxicity in WS since, in the same study, pathologies occurred in WS fed lower concentrations of SeMet (Zee et al., 2016a) and effects on the cortisol response to stress were not observed at these concentrations.

As with cortisol, there were significant differences in concentrations of plasma glucose and lactate in WS exposed to 104.4 μg Se/g dm. At 0 h, plasma glucose levels in WS fed the diet containing 104.4 μg Se/g dm were significantly lower compared to controls, suggesting depletion of glucose as a result of increased energy demands. Since cortisol is known to regulate energy metabolism, it is likely that the elevation in basal cortisol levels stimulated greater glucose mobilization for use as an energy substrate, which lead to a decrease in plasma glucose levels (Wendelaar Bonga 1997; Mommsen et al., 1999). Transcript abundance of *pepck* appeared to increase with greater SeMet exposure, indicating enhanced gluconeogenic activity in the liver (Mommsen et al., 1999). This is consistent with the elevated basal plasma cortisol levels in WS fed the highest dose of SeMet, since cortisol has been found to positively regulate *pepck* mRNA abundance (Sathiyaa and Vijayan, 2003). Low glucose concentrations despite increased gluconeogenesis could be a result of a high turnover rate of glucose (Mommsen et al., 1999). Lower glucose levels could also be a result of fasting, since food aversion occurred in all high dose WS by day 21 of the exposure (Vijayan and Moon, 1994; Zee et al., 2016a). Furthermore, plasma

lactate concentrations were significantly greater in the highest exposure group, suggesting glucose was metabolized under anaerobic conditions, since lactate is a by-product of anaerobic glycolysis. Thomas et al. (2013), also observed significantly greater lactate concentrations in zebrafish fed 27.5 µg Se/g dm, compared to controls. Like cortisol, basal levels of glucose and lactate appear to be variable even within the same species. Basal levels of plasma glucose and lactate in this study were comparable to concentrations in some rainbow trout (Gagnon et al., 2006; Gravel and Vijayan, 2007; Miller et al., 2007), but greater than those found in previous sturgeon studies (Barton et al., 2000; Zuccarelli et al., 2008). Basal plasma lactate concentrations were also significantly lower in WS compared to trout in the study by Wiseman et al. (2011). Again, the variability is likely a result of differences between species and experimental methods used in each study.

Exposure to SeMet affected glycogen concentrations in liver tissue. There was a trend of decreasing glycogen concentrations in livers, with WS fed 104.4 µg Se/g dm having significantly lower levels than controls. These findings are consistent with other studies where WS exposed to > 40.0 µg Se/g dm had significant glycogen depletion in liver tissues (Tashjian et al., 2006; De Riu et al., 2014). Depletion of energy stores in the liver, but not in the muscle, could suggest that liver glycogen was a more significant energy source in WS exposed to SeMet. Increased liver glycogen mobilization as a result of greater catecholamine and cortisol secretion can also explain the depletion of energy stores in high dose WS (Vijayan and Leatherland, 1989; Reid et al., 1998; Mommsen et al., 1999). However, it is difficult to determine whether the significant depletion of liver energy stores in high dose WS sampled at 0 h, is a direct effect of SeMet exposure or an indirect effect caused by aversion to Se laced food.

There was a typical response to the acute handling stressor with glucose and lactate levels in blood plasma from control, low and medium dosed fish increasing at 2 h and returning to pre-stressor levels at 24 h, which is consistent with responses in other fish species (Mommsen et al., 1999; Gravel and Vijayan, 2007; Wiseman et al., 2011a; Sandhu et al., 2014). Acute stressor-induced increases in plasma glucose and lactate concentrations in fish seem to be affected variably by contaminants. Previously, SeMet in rainbow trout was found to disrupt the stressor-induced increase in plasma glucose, while plasma lactate levels were not affected (Wiseman et al., 2011a). Similarly, exposure to salicylates had no effect on concentrations of plasma glucose or lactate in rainbow trout subjected to an acute handling stressor (Gravel and Vijayan, 2007). However,

stressor-induced increases in plasma glucose and lactate were attenuated in rainbow trout exposed to cadmium (Sandhu et al., 2014), while the plasma lactate response to an acute handling stressor increased in rainbow trout exposed to Aroclor 1254 (Wiseman and Vijayan, 2011). The lack of effects of SeMet on acute stressor-induced increases in plasma lactate and glucose further supports the idea that the physiological response to stress is not greatly modulated in WS exposed to < 22.4 μg Se/g dm.

Effects of the acute handling stressor on muscle glycogen concentrations were attenuated by exposure to SeMet. The significant decrease in muscle glycogen of control WS at 2 h is consistent with increased plasma glucose concentrations at 2 h, in the same fish. When exposed to an acute stressor, stimulation of glycogenolysis by catecholamines results in a rapid increase in glucose production to meet energy demands of recovering from stress (Mommsen et al., 1990; Reid et al., 1998). Muscle glycogen concentrations in WS given diets containing the low and medium concentrations of SeMet and sampled at 2 h, did not differ from concentrations in WS at 0 h, even though plasma glucose concentrations increased in the same fish. This could suggest an effect on their ability to utilize muscle glycogen as an energy source. The increased plasma glucose at 2 h also was not a result of breakdown of liver glycogen, but the significant decrease in liver glycogen at 24 h suggests it likely was a source of energy used to fuel recovery from the acute stressor. Contrary to these results, Wiseman et al. (2011) found no effect of handling stressor or SeMet diet on liver glycogen concentrations in rainbow trout, while muscle glycogen decreased in trout sampled 24 h post-stressor. Trout exposed to SeMet also had greater overall concentrations of muscle glycogen compared to controls (Wiseman et al., 2011a). Greater whole body concentrations of glycogen have also been reported in zebrafish exposed to SeMet (Thomas and Janz, 2011) and in northern pike (*Esox lucius*) sampled from metal-mining contaminated lakes (Kelly and Janz, 2008), while whole body glycogen and liver glycogen concentrations decreased in fathead minnows (McPhee and Janz, 2014) and Sacramento splittails (*Pogonichthys macrolepidotus*) (Teh et al., 2004) exposed to dietary Se. The variability of effects on energy storage further suggests differences in species sensitivity to SeMet exposure.

Overall findings from the current study are consistent with previous studies that show deleterious effects start to occur in WS exposed to ≥ 20 μg Se/g in their diet (Tashjian et al., 2006; De Riu et al., 2014; Zee et al., 2016a), placing WS among the more tolerant species to SeMet exposure with regard to the endpoints investigated here (Hamilton, 2004; USEPA, 2016). Zee et

al. (2016a) found WS in the same study to be amongst the most sensitive species when considering mild edema as an endpoint, although the biological relevance of this is unknown. WS appear to be less sensitive to SeMet in regard to effects on the physiological stress response and energetics, compared to other species of fish such as rainbow trout and zebrafish (Thomas and Janz, 2011; Wiseman et al., 2011a). However, further investigation is warranted to determine effects of SeMet on the physiological stress response at concentrations between 22.4 and 104.4 µg/g dm, since exposure to dietary concentrations within this range appear to adversely affect the performance of juvenile WS (Tashjian et al., 2006; De Riu et al., 2014; Zee et al., 2016a). In addition to possessing a physiological stress response that is less sensitive to SeMet than the response of teleost fishes, WS appear to have an overall lower capacity to respond to acute stress, which could have consequences on their ability to cope with other environmental stressors.

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CHAPTER 3: WHOLE TRANSCRIPTOMIC RESPONSE OF JUVENILE WHITE STURGEON (*ACIPENSER TRANSMONTANUS*) TO DIETARY SELENOMETHIONINE

3.0 Preface

Chapter 2 revealed that exposure to a high dose of dietary SeMet can alter endpoints related to the physiological stress response and energetics in WS; however, these alterations are likely not responsible for the pathologies that were observed in WS by the end of the 72 d study, including edema, as these effects were observed to some degree in WS from all SeMet dosed groups. Therefore, in this research, Illumina sequencing was conducted to investigate transcriptome level responses that could be indicative of other unknown sublethal effects of dietary SeMet exposure in WS, as well as to identify molecular initiating events that could be linked to the pathological endpoints observed at the end of the study and discussed in Chapter 2 and a parallel study (Zee et al., 2016a). Chapter 3 discusses the results of the Illumina sequencing and the potential pathways of SeMet toxicity in WS.

This chapter will be submitted as a manuscript to *Aquatic Toxicology* under joint authorship with Jenna Zee (University of Saskatchewan), Song Tang (Chinese Center for Disease Control and Prevention), Steve Wiseman (University of Saskatchewan, University of Lethbridge) and Markus Hecker (University of Saskatchewan). The tables, figures and references cited have been reformatted here to the thesis style. References cited in this chapter are listed in the references section of this thesis.

The author contributions to Chapter 3 were as follows:

- Sarah Patterson (University of Saskatchewan) conducted the SeMet exposure, collected the samples, extracted mRNA, performed the library preparation for Illumina sequencing, performed qPCR analysis, assisted with RNA-seq data analysis, conducted the ClueGO pathway analysis, and wrote the manuscript.
- Jenna Zee (University of Saskatchewan) co-managed the SeMet exposure, assisted with sample collection, assisted with extracting mRNA and library preparation for

Illumina sequencing, and will review the manuscript before submission.

- Song Tang (Chinese Center for Disease Control and Prevention) provided training and support for the RNA-seq data analysis, assisted with ClueGO analysis, and will review the manuscript before submission.
- Steve Wiseman (University of Saskatchewan, University of Lethbridge) provided guidance and assistance in conducting the exposure and collecting samples, as well as, provided guidance and assistance in analyzing the qPCR and transcriptome data, and he will provide revisions prior to submission of the manuscript.
- Markus Hecker (University of Saskatchewan) provided funding to conduct this research and provided guidance and assistance with the experimental design, collection of samples, and data analysis, and reviewed and revised the manuscript.

3.1 Abstract

Although selenium (Se) is an essential micronutrient, it is also considered a contaminant of great concern for aquatic systems due to its ability to bioaccumulate into the food chain, primarily in its organic form, selenomethionine (SeMet). White sturgeon (WS; *Acipenser transmontanus*) are potentially more susceptible to bioaccumulative contaminants such as SeMet, due to their long lifespan and benthic lifestyle. Oxidative stress has been hypothesized as the primary mechanism causing SeMet toxicity in teleost fish; however, a parallel study found little evidence of this in WS, and little else is known about the mechanism(s) leading to dietary SeMet toxicity in WS. Therefore, the goal of this study was to identify pathways of SeMet toxicity in juvenile WS, after a 10 d exposure period, by use of an unbiased RNA-sequencing (RNA-seq) approach to determine changes in gene expression across the whole transcriptome. Juvenile WS were fed either a control diet containing 1.4 µg Se/g dry mass (dm), or a spiked diet containing 5.6, 22.4 and 104.4 µg Se/g dm, for 72 d. After 10 d, a sub-set of fish were euthanized, and liver samples were collected for use in Illumina sequencing. The study continued for 72 d with the remaining fish, and any adverse effects reported in a parallel study were correlated with molecular toxicity pathways characterized in this study, to establish links between the pathways and actual adverse effects. Out of 13 900 contigs, a total of 178 and 147 transcripts were significantly down- and up-regulated, respectively, by dietary SeMet in the livers of WS. Pathway analysis, conducted using the Cytoscape plug-in ClueGO, identified a greater proportion of pathways associated with down-regulated genes. Pathway analysis revealed several physiological processes altered by SeMet exposure, most of which were associated with energy, cholesterol/lipid, and protein metabolism. These pathways could be associated with the reduction in hepatic energy stores (lipid and glycogen), reduced growth and increased edema and mortality observed in WS later on in the exposure, suggesting these pathways may play an important role in SeMet toxicity in this species.

3.2 Introduction

Selenium (Se) is a naturally occurring trace element whose environmental contamination is of significant concern for aquatic ecosystems (Lemly and Smith, 1987; Lemly, 1997a; Janz, 2012). The issue is confounded by Se being an essential micronutrient that is critical to the formation of selenoproteins in vertebrates, such as fish (reviewed in Janz, 2012). Fish require between 0.1 and 0.5 μg Se/g dry mass (dm) through the diet (Hodson and Hilton, 1983; Lemly, 1997b); however, dietary concentrations greater than 3 μg Se/g dm have been found to cause adverse effects in certain species of fish, indicating a narrow margin between essentiality and toxicity (Lemly, 1997a; Hamilton, 2004). One organic form of Se, selenomethionine (SeMet), is of particular concern because it persists in the environment and bioaccumulates in the food chain, leading to the transfer of potentially toxic concentrations to more sensitive, higher trophic level organisms (Lemly and Smith 1987; Stewart et al., 2010; Janz, 2012). Selenium can cause a wide range of adverse effects in fish including larval deformities (skeletal, craniofacial and fin malformations), reduced growth, reproductive impairment, tissue pathologies, and mortality (Lemly, 2002; Hamilton, 2004).

Although Se has been studied extensively in selected teleost species, such as zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynnchus mykiss*), among others, there is increasing interest in studying the sensitivity of native species of concern such as white sturgeon (WS; *Acipenser transmontanus*). White sturgeon are an endangered species of fish that have been found to be more susceptible to certain bioaccumulative contaminants than other fish species, because of their longevity and benthic lifestyle (Little et al., 2012; Vardy et al., 2011, 2013). High levels of Se have been detected in the tissues of WS in the Kootenai River (Kruse and Scarnecchia, 2002) and the San Francisco (S.F.) Bay-Delta (White et al., 1988; Urquhart and Rigaldo, 1991; Linville et al., 2002; Linares-Casenave et al., 2014), as well as in filter-feeding bivalves, such as the Asian clam (*Potamocorbula amurensis*), which are a major component of the WS diet in the S.F. Bay-Delta (Linville et al., 2002). Therefore, increased risk of Se inputs into WS habitats, particularly the Kootenay River and San Francisco (S.F.) Bay-Delta, may be detrimental to the survival of this endangered species in these environments (Presser and Luoma, 2006; Dessouki and Ryan, 2010).

Previously, WS have been reported to be sensitive to effects of dietary Se exposure, displaying a range of apical effects including decreased growth, activity and feeding behavior, reduced energy reserves, edema, histopathological alterations, and mortality (Linville, 2006; Tashjian et al., 2006; De Riu, 2014; Zee et al., 2016a; Patterson et al., 2017).

Despite the increasing body of literature on Se toxicity in WS and other fishes, the specific mechanism(s) of SeMet toxicity in fish is still poorly understood. Previous research suggested that Se toxicity resulted from substitution of Se for sulfur in the amino acids methionine and cysteine, preventing the formation of disulfide bonds (S-S) and causing alterations in protein structure and function (Lemly, 2002; Janz, 2012). This theory is no longer supported, and more recent research has indicated that oxidative stress likely plays an important role in Se toxicity in fish (Palace et al., 2004; Misra et al., 2010, 2012a). However, Zee et al. (2016b) did not find significant evidence of oxidative stress in juvenile WS exposed to SeMet, suggesting this pathway might not be critical to SeMet toxicity in this species. Juvenile WS also responded differently to SeMet exposure than other fishes based on the development of edema at low concentrations, as well as the lack of effects on the physiological response to stress at low concentrations (Zee et al., 2016a; Patterson et al., 2017). Given the different responses of WS to SeMet, with respect to multiple endpoints, compared to teleost fishes, and the limited knowledge of the mechanism(s) of Se toxicity in WS, it is important to provide further insight into the mechanisms of SeMet toxicity in this species. Molecular analysis is a valuable tool for investigating toxicity of contaminants, given that alterations at the molecular level, such as mRNA abundance, represent early signs of a toxic response and can provide mechanistic information for alterations at the physiological level.

To date, studies assessing molecular level effects of SeMet have primarily been conducted in teleost species such as rainbow trout and have utilized quantitative real-time PCR (qPCR) or microarray analysis (Palace et al., 2004; Misra et al., 2010, 2012a; Knight et al., 2016; Pacitti et al., 2016). These technologies are limited as they require prior knowledge about sequences to construct transcript-specific probes or primers and are not ideal for characterizing gene expression in non-model species such as WS (Reviewed in Qian et al., 2014). In contrast, next-generation sequencing technologies, such as RNA-

sequencing (RNA-seq), enable unbiased analysis of whole transcriptomes. Obtaining an unbiased view of the transcriptional profile allows for the discovery of novel molecular responses and improves the ability to identify relationships between molecular responses and adverse biological effects, making RNA-seq a powerful tool to investigate the impact of contaminants on non-model species, with limited genetic information available (Mehinto et al., 2012; Illumina, 2017).

Therefore, the goal of this study was to characterize molecular responses in juvenile WS using an RNA-seq approach, to identify potential novel pathways of SeMet toxicity that will inform future studies for specific toxicity endpoints. Links between the initial molecular responses to SeMet exposure and adverse effects observed after 72 days of exposure (Zee et al, 2016a; Patterson et al., 2017) were also investigated.

3.3 Materials and methods

3.3.1 Test chemical and species

Seleno–L-methionine (SeMet) (purity > 98%) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Approximately 3 year old, juvenile WS were reared in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan, from eggs donated by the Kootenay Trout Hatchery (Fort Steele, BC, Canada). Sturgeon cultures were maintained at approximately 12°C in 712 L flow-through tanks and fed approximately 1.5% of their body weight of trout chow (Martin, Profishent Aquaculture Nutrition 6PT; Elmira, ON, Canada).

3.3.2 Experimental design

As previously described in Patterson et al. (2017) and Zee et al. (2016a), WS (n = 5 per replicate tank) were randomly assigned to 28, 712 L tanks, with 7 replicate tanks per dose. Water temperature was maintained at 13.4 ± 0.4°C (mean ± SD) with a minimum flow rate of 50L/h in each tank and a 16 h light: 8 h dark photoperiod. After 1 week of

acclimation, sturgeon were switched from control feed to the respective spiked feed. Feed was spiked with nominal concentrations of 0, 5, 25 and 125 μg Se/g dry mass (dm) in the form of SeMet, and average total Se concentrations were determined to be 1.4, 5.6, 22.4, and 104.4 μg/g dm by use of inductively coupled plasma-mass spectrometry (ICP-MS) (Zee et al., 2016a). Fish were fed 1.5% of their body weight, once daily, 6 times a week, for 72 d. Ten days following initiation of the exposure, a sub-sample of 28 fish (1 from each tank) were euthanized with a quick blow to the head and livers were dissected, flash frozen, then stored at -80°C for molecular analysis. Fish sampled at 72 d were used for analysis of biological and pathological effects such as the cortisol response to stress and histopathology (Zee et al., 2016a; Patterson et al., 2017). The results from those studies were compared to molecular changes observed on day 10.

3.3.3 Library preparation and RNA-sequencing

Liver tissues collected from WS on day 10 of the exposure were analyzed following methods similar to those presented in Doering et al. (2016) by RNA-seq, using in house *Illumina* Mi-Seq technology. Liver samples from 3-4 fish were pooled for both the control and medium dose (22.4 μg Se/g dm) groups. Due to the high cost of Mi-Seq analysis, only 2-pooled samples, one from the control group and medium dose, were analyzed using RNA-seq. However, confirmation of selected target genes by qPCR was conducted on six or seven individuals per treatment group to verify sequencing results (see section 3.3.6). Total RNA was extracted from liver tissue using an RNeasy lipid mini kit with the addition of an on-column DNase digestion step (Qiagen, Mississauga, ON, Canada), following the manufacturer's protocol and quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Equal amounts of RNA were pooled to obtain a sample of 4 μg RNA per treatment. One RNA-seq library was prepared for each treatment, using a Tru-Seq RNA Sample Prep Kit (*Illumina*, San Diego, CA, USA) according to manufacturer's protocol. Library quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and total DNA concentrations were determined by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop

Technologies). Each library was loaded separately onto Mi-Seq Reagent Kits v2 (*Illumina*) and run as 75-base pair (bp) paired-end reads on a Mi-Seq sequencer (*Illumina*), at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK). Raw reads were made available online in the National Centre for Biotechnology Information (NCBI) Gene expression Omnibus (GEO) (Accession # GSE79624).

The quality of raw reads was assessed using FASTQC on the open, web-based platform, Galaxy (http://galaxy-qld.genome.edu.au/galaxy). Reads from the control and medium dose liver samples were aligned to a WS reference transcriptome, with 75% alignment. The reference transcriptome was constructed on CLC Genomics Workbench v.5.0 (CLC Bio, Aarhus, Denmark) by performing 3 rounds of *de novo* assembly on paired-end reads of RNA isolated from sturgeon livers from this study and other studies described previously (Doering et al., 2014, 2016). This reference transcriptome was made available online in the NCBI GEO (Accession #GSE79624).

3.3.4 Differential expression analysis

Mapped reads were filtered based on counts per million (CPM) of > 5 for at least one sample (reads with CPM < 5 removed for one sample) using the R software v.3.1.2 package, edgeR (R Foundation for Statistical Computing, Vienna, Austria). Reads with zero CPM in one sample were also removed. After filtering, the control and SeMet treatment libraries consisted of 14 051 774 and 17 834 683 reads, respectively, and there were 13 900 contigs in total. CPM for contigs in the SeMet treated sample were compared to CPM in the control to calculate the fold-change for each contig. A false discovery rate (FDR - adjusted p-value) of 0.05 was used as the cutoff for significance of differentially expressed contigs. The change in abundance in the SeMet treated group was considered significant if it was ≥ ± 2-fold, relative to controls.

3.3.5 Annotation and pathway analysis

Differentially expressed contigs were annotated using Local Blast in Blast2GO v.2.5.0 software. White sturgeon contig IDs were blasted against the zebrafish genome, due to the lack of WS annotation information, which produced a list of equivalent zebrafish protein accession number IDs. BioDBnet software was then used to convert zebrafish protein accession number IDs to Refseq mRNA IDs, for use in pathway analysis.

ClueGO v.2.1.7 (Bindea et al., 2009), a Cytoscape v.3.2.1 (Shannon et al., 2003) plug-in, was used for the functional analysis and visualization of significantly up- and down-regulated transcripts by creating a Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome and Biocarta pathway term network. Refseq mRNA IDs associated with up- and down-regulated transcripts were loaded as 2 separate clusters on ClueGO and annotated with ontology and pathway data for zebrafish to create a functionally grouped annotation network. The statistical test performed by ClueGO was a right-sided hypergeometric test with a Bonferroni (step down) p value correction and a kappa score threshold of 0.4.

3.3.6 RNA-seq validation by quantitative real-time PCR

A subset of 12 transcripts was analyzed using qPCR to validate the RNA-seq data. Total RNA was isolated from liver samples from 6 control fish and 7 medium dose fish following the manufacturer's protocol using a Qiagen RNeasy lipid mini kit (Qiagen, Mississauga, ON, Canada) and quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol.

Quantitative real-time PCR (qPCR) was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 70 μL reaction mixture, consisting of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen), 3.5 μL of cDNA, 3.5 μL of gene-specific PCR primers (Table A1) and nuclease free water, was prepared for each cDNA sample and primer combination. Reactions were performed in triplicate with 20 μL reaction volumes per well. Before the first PCR cycle, the reaction mixture was denatured at 95°C for 10 min. The thermal profile consisted of denaturing at

95°C for 10 s and extension for 1 min at 60°C for a total of 40 PCR cycles. Dissociation curves were generated for all samples to ensure amplification of a single PCR product. Transcript abundance of target genes was normalized to β-actin according to methods previously described by Simon (2003).

3.4 Results and discussion

3.4.1 Global changes in transcript abundance

Exposure to dietary SeMet led to significant alterations in transcript abundance in the livers of juvenile WS. RNA-seq analysis revealed that out of approximately 13 900 contigs, 725 contigs were identified as significantly altered by ≥ 2-fold, relative to controls in the livers of WS. The expression of 348 contigs was ≥ 2-fold down-regulated relative to controls and expression of 377 contigs was ≥ 2-fold up-regulated. Out of the 725 contigs significantly altered, 147 up-regulated transcripts and 178 down-regulated transcripts matched to unique zebrafish protein accession number IDs and could be analyzed using the Cytoscape plug-in, ClueGO.

Fold-change analysis of selected transcripts via qPCR was performed to confirm the results from RNA-seq analysis, since RNA-seq could only be performed on one pooled sample for both the control and medium dose group. The fold-change in transcript abundance as determined by qPCR showed a good congruency (83% of the genes were confirmed to show the same trend in change) to RNA-seq (Table 3.1), confirming the reliability of the RNA-seq analysis. Although the fold-change in abundance varied between RNA-seq and qPCR, the direction of expression (up- or down-regulated) was the same for majority of the transcripts. Previously, transcript level measurements in fish by RNA-seq were found to be reliable and comparable to results obtained from microarrays (Oleksiak et al., 2011) and qPCR (Brinkmann et al., 2016; Doering et al., 2016), which is in accordance with our observations.

Table 3.1. Selected transcripts altered by at least 2-fold in livers of WS exposed to 22.4 µg Se/g for 10 days. Fold-change in transcript abundance as determined by RNA-seq analysis versus qPCR.

Gene Name	Gene ID	Biological Function	RNA-seq Fold-Change	qPCR Fold-Change
phosphoenolpyruvate carboxykinase	*pepck*	gluconeogenesis	+57	+29
fructose- 1,6-bisphosphatase	*fbp1a*	gluconeogenesis	+8.7	+2.2
glucose-6-phosphatase	*g6pca*	gluconeogenesis	+2.7	+1.0
apolipoprotein a-1a	*apoa1a*	cholesterol homeostasis	-909	-5.7
cholesterol 7-alpha-monooxygenase	*cyp7a1a*	cholesterol homeostasis	-3.2	-2.9
ATP-binding cassette, sub family B member 11	*abcb11b*	bile acid/salt transport	-3.9	-2.1
proprotein convertase subtilisin kexin type 5	*pcsk5*	serine-type endopeptidase activity	+5.3	-1.1
glutathione-s-transferase	*gst*	xenobiotic metabolism	+2.8	+1.9
cytochrome p450 1a	*cyp1a*	xenobiotic metabolism	-2.7	-1.8
apoptosis inducing factor	*aif*	apoptosis	-3.4	+1.1
ATP-binding cassette, sub family D member 3	*abcd3a*	peroxisomal ABC transporters	-2.9	-2.2
fatty acid binding protein 7, brain	*fabp7b*	PPAR signaling pathway	-4.3	-1.6

To our knowledge, this is the first study to use RNA-seq analysis to assess responses to potentially toxic levels of dietary SeMet across the whole transcriptome in fish. Most studies to date have used microarray or qPCR analysis to investigate transcript level effects of Se in fish. In a previous study, microarray analysis identified 449 differentially expressed genes in livers of juvenile rainbow trout exposed to varying doses of dietary organic Se (selenized yeast) for 60 d, relative to controls, with majority of those genes identified in fish fed the two highest Se diets (178 genes and 254 genes in trout exposed to

19.5 and 31.8 mg/kg Se dw, respectively) (Knight et al., 2016). All Se treated groups, had a greater number of up-regulated genes than down-regulated genes, relative to controls (Knight et al., 2016). Pacitti et al. (2016) assessed transcriptomic responses in liver and head kidney tissue of rainbow trout fed diets containing 6.35 mg/kg of organic Se, as Se-yeast, for 10 weeks. Microarray analysis identified 133 differentially expressed (≥ 2-fold) genes in the liver and 539 in the head kidney, relative to controls. In the liver, 71 genes were up-regulated and 62 were down-regulated; while 417 were up-regulated and 122 down-regulated in the head kidney (Pacitti et al., 2016). Dietary Se caused a slight increase in overall gene expression in the livers of rainbow trout, which is similar to the findings in WS from the current study, based on the number of up- versus down-regulated contigs. However, following annotation, a greater number of down-regulated transcripts were identified. ClueGO analysis also identified more physiological processes associated with down-regulated genes, which is in contrast to the above studies. The difference in the proportion of up- versus down-regulated genes could be due to not all genes being annotated and included in the pathway analysis. A similar number of genes were differentially expressed in WS and rainbow trout livers, after Se exposure (Knight et al., 2016; Pacitti et al., 2016). The difference in proportions of up- versus down-regulated genes between the above discussed rainbow trout studies and WS in the current study, could be explained by the use of different methodologies (RNA-seq versus microarray) since microarray studies are limited in their analysis and require prior selection of genes. Differences between species and in study design (exposure length and level of Se treatment) could also explain this difference. Transcriptome responses were analyzed in trout after 60 and 70 d in Knight et al. (2016) and Pacitti et al. (2016), respectively, whereas RNA-seq was conducted on WS livers after 10 d of Se exposure, in order to capture the initial molecular responses. With increasing exposure time, gene expression patterns tend to shift towards compensatory processes. The number of genes that were able to be annotated in the current study may have also impacted this difference.

3.4.2 Pathway Analysis

The Cytoscape plug-in, ClueGO, was used to create a GO/KEGG/Reactome

pathway term network, which allowed for the visualization of physiological processes in WS significantly altered by SeMet (Fig. 3.1). ClueGO analysis identified 63 altered terms or pathways (most significant terms shown in Fig. 3.2, list of all in Fig. A.1 and Fig. A.2), with many associated with a higher proportion of down-regulated genes. The main processes altered by dietary SeMet included those involved with energy, cholesterol/lipid, protein and xenobiotic metabolism.

The overall trend towards a greater number of down-regulated biological processes is consistent with the significant pathologies observed in WS after 72 d of exposure to 22.4 µg Se/g dm in the diet, specifically the occurrence of edema in 41% of WS leading to mortality in 22% of these fish, relative to the controls, which had no incidents of edema or mortality (Zee et al., 2016a).

The pathway analysis results are separated into up- versus down-regulated pathways, based on the directional change of the majority of genes associated with each pathway. However, given the complexity of gene expression regulation, it should be noted that the direction of change in expression may not be as important or as reliable of an endpoint as the overall change, considering gene expression patterns can represent compensatory mechanisms.

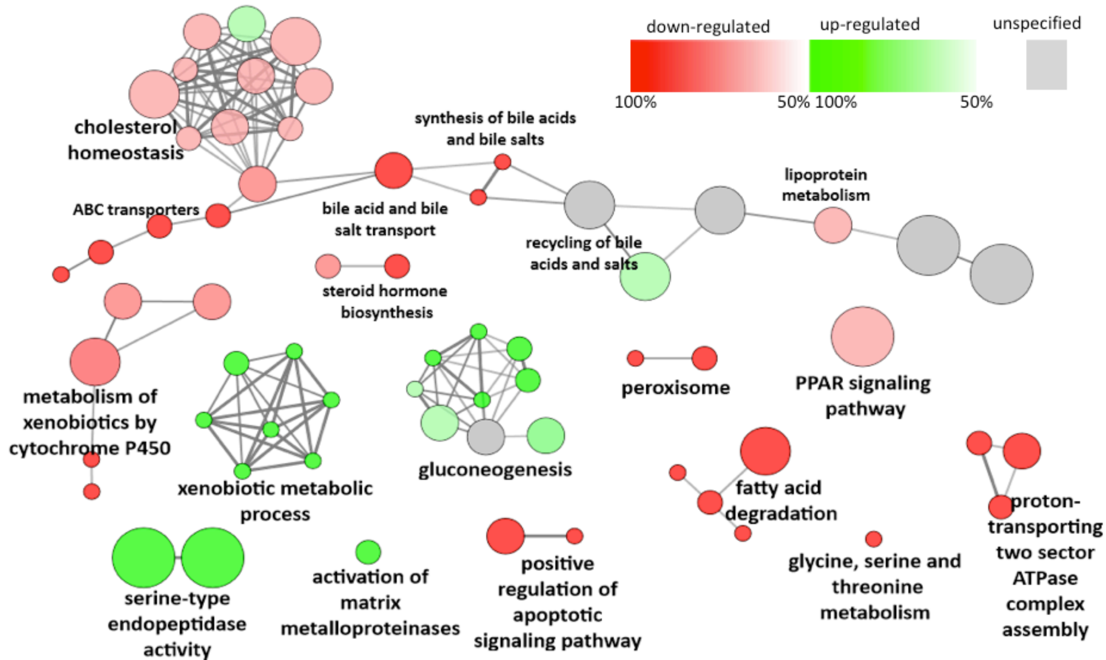

Figure 3.1. Visualization of altered GO terms and KEGG/Reactome pathways in the liver of WS exposed to SeMet, by use of the Cytoscape plug-in, ClueGO (Bindea et al., 2009). Red and green nodes indicate terms associated with down-regulated and up-regulated genes, respectively. The color gradient shows the proportion of up and down-regulated genes associated with each term. Lines connecting different nodes indicate shared genes among these categories. Grey nodes indicate a 50/50 ratio of up- and down- regulated genes. The size of the nodes reflects the enrichment significance of terms.

Figure 3.2. GO/KEGG/Reactome pathway terms associated with a greater proportion of up-regulated (A) or down-regulated (B) genes, identified in livers of juvenile WS fed a diet with 22.4 µg Se/g dm, relative to controls. The name of each group represents the most significant term in the group. Bars represent the percentage of genes found from the uploaded cluster, compared to all the genes associated with the term. Numbers at the end of each bar indicate the number of genes assigned to each term. Only the most significant terms are shown ($p < 0.05$ after Bonferroni correction) (Bindea et al., 2009). A complete list can be found in Appendix A (Fig. A.1 and Fig A.2).

3.4.3 Up-regulated pathways

ClueGO analysis identified 22 physiological processes that were up-regulated in the livers of WS exposed to dietary SeMet (Fig. A.1). Nine of these were considered significant ($p < 0.05$ after Bonferroni correction) (Fig. 3.2). While many pathways were altered, the majority appear to be associated with energy (gluconeogenesis, gluconeogenesis/glycolysis and glucose metabolic process) and protein metabolism (serine type peptidase/endopeptidase activity).

Gluconeogenesis is responsible for glucose synthesis in the liver and this pathway is typically enhanced in response to stress to meet increased demands for energy production (Moon et al., 1987; Mommsen et al., 1999). Genes that encode for key enzymes in this pathway including, fructose-1,6-biphosphatase 1a (*fbp1a*), glucose-6-phosphatase a (*g6pca*) and phosphoenolpyruvate carboxykinase (*pepck*) were significantly up-regulated (Table 3.1). While gluconeogenesis did not appear enhanced in medium dose WS at the end of the 72 d SeMet exposure, there were significant alterations in energy metabolism in the high dose group from the same experiment, that are indicative of increased demands on energy production and increased gluconeogenic activity (Patterson et al., 2017). White sturgeon in the high dose group had significantly depleted energy stores, including liver glycogen and plasma glucose, as well as significantly increased plasma cortisol and lactate levels, and reduced growth, suggesting increased mobilization of energy stores to meet the increased metabolic demands of repairing damage from SeMet (Zee et al., 2016a; Patterson et al., 2017). There was limited evidence that the medium dose of SeMet hindered the ability of WS to utilize muscle glycogen as an energy source after exposure to a secondary stressor; however, the lack of significant effects on other energy substrates in medium dose WS, despite changes at the level of the transcriptome, suggest that the majority of WS were still able to maintain energy homeostasis at this dose. Selenium tissue concentrations also did not reach steady state in medium dose fish by the end of the exposure. Based on Se analysis in muscle tissue of high dose mortalities, Se concentrations plateaued between 60 and 70 µg/g dm around day 40 of the study, while Se muscle tissue concentrations in medium dose fish were only approximately 20 µg/g on day 72 (Zee et al., 2016a). Regardless, molecular response patterns pertaining to energy homeostasis, determined

early during this exposure experiment were indicative of later adverse effects in higher dose-groups, and as such appear to represent useful indicators of long-term apical outcomes.

Altered energy metabolism is a common feature of dietary SeMet exposure in fish, however the responses are varied. Wiseman et al. (2011a) suggested that increases in muscle glycogen production in rainbow trout after exposure to dietary SeMet could be associated with enhanced gluconeogenic activity. Studies in zebrafish have found SeMet to cause both an increase (Thomas and Janz, 2011) and decrease (McPhee and Janz, 2014) in whole body concentrations of glycogen. Liver glycogen stores also decreased in WS after exposure to dietary SeMet (Tashjian et al., 2006; De Riu et al., 2014). In contrast to the current study and others, there were no significant differences in transcript abundance of genes related to glycolysis and gluconeogenesis, including phosphoenolpyruvate carboxykinase and fructose 1,6-bisphosphatase, as well as genes related to other energy production processes (citric acid cycle, pentose phosphate pathway and fatty acid synthesis) in rainbow trout exposed to organic Se (Knight et al., 2016). Liver glycogen concentrations in trout fed Se were variable but not different from controls (Knight et al., 2016). Differences in species physiology and study design could result in these variable responses. In the future, endpoints such as gluconeogenic enzyme activity should be analyzed to fully elucidate these responses to SeMet.

Serine type endopeptidase/peptidase are proteolytic enzymes involved with various processes such as digestion, immune function, blood coagulation, fertilization and apoptosis (Neurath, 1984; Page and Di Cera, 2008). Genes encoding for proteolytic enzymes that were up-regulated included elastase 2 like (*ela2l*), proprotein convertase subtilisin/kexin type 5b (*pcsk5b*), tripeptidyl peptidase 1 (*tpp1*), chymotrypsin-like (*ctrl*) and trypsin-like (*try*). Enhanced protein degradation could increase the free supply of amino acids available as a substrate for gluconeogenesis (Mommsen et al., 1999), which would be in accordance with the greater energy metabolic activity observed in this study (Patterson et al. 2017). This is consistent with WS in Tashjian et al. (2006) as well, where whole body protein content decreased with increasing dietary SeMet. Proteolytic activity could also be enhanced to increase the removal of damaged proteins and be an indicator of oxidative stress; however, there was little evidence of hepatic oxidative stress in WS from

the same study (Zee et al., 2016b). Protein degradation could also be associated with the occurrence of edema and exophthalmos in WS from the same treatment group (Zee et al. 2016a); however, further investigation is required to assess the potential link between these adverse effects and altered expression of proteolytic enzymes.

Other altered processes included recycling of bile acids and salts, neuron projection regeneration, and scavenging of heme from plasma (Fig. 3.2). Recycling of bile acids and salts was associated with an up-regulation of genes that encode for transport proteins, including fatty acid binding protein (*fabp*) and vitamin D-binding protein (*gc*), which is also involved with binding fatty acids (Chun, 2012). Up-regulation of these genes, which are important for bile, fatty acid and lipid homeostasis, could be associated with the increased utilization of energy stores in response to SeMet exposure and with the reduced hepatic lipid stores observed in WS after SeMet exposure (Zee et al., 2016a). Effects on bile and lipid processes are discussed in more detail in section 3.4.4. Up-regulation of the gene, major vault protein (*mvp)*, was associated with neuron projection regeneration. The function of *mvp* is not well understood in aquatic species, but in mammals it has been associated with cell signaling, drug metabolism/resistance and tumor formation (Margiotta et al., 2017). Some research in fish has found *mvp* mRNA is highly expressed in macrophages and liver lesions and it is induced by xenobiotics causing DNA damage (Margiotta et al., 2017). Up-regulation of *mvp* in the current study could be a response to cell damage caused by SeMet and this may be associated with the signs of liver toxicity observed in high dose WS at the end of the exposure (Zee et al., 2016a). Scavenging of heme from plasma prevents over accumulation of free heme following hemolysis, which is otherwise damaging to tissues (Nielsen et al., 2010). Given the significant edema observed in WS in the same study, it would be important to investigate processes related to hemolysis in the future. Increased expression of hemoglobin, alpha adult 1 (*hbaa1*), was associated with this biological process. *Hbaa1* is involved with oxygen transport from gills to peripheral tissues and its induction could be indicative of increased respiratory demands in WS (Lemly, 2002; Kulkeaw and Sugiyama, 2012). This supports the finding that there are high metabolic demands required of WS to maintain homeostasis following SeMet exposure (Zee et al., 2016a; Patterson et al., 2017). Further studies are required to fully assess these pathways.

3.4.4 Down-regulated pathways

ClueGO analysis identified 41 physiological processes that were down-regulated by dietary SeMet in the livers of WS (Fig. A.2), 18 of which were considered significant ($p < 0.05$ after Bonferroni correction) (Fig. 3.2). The main processes identified were associated with cholesterol and lipid metabolism, specifically: cholesterol metabolic process; cholesterol/sterol homeostasis; bile acid and bile salt transport; organic hydroxy compound transport; acylglycerol/triglyceride/neutral lipid metabolic process; lipid digestion, mobilization and transport; lipoprotein metabolism; fatty acid degradation; and the PPAR (peroxisome proliferator activated receptor) signaling pathway (Fig. 3.2).

Significantly down-regulated genes associated with cholesterol metabolism and biliary transport processes were apolipoprotein A-1a (*apoa1a*), cholesterol 7-alpha-monooxygenase (*cyp7a1a*), and ATP binding cassette, sub family B member 11 (*abcb11b*) (Table 3.1). *Apoa1a* mediates cholesterol efflux from tissues to the liver for excretion (Goedeke and Fernandez-Hernando, 2012). *Cyp7a1* catalyzes the conversion of cholesterol to 7-alpha hydroxycholesterol which is the rate limiting step in the synthesis of bile acid (Cuperus et al., 2014). *Abcb11b* encodes the bile salt export pump, which is responsible for transporting bile salts from hepatocytes to the bile (Reviewed in Ferreira et al., 2014). Cholesterol is an important constituent of cell membranes that is responsible for regulating membrane fluidity and permeability, and it is the precursor of metabolites including, steroid hormones and bile salts (Goedeke and Fernandez-Hernando, 2012). Therefore, altered expression of genes associated with cholesterol homeostasis could have widespread physiological effects. Down-regulation of these genes could also reduce cholesterol removal and bile flow (cholestasis) leading to over-accumulation of bile salts in the liver causing liver damage (Cuperus et al., 2014; Elferink 2003; Trauner et al., 1998), evidence of which has previously been observed in WS after exposure to dietary SeMet (Linville, 2006; Tashjian et al., 2006).

Down-regulated genes associated with lipid metabolism (acylglycerol/ triglyceride/ neutral lipid metabolic process; lipid digestion, mobilization and transport; lipoprotein metabolism; fatty acid degradation; PPAR signaling pathway) included *apoa1a*, *cyp7a1a*, diacylglycerol O-acyltransferase 2 (*dgat2*), brain-type fatty acid binding protein 7b

(*fabp7b*), hepatic lipase (*lipc*), acetyl-coenzyme A acyltransferase 2 (*acaa2*), long chain fatty acid ligase (*acsbg2*), alcohol dehydrogenase class 3 (*adh5*), and carnitine palmitoyltransferase (*cpt2*). Down-regulation of genes related to lipid and fatty acid metabolic processes likely represent compensatory responses to the increased demands on energy production and utilization of energy stores to maintain homeostasis during SeMet exposure, given that WS from the same study were found to have reduced hepatic lipid stores at the end of the exposure.

Based on histological analysis of WS livers, there appeared to be no significant effects on cholesterol or lipid metabolic processes at the end of the 72 d study in WS fed 22.4 µg Se/g (Zee et al., 2016a), despite alterations at the transcriptome level early on in the exposure. However, there was an increase in the size and frequency of melanomacrophage aggregates with increasing SeMet exposure (only significant in the high dose), and a decrease in hepatic lipid stores in sturgeon exposed to 104.4 µg/g dm, which indicates liver toxicity. Growth in high dose WS was also significantly lower than the other fish and was thought to be associated with the depleted lipid stores (Zee et al., 2016a). Unfortunately, biochemical endpoints related to these pathways, such as triglyceride content in the liver, were not analyzed.

Previous studies have found dietary SeMet affects biliary transport and lipid storage in WS. Linville (2006) determined that dietary exposure to 20, 36 and 53 µg Se/g in the form of selenized yeast, caused cholestasis in juvenile WS, based on histopathological changes observed in their livers, including proliferation of biliary ductules and ducts, and an increase in melanomacrophage aggregates. Hepatocellular and bile duct hyperplasia was observed in juvenile WS fed above 41.7 µg/g of SeMet in the diet (Tashjian et al., 2006). Although Zee et al (2016b) did not observe biliary stasis or hyperplasia in WS from the current study, pathologies described from histological analysis in WS have been inconsistent, likely due to the lack of baseline information available for normal cells, and therefore further investigation into these effects is still warranted. Tashjian et al. (2006) and De Riu et al. (2014) found a decrease in whole body lipid content of WS with increasing dietary SeMet, as well as decreased growth, which is similar to findings in WS from this study. Knight et al. (2016) also found that Se disrupts hepatic energy storage, based on lower levels of liver triglycerides and decreased growth in rainbow trout that

received 19.5 and 31.8 mg Se/kg. These adverse effects were associated with up-regulation of cell processes involving lipids (long chain fatty acid transport, fatty acid transport, low density lipid oxidation), epidermal growth factor and Notch signaling, in the liver. Pacitti et al. (2016) found that lipid/steroid metabolism was the main pathway altered in the livers of rainbow trout fed a diet with up to 6.35 mg Se/kg. The results from the current study taken together with previous findings, indicate that SeMet causes liver toxicity in WS, potentially through mechanisms involving disruption of cholesterol, bile and lipid processes. Given the importance of cholesterol to cellular membrane permeability, it is possible that alterations in cholesterol/lipid homeostasis could disrupt cell membrane permeability, leading to membrane damage and potentially edema (Goedeke and Fernandez-Hernando, 2012), which represented one of the main pathologies observed in WS in a parallel study (Zee et al., 2016a). Possible links between altered cholesterol/lipid homeostasis and edema need to be studied further. Selenomethionine also alters hepatic lipid production, transport and storage processes in fishes. Alterations in these processes early on in the SeMet exposure are likely associated with the decrease in hepatic lipid storage and growth in high dose WS after 72 d of exposure.

Selenomethioine exposure altered glutathione metabolism, metabolism of xenobiotics by cytochrome p450, and drug metabolism in WS. These processes were associated with down-regulated expression of genes that encode for key xenobiotic biotransformation enzymes, such as cytochrome p450 1a (*cyp1a*) and *adh5*, while glutathione-S-transferase (*gst*) expression was up-regulated (Table 3.1). Detoxification capabilities could be reduced or impaired with altered regulation of these biological processes (Hoffman, 2002). However, signs of oxidative stress and damage such as increased levels of ROS and tissue damage, would be expected if this were the case (Hoffman, 2002). Zee et al. (2016b) found limited evidence of hepatic oxidative stress in WS from the same study, based on the results of gene expression, lipid hydroperoxide and histological analysis in liver tissue. Some of these pathways were also found to be up-regulated but were not considered significant. The lack of evidence of oxidative stress, despite indications at the molecular level, suggest that WS were still able to maintain their detoxification and antioxidant systems (Zee et al., 2016b). Similarly to WS, rainbow trout in a recent study were found to not have signs of oxidative stress following exposure to organic Se concentrations up to 31.8 mg/kg (Knight

et al., 2016). These studies contradict others that have shown Se exposure induces oxidative stress in fish (Palace et al., 2004; Misra et al., 2010, 2012a; Pacini et al., 2013; Elia et al., 2014). These findings highlight the need for further studies to confirm the lack of oxidative stress in WS following SeMet exposure and to understand the difference in responses among species.

Proton transporting two-sector ATPase complex assembly was altered in SeMet treated WS, based on decreased expression of ATPase h+transporting lysosomal v0 subunit a lb (*atp6v0a1b*) and ATP synthase mitochondrial F1 complex assembly factor 2 (*atpaf2*). The ATPase complex catalyzes the synthesis and hydrolysis of ATP and regulates ion transport across membranes (Ives and Rector, 1984). This process is associated with various broad cell functions so further investigation is needed to determine how alterations could specifically affect WS. The biological process, positive regulation of apoptotic signaling pathways, was associated with down-regulated expression of dipeptidyl peptidase 1 (*dpp1*), also known as cathepsin C (*ctsc*), which encodes for cysteine proteases that activate serine proteases and are involved with protein degradation, cell growth, and immune and inflammatory responses (Wang et al., 2018). Effects on serine proteases were highlighted in the previous section and this further supports the finding that dietary SeMet alters pathways related to protein metabolism that could be associated with edema in WS; however, further studies are required to fully understand the implications of this.

3.5 Conclusions

RNA-seq analysis revealed a greater number of down-regulated genes than up-regulated genes in WS fed diets containing 22.4 μg Se/g dm, relative to controls, which was in contrast to findings from previous transcriptome studies in rainbow trout (Knight et al., 2016; Pacitti et al., 2016). ClueGO analysis also identified a greater number of affected biological pathways associated with down-regulated genes (Fig. 3.1 and 3.2), which could be associated with the pathological effects observed in WS at the end of the study (Zee et al., 2016a).

Although several liver pathways were altered by SeMet, the majority of pathways appear to be associated with energy, cholesterol/lipid and protein metabolism. Significant

alterations in cholesterol homeostasis, bile acid transport/synthesis, serine peptidase/endopeptidase activity, gluconeogenesis, and lipid metabolism, suggest these pathways could be involved in mediating SeMet toxicity in WS and further analysis is warranted to investigate links between these molecular responses and adverse effects. Pathways related to other tissues such as the kidney, should also be investigated, as previous studies have found significant accumulation of Se and histopathological lesions in the kidneys of WS after dietary SeMet exposure (Tashjian et al., 2006; De Riu et al., 2014).

CHAPTER 4: GENERAL DISCUSSION

4.1 General discussion

This research, along with the research by Zee et al. (2016a, 2016b), was conducted to assess the sensitivity of juvenile white sturgeon (WS; *Acipenser transmontanus*) to dietary SeMet and to provide insight into the physiological effects of SeMet exposure in this species, by establishing links between molecular toxicity pathways and pathological endpoints. The objectives of the research presented in this thesis were to determine the effects of dietary SeMet on the physiological stress response of WS, specifically the effects on their ability to mount a cortisol response to a secondary stressor, and to identify potential alternative and unknown pathways of SeMet toxicity in WS, by analyzing gene expression changes across the whole transcriptome.

Although Se has been studied extensively in teleost species such as rainbow trout (*Oncorhynchus mykiss)*, research is limited in other fish species, including endangered sturgeon. White sturgeon have been shown to be more vulnerable to bioaccumulative contaminants than other species, due to their longevity and proximity to the sediment (Little et al., 2012; Vardy et al., 2011, 2013). Focus on the impact of Se on WS is important for future risk assessments of Se impacted regions, some of which are inhabited by threatened or endangered WS populations.

The present study found that juvenile WS are less sensitive to dietary SeMet than teleost species, based on sublethal endpoints including effects on the physiological stress response and energetics (Hamilton, 2004; USEPA, 2016; Patterson et al., 2017). There was limited evidence that a medium level of dietary SeMet (22.4 µg Se/g) impacted the ability of WS to utilize muscle glycogen stores following exposure to an acute handling stressor; while high levels of dietary SeMet (104.4 µg Se/g) altered cortisol dynamics and energy substrates, specifically basal levels of cortisol, glucose and lactate in blood plasma, as well as glycogen levels in liver tissue. Selenomethionine did not, however, impact the cortisol response to a handling stressor in WS (Patterson et al., 2017). This was in contrast to rainbow trout, where the cortisol response to a handling stressor was attenuated in the fish exposed to dietary SeMet (Wiseman et al., 2011a). The magnitude of the cortisol response

to stress was also significantly lower in WS than in rainbow trout and other teleost species studied to date (Barton and Iwama, 1991; Barton, 2002; Aluru and Vijayan, 2006; Gravel and Vijayan, 2007; Wiseman et al., 2007; Pankhurst, 2011; Wiseman et al., 2011a; Wiseman and Vijayan, 2011; Patterson et al., 2017). This was an interesting discovery as it could suggest an overall lowered ability for WS to respond to acute stress which could have implications on their ability to cope with environmental stressors. The stress response of WS could be less sensitive than that of teleost species due to significant differences in their physiology and life history. Perhaps WS have less of a need to respond rapidly to stressors in the wild due to the lack of natural predators and therefore, have not developed the same capacity for responding to stress as teleost fish (Barton et al., 2002). It is possible that the time it takes for blood plasma cortisol levels to peak varies between species, therefore the peak in plasma cortisol levels might not have been captured in WS in the current study. Variations in study design could also lead to differences in the cortisol responses. Based on the results of this study, effects on endpoints related to the stress response were likely not the primary driver of SeMet toxicity in WS, given that pathologies such as edema occurred in WS at all SeMet treatments, while stress response endpoints were only significantly affected at the high dose (Zee et al., 2016a; Patterson et al., 2017).

It should be noted that the use of artificially spiked SeMet diets in this experiment, versus the use of naturally Se rich diets, could have impacted the apparent toxicity of SeMet to WS. In the environment, SeMet is incorporated into proteins in biota, while SeMet in a spiked diet would mostly be free and potentially more bioavailable, leading to the appearance of higher sensitivity relative to what would be observed in the wild. Other species of Se would also be present in natural food sources, in addition to SeMet, which could alter the toxicity given that there are likely differences in metabolism between Se species. As a result, WS may have been less sensitive to SeMet from a natural diet. However, a natural diet may contain additional contaminants that could alter toxicity of SeMet as well. Although spiked diets may not be ideal, this type of laboratory toxicity testing is especially useful as a first step for non-model species, such as WS, where there is limited toxicity data available. Future studies should assess the potential differences in sensitivity of WS to diets naturally rich in SeMet versus SeMet spiked diets.

The low sensitivity of stress response related endpoints to SeMet in WS, despite the high sensitivity of some pathologies such as edema (Zee et al., 2016a), relative to teleosts, suggests that SeMet may have different mechanisms of action in WS than in teleosts. In hopes of providing further insight into the potential mechanisms of SeMet toxicity in WS, RNA-seq analysis was conducted to assess gene expression changes in response to SeMet exposure, across the entire transcriptome. The goal was to identify potential novel toxicity pathways of SeMet in WS, based on RNA-seq data, that could be linked to potential adverse outcomes and be used to inform future studies for specific toxicity endpoints for SeMet. RNA-seq data was also assessed to establish links between initial molecular responses to SeMet and the adverse pathological effects observed in WS at the end of the exposure (Zee et al., 2016a; Patterson et al., 2017). RNA-seq analysis revealed that short-term exposure to dietary SeMet at 22.4 μg/g dm significantly altered gene expression across the whole transcriptome in WS, relative to controls. The results from RNA-seq were verified by analyzing select transcripts using qPCR. The results were found to be comparable to qPCR, confirming the reliability of RNA-seq analysis as a valuable tool for assessing molecular responses. Several physiological pathways were altered by SeMet exposure, many of which were found to be down-regulated based on the expression of genes associated with these pathways. The majority of the altered pathways were associated with energy, cholesterol/lipid and protein metabolism. The molecular responses associated with these pathways are indicative of adverse effects that occurred in WS later on in the exposure, including depletion of hepatic energy stores, decreased growth, and edema (Zee et al., 2016a; Patterson et al., 2017), and therefore appear to represent useful indicators of long-term apical outcomes. However, further research is required to fully elucidate these pathways and confirm specific links and relationships between molecular responses and adverse effects.

Oxidative stress has been hypothesized as one of the primary drivers of SeMet toxicity in teleost species (Palace et al., 2004; Misra et al., 2010, 2012a); however, the results from RNA-seq analysis and Zee et al. (2016b) found little evidence that SeMet induces hepatic oxidative stress in WS, and therefore it was likely not critical to the development of pathologies after SeMet exposure (Zee et al., 2016a). Alternatively, energy, cholesterol/lipid and protein metabolism pathways appeared to be significantly

altered by SeMet exposure suggesting that disruption of these processes could play an important role in toxicity. Altered expression of genes related to energy and lipid metabolic processes and proteolysis appear associated with the increase in energy substrate mobilization, leading to depletion of hepatic energy stores (lipid and glycogen), decreased growth and increased mortality as observed in the high dose WS. This is similar to findings by Knight et al. (2016) who proposed that the disruption of metabolites associated with triglyceride production and storage, and up-regulation of pathways related to cell growth and proliferation (epidermal growth factor and Notch signaling), were responsible for the adverse effects related to growth in rainbow trout; however, they did not find any effects on genes related to energy production (gluconeogenesis or glycolysis), as was found in WS. Increased edema and exophthalmos, leading to mortality in medium and high dose fish, may have been caused by enhanced proteolysis or altered cholesterol/lipid homeostasis affecting cell permeability and causing cell damage. It could also be due to disruption of osmotic regulation through kidney or gill damage; however, this was not assessed in the current study but should be investigated in the future.

4.2 Conclusions

Overall, WS appear to be sensitive to dietary SeMet concentrations ≥ 22.4 μg/g based on the biochemical and molecular endpoints discussed in this thesis. This is consistent with previous research that has observed adverse effects in WS at dietary Se concentrations > 20 μg/g (Tashjian et al., 2006; De Riu et al., 2014; Zee et al. 2016a), which is higher than concentrations that have caused toxicity in teleosts, such as rainbow trout, chinook salmon (*Oncorhynchus tshawytscha*) and fathead minnow (Lemly, 1997a; Hamilton, 2004). Based on these results, the maximum acceptable toxicant concentration (MATC), which is the geometric mean of the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC), of SeMet in juvenile WS is 11.2 μg/g dm in the diet (based on a NOEC of 5.6 μg/g dm and a LOEC of 22.4 μg/g dm) and 11.2 μg/g dm in muscle tissue (based on a NOEC of 5.3 μg/g dm and a LOEC of 23.5 μg/g dm) (Zee et al., 2016a). These values are above the BC chronic dietary and tissue guidelines for Se (4 μg/g in the diet; 4 μg/g in muscle – interim) and just below the USEPA Se criterion

of 11.3 μg/g in muscle tissue (BC Ministry of Environment, 2014; USEPA, 2016). However, if these values are based on the occurrence of mild edema in WS from the same study (Zee et al., 2016a), the MATC of SeMet would be 2.8 μg/g dm in the diet (based on a NOEC of 1.4 μg/g dm and a LOEC of 5.6 μg/g dm) and 2.4 μg/g dm in muscle tissue (based on a NOEC of 1.1 μg/g dm and a LOEC of 5.3 μg/g dm), which is below the BC chronic dietary and tissue guidelines for Se and the USEPA criterion for Se, and places WS among the more sensitive species. Testing of earlier life stages of WS may also reveal greater sensitivity, since embryonic and larval stages of fish are considered to be the most sensitive life stages. Depending on the biological relevance of mild edema, the BC guidelines and USEPA criterion may not be protective of juvenile or earlier life stage WS originating from the Columbia River, which warrants further investigation.

Consistent findings between this research and the parallel studies by Zee et al. (2016a, 2016b) are that WS differ significantly from teleosts in their responses to SeMet. Although WS appeared less sensitive to dietary SeMet than teleosts, based on effects related to the stress response and energetics, they were considered among the most sensitive species based on the development of mild edema. The presence of edema leading to exophthalmos was also a surprising response that is not commonly observed in teleosts. Although the cortisol response to stress in WS did not appear as sensitive to SeMet as in teleosts, the overall response was lower in magnitude, suggesting WS might have a lower ability to respond to stress. The mechanism(s) by which toxicity is manifested in WS also differs from those described in previous work for teleost fishes. Where oxidative stress has been suggested as the critical mechanism causing toxicity in teleosts, pathways associated with energy, cholesterol/lipid and protein metabolism appear to be more critical in WS. These differences between WS and teleosts should be taken into consideration when conducting future risk assessments for Se to ensure protection of all sensitive species.

The unique evolution and physiology of WS may provide some explanation for the different responses observed between WS and teleosts. Sturgeons are prehistoric fishes that are thought to have diverged approximately 300 million years ago from the ancient lineage leading to teleosts (Hurley et al., 2007). Sturgeons have changed very little morphologically over time and they have a low molecular evolution rate (Krieger and Fuerst, 2002). Given this and their slow growth rate, sturgeon likely cannot adapt as easily

or as quickly to environmental changes as teleosts. In addition, WS may not have developed a capacity for a sensitive stress response, due to their size, long lifespan and their lack of natural predators. Female sturgeons produce between 700 000 and 4 million eggs at each spawning event, depending on their size (BC Ministry of Environment, 1997), and therefore only a few offspring need to survive and reach maturity to maintain the population, which contributes to the theory that they lack an evolutionarily need for a sensitive stress response. In comparison, female rainbow trout only produce 2000 to 3000 eggs and they have a maximum lifespan of 11 years (Tyler et al., 1996). As a result, rainbow trout may have needed to develop a more sensitive stress response to ensure survival. The low evolution rate of WS also suggests that their physiological functions may be more primitive relative to teleosts, which are highly evolved and diverse. This may play a role in why the mechanisms of action of SeMet and the subsequent responses to exposure differ between these species.

4.2 Future work

Future studies should assess the effects of dietary SeMet at concentrations between 22.4 and 104.4 µg Se/g, as most adverse effects occurred in this range. This will provide a greater understanding of the toxicity threshold of SeMet in WS. Additionally, more sampling time points to better establish the cortisol response to stress are needed to confirm that the primary stress response is not affected by SeMet in WS. Proteomics and enzyme activity analysis should be conducted to confirm whether alterations in gene expression identified through RNA-seq analysis are representative of actual physiological changes. This would provide support for creating links between changes in gene expression and adverse pathological effects. Alterations in biochemical endpoints associated with the pathways identified by ClueGO analysis need to be investigated to link alterations in specific pathways to adverse physiological effects. The histopathology, as well as molecular and biochemical responses in different tissues such as the kidney, gill and brain could also be assessed. Fish have been found to accumulate significant levels of Se in these tissues after exposure to dietary SeMet (Tashjian et al., 2006; Misra et al., 2012b; De Riu et al., 2014) and Se accumulation in the kidney has previously been linked to

histopathological lesions in WS (Tashjian et al., 2006; De Riu et al., 2014). Analysis of gill and kidney tissues would provide insight into possible effects on osmotic regulation, which might have been associated with the development of edema in WS in the current study. Brain tissue is usually overlooked, however, since previous studies have found Se accumulates in fish brain tissue, it would be interesting to assess whether changes in the brain are associated with altered behavior (feeding and swimming activity) in WS, as there is some evidence that SeMet toxicity in the brain is associated with cognitive impairment in zebrafish (Naderi et al., 2017). Future studies should also include embryo and larval stages of WS as they are the most sensitive life stages and SeMet has been shown to cause teratogenicity in other fish species (Lemly, 1993; Janz et al., 2012).

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APPENDIX: SUPPLEMENTARY MATERIAL

Table A1. Sequences, efficiency, annealing temperatures and GenBank accession numbers of oligonucleotide primers used in quantitative real-time PCR.

Gene Identity	Accession #	Sequence (5'–3')	Efficiency (%)	Annealing temp. (°C)
β-actin	FJ205611	F: CCGAGCACAATGAAAATCAA R: ACATCTGCTGGAAGGTGGAC	96	60
pepck	NA	F: TCAATCCAGAGAACGGCTTT R: TGGTTCTTCCAGGAGGTCAC	100	60
fbp1a	NA	F: CAATGGTGGCTGATGTTCAC R: GTGGATGCACTCTGGCTGTA	102	60
g6pca	NA	F: GCTGTGCTGTGGTCAGTGTT R: AGATCAGGAACAGGGTGGTG	104	60
apoa1a	NA	F: CTCGCTCTCTTTAACGCACC R: TTGGACAAGCAGCAACACAA	105	59
cyp7a1a	NA	F: GCCATTGAAACCTCAAGGAA R: AGTCCTTCTGTGGTCCATGC	103	60
abcb11b	NA	F: CAAGAGCCTGTGCTGTTTGA R: GATGGCAATCCGTTGTTTCT	103	60
pcsk5	NA	F: CGCGGAATGATTTCAATTTT R: GGTCTGGGTGGTTTTTCTCA	105	60
gst	NA	F: CTCCAGGATGAAAACCTTGG R: ACTCAATCCCATGCAAAAGG	107	60
cyp1a	JQ660369	F: GATCCCTCCACCTTCTCTCC R: GCCGATAGACTCACCAATGC	99	60
aif	NA	F: ATCGTGGGTGGAGGATTTG R: GCCCCTACGTTGTGATGGA	104	60
abcd3a	NA	F: GGCTATGGCACGTCTGTTTT R: CTTCCATCCATGTGCAGGTA	100	60
fabp7b	NA	F: AGGGAGATAAGGTTGTGGTGA R: AAATGTGGTCTCCTTGCCGT	100	59

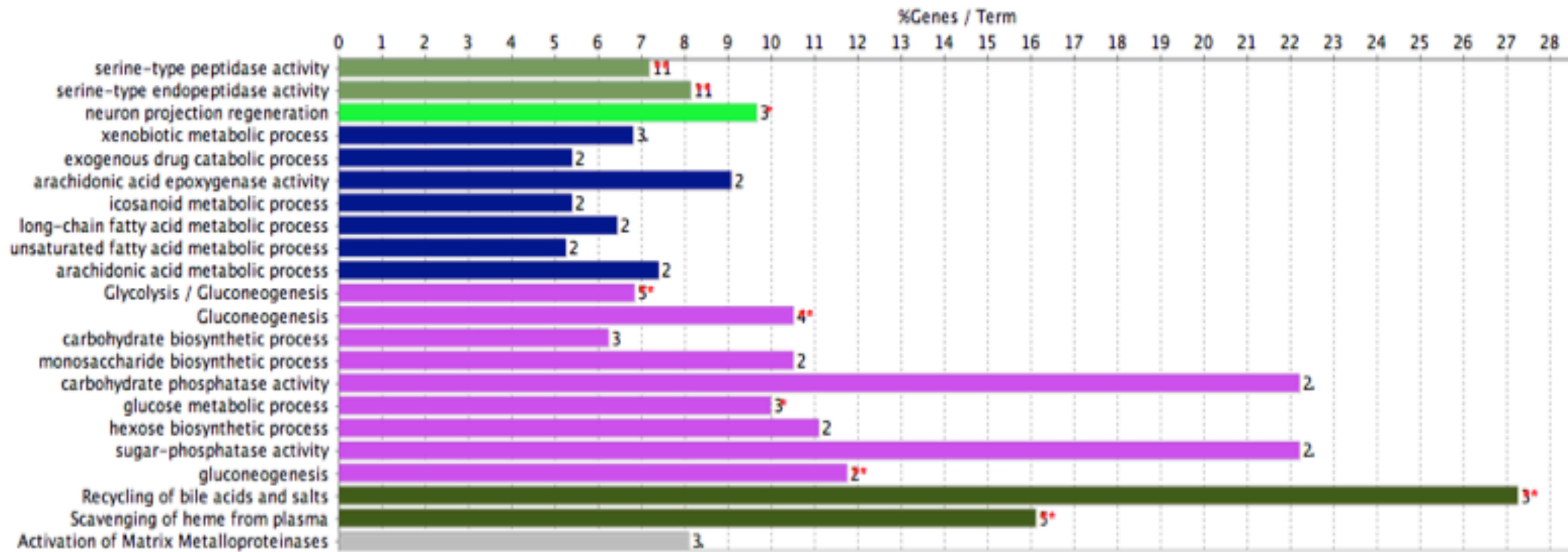

Figure A1. GO/KEGG/Reactome pathway terms associated with a greater proportion of up-regulated genes identified in livers of juvenile WS fed a diet with 22.4 µg Se/g dm relative to controls. The name of each group represents the most significant term in the group. Bars represent the percentage of genes found from the uploaded cluster, compared to all the genes associated with the term. Numbers at the end of each bar indicate the number of genes assigned to each term (Bindea et al., 2009).

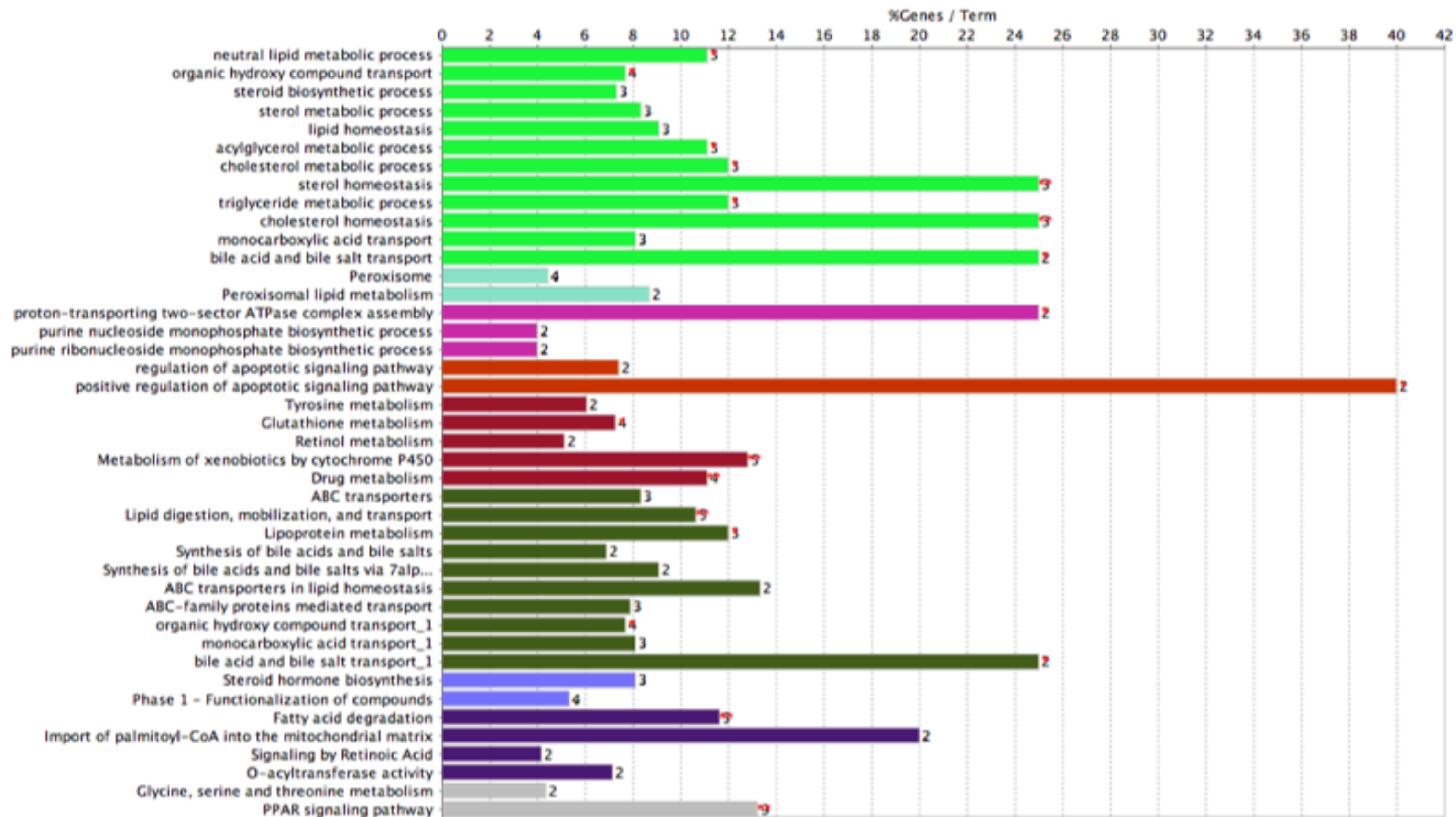

Figure A2. GO/KEGG/Reactome pathway terms associated with a greater proportion of down-regulated genes, identified in livers of juvenile WS fed a diet with 22.4 µg Se/g dm, relative to controls. The name of each group represents the most significant term in the group. Bars represent the percentage of genes found from the uploaded cluster, compared to all the genes associated with the term. Numbers at the end of each bar indicate the number of genes assigned to each term (Bindea et al., 2009).

Table A2. Significant GO/KEGG/Reactome pathway terms identified by use of ClueGO analysis, followed by the percentage of genes found from the uploaded cluster compared to all the genes associated with the term, the percentage of up and down-regulated genes associated with each term, the Gene IDs, the number of genes, and the significance of each term ($p < 0.05$ after Bonferroni correction).

Up-regulated pathways								
UNIQUE ID	**GO Term**	**% Associated Genes**	**% Down-Regulated Genes**	**% Up-Regulated Genes**	**Down-Regulated Genes**	**Up-Regulated Genes**	**Number of Genes**	**Term *p*-value Corrected with Bonferroni step down**
GO:0008236	serine-type peptidase activity	7.2	0	100		ctrl, ela2l, pcsk5b, tpp1, try, zgc:112160, zgc:136461, zgc:158446, zgc:92041, zgc:92590, zgc:92745	11	1.28E-06
GO:0004252	serine-type endopeptidase activity	8.1	0	100		ctrl, ela2l, pcsk5b, tpp1, try, zgc:112160, zgc:136461, zgc:158446, zgc:92041, zgc:92590, zgc:92745	11	3.52E-07
GO:0031102	neuron projection regeneration	9.7	33.3	66.7	apoa1a	apoa4, mvp	3	0.049098031
KEGG:00010	Glycolysis / Gluconeogenesis	6.8	40	60	adh5, pgm1	fbp1a, g6pca.2, pck1	5	0.009007964
REACTOME:6298404	Gluconeogenesis	10.5	25	75	slc25a11	fbp1a, pck1, slc25a1	4	0.007575284
GO:0006006	glucose metabolic process	10.0	33.3	66.7	pgm1	g6pca.2, pck1	3	0.047670745
GO:0006094	gluconeogenesis	11.8	0	100		g6pca.2, pck1	2	0.117712939
REACTOME:6299015	Recycling of bile acids and salts	27.3	33.3	66.7	abcb11b	fabp6, gc	3	0.002920028
REACTOME:6299597	Scavenging of heme from plasma	16.1	40	60	apoa1a, zgc:152945	LOC798813, gc, hbaa1	5	1.64E-04
Down-regulated pathways								
UNIQUE ID	**GO Term**	**% Associated Genes**	**% Down-Regulated Genes**	**% Up-Regulated Genes**	**Down-Regulated Genes**	**Up-Regulated Genes**	**Number of Genes**	**Term *p*-value Corrected with Bonferroni step down**
GO:0006638	neutral lipid metabolic process	11.1	66.7	33.3	apoa1a, dgat2	apoa4	3	0.036051942
GO:0015850	organic hydroxy compound transport	7.7	75.0	25.0	abcb11b, apoa1a, zgc:92111	apoa4	4	0.023552964
GO:0006639	acylglycerol metabolic process	11.1	66.7	33.3	apoa1a, dgat2	apoa4	3	0.036051942
GO:0008203	cholesterol metabolic process	12.0	66.7	33.3	apoa1a, cyp7a1a	apoa4	3	0.029563641
GO:0055092	sterol homeostasis	25.0	66.7	33.3	apoa1a, cyp7a1a	apoa4	3	0.003779345
GO:0006641	triglyceride metabolic process	12.0	66.7	33.3	apoa1a, dgat2	apoa4	3	0.029563641
GO:0042632	cholesterol homeostasis	25.0	66.7	33.3	apoa1a, cyp7a1a	apoa4	3	0.003779345
GO:0015721	bile acid and bile salt transport	25.0	100.0	0.0	abcb11b, zgc:92111		2	0.047540786
GO:0070071	proton-transporting two-sector ATPase complex assembly	25.0	100.0	0.0	atp6v0a1b, atpaf2		2	0.047540786
GO:2001235	positive regulation of apoptotic signaling pathway	40.0	100.0	0.0	ctsc, zgc:194314		2	0.020684918
KEGG:00480	Glutathione metabolism	7.3	75.0	25.0	idh1, zgc:158387, zgc:162356	gstp1	4	0.028317065
KEGG:00980	Metabolism of xenobiotics by cytochrome P450	12.8	80.0	20.0	adh5, cyp1a, zgc:158387, zgc:162356	gstp1	5	5.17E-04
KEGG:00982	Drug metabolism	11.1	75.0	25.0	adh5, zgc:158387, zgc:162356	gstp1	4	0.006278306
REACTOME:6298829	Lipid digestion, mobilization, and transport	10.6	60.0	40.0	apoa1a, fabp7b, lipc	fabp6, gc	5	0.001253951
REACTOME:6298917	Lipoprotein metabolism	12.0	66.7	33.3	apoa1a, lipc	gc	3	0.029563641
GO:0015850	organic hydroxy compound transport	7.7	75.0	25.0	abcb11b, apoa1a, zgc:92111	apoa4	4	0.023552964
KEGG:00071	Fatty acid degradation	11.6	100.0	0.0	acaa2, acsbg2, adh5, cpt2, si:ch211-236l14.3		5	8.25E-04
KEGG:03320	PPAR signaling pathway	13.2	66.7	33.3	acsbg2, apoa1a, cpt2, cyp7a1a, fabp7b, si:ch211-236l14.3	adipoql, fabp6, pck1	9	1.18E-07