SPECIES AND LIFE-STAGE SPECIFIC DIFFERENCES IN THE SENSITIVITY OF RAINBOW TROUT AND WHITE STURGEON TO CADMIUM AND COPPER: A MECHANISTIC STUDY

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ABSTRACT

Marked differences in the sensitivity among fish life-stages and species have been observed for cadmium (Cd) and copper (Cu), two contaminants of particular concern in Canadian surface waters. White sturgeon (*Acipenser transmontanus*) are highly sensitive to metals such as Cu. Moreover, white sturgeon have also shown significant life-stage-specific differences in sensitivity to metals. When compared to rainbow trout (*Oncorhynchus mykiss*), white sturgeon are more sensitive to waterborne acute Cu exposure, whereas the trend in sensitivity is reversed with Cd exposure. The mechanisms of life-stage- and species-specific differences in sensitivity among fish species are not well understood, especially for data-poor, non-model species such as white sturgeon. Hence, the overall objective of this study was to characterize the physiological, biochemical and molecular drivers of species-specific differences in the sensitivity of white sturgeon and rainbow trout to Cd and Cu over three different early life-stages.

During acute waterborne exposure to Cd and Cu, the most important mechanisms of toxicity are the disruption of calcium (Ca) and sodium (Na) homeostasis, respectively. Therefore, I compared the effect of Cd and Cu on uptake and whole-body levels of Ca and Na, respectively, between rainbow trout and white sturgeon across multiple early life-stages. I showed that the greater sensitivity of white sturgeon to Cu was explained by greater reduction of its sodium Na uptake compared to rainbow trout, when exposed to same waterborne Cu concentration. Similarly, higher sensitivity of rainbow trout to Cd was explained by significantly higher reduction in Ca uptake relative to white sturgeon. With both Cu and Cd, reduction in whole-body ion uptake resulted in reduction of whole-body levels of Na and Ca, respectively, and the response level was significantly higher in the more sensitive species.

Additionally, I analysed Cd/Cu accumulation, Cd/Cu induced oxidative stress, metallothionein and heat shock protein responses and compared these parameters between rainbow trout and white sturgeon across multiple early life-stages. Species-specific differences in these parameters were evaluated because they are known to be important pathways through which Cd and Cu cause toxicity. In larval and swim-up life-stages, white sturgeon showed greater Cu-induced oxidative damage (lipid hydroperoxide (LPO) induction) than in rainbow trout, which explained the greater sensitivity of white sturgeon to Cu in early life-stages. Similarly, exposure to Cd showed that rainbow trout, the more sensitive species to Cd, had significantly greater LPO

induction at the swim-up and juvenile life-stages as compared to LPO levels in the respective life-stages of white sturgeon. Mechanisms such as antioxidant enzymes, antioxidant molecules, metal accumulation and metallothionein could only partially explain the patterns observed in Cd or Cu induced LPO levels with no consistent response across life-stages. Regardless of the underlying mechanism, LPO seems to be a good indicator of species-specific differences in the sensitivity to Cd and Cu between rainbow trout and white sturgeon in selective life-stages.

In conclusion, my study showed that ion physiology parameters such as ion uptake and whole-body ion levels are good indicators of species-specific differences in the sensitivity of rainbow trout and white sturgeon to Cd and Cu, and possibly other metals as well. In addition, LPO levels in the gills also generally explained the species-specific differences in sensitivity to Cd and Cu between rainbow trout and white sturgeon. The parameters identified in this study could be good surrogates for testing species-specific differences in the sensitivity to metals. Moreover, the parameters identified in this study are also good candidates for developing predictive approaches for toxicity testing of metals, after further characterization in more species.

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LIST OF ABBREVIATIONS

°C	degree Celsius
µCi	microcurie
µg	microgram
µm	micrometer
µM	micromolar
18S	18 Svedberg rRNA
ADP	adenosine diphosphate
Ag	silver
ANOVA	analysis of variance
ASC	ascorbate
ATRF	Aquatic Toxicology Research Facility
b-actin	beta actin mRNA sequence
Ca	calcium
CA	carbonic anhydrase
Ca^{45}	calcium-45 radioisotope
Ca-ATPase	calcium ATPase enzyme
$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
CAS	chemical abstracts service
CAT	catalase
CCME	Canadian Council of Ministers of the Environment
Cd	cadmium
Cd^{109}	cadmium-109 radioisotope
Cd^{2+}	divalent cadmium cation
$CdCl_2$	cadmium chloride
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
Cl	chloride
COSEWIC	Committee on the Status of Endangered Wildlife in Canada

CPM	counts per minute
CPM_{wb}	counts per minute in whole body
C_t	cycle threshold in PCR
Cu	copper
CV	coefficient of variation
DF	Degree of freedom
DMT	divalent metal transporter
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DOC	dissolved organic carbon
DOLT-4	dogfish liver certified reference material for trace metals
dph	days post hatch
DRP2	DNA directed RNA polymerase II subunit I
E	efficiency (primer PCR)
ECaC	epithelial calcium channel
EDTA	ethylenediaminetetraacetic acid
EF1a	elongation factor-1 alpha
g	gram
g	acceleration of gravity
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GOI	gene of interest
GPX/GPX4a1	glutathione peroxidase
GSH	glutathione
GSSG	oxidized glutathione
GST	glutathione-s-transferase
h	hours
H^+	hydrogen ion
HAT	H^+-ATPase
HDPE	high density polyethylene
HIF1A	hypoxia inducible factor 1

HPRT	hypoxanthine phosphoribosyltransferase
HSP/HSP70/70a/70b	heat shock protein 70/70a/70b
J_{in}	inward uptake rate
J_{max}	maximum uptake rate
kg	kilogram
K_m	affinity of uptake
L	litre
LC_{50}	median lethal concentration
LL.2	log-logistic function in DRC package of R
log $K_{Cd\text{-}gill}$	logarithm of affinity constant of gill Cd uptake
log $K_{Cd\text{-}wb}$	logarithm of affinity constant of whole-body Cd uptake
LPO	lipid hydroperoxide
mCi	millicurie
mg	milligram
Mg	magnesium
min	minutes
mL	millilitre
MM.2	Michaelis-Menten function in DRC package of R
Mmol	millimole
mRNA	messenger ribonucleic acid
MT/MTa/MTb	metallothionein
n	sample size
N	normal (concentration)
Na	sodium
Na^{22}	sodium-22 radioisotope
NADH	reduced nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NKA	Na^+/K^+-ATPase
nM	nanomolar
nm	nanometer
nmol	nanomole

OPT	o-phthalaldehyde
p	calculated probability
PCR	polymerase chain reaction
pH	potential of hydrogen
pmol	picomole
qPCR	reverse transcription quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RPL8	ribosomal protein l8
rRNA	ribosomal ribonucleic acid
SARA	Species at Risk Act (2002, Canada)
S_{aw}	specific activity of water
SD	standard deviation
SLRS-6	river water certified reference material for trace metals and other constituents
SO_4	sulphate
SOD/SOD 1/SOD2	superoxide dismutase enzyme
SYBR	SYBR green dye for PCR
t	time
TRV	toxicity reference values
USEPA	United States Environmental Protection Agency
UV-Vis	ultraviolet-visible
Zn	zinc

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for a manuscript-style thesis. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 7 contains a general discussion and overall conclusion. Chapters 2, 3, 4, 5, and 6 of this thesis are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 2 was published in the journal, Environmental Science and Technology, Chapters 3 and 4 were published in Aquatic Toxicology, Chapter 5 was published in Science of the Total Environmental. Finally, Chapter 6 has been submitted to Ecotoxicology and Environmental safety and it is currently under review. Full citations for the research papers and a description of author contributions are provided following the preface of each chapter. As a result of the manuscript style format, there is some repetition of material in the introduction and material and methods sections of the thesis. The tables, figures, supporting information, and references cited in each chapter have been reformatted here to a consistent thesis style. References cited in each chapter are combined and listed in the 'References' section of the thesis. Supporting information associated with research chapters are presented in the 'Appendix' section at the end of this thesis as Cx.Sy format, where 'Cx' indicates chapter number and 'Sy' indicates figure or table number.

1 CHAPTER 1: GENERAL INTRODUCTION

PREFACE

Chapter 1 is a general introduction and literature review of the topics of metal toxicity in fish and the life-stage and species-specific differences in the sensitivity of fishes to metals. More specifically, the chapter deals with the toxicity of cadmium (Cd) and copper (Cu) in rainbow trout and white sturgeon. Chapter 1 also includes the overall objectives of the project, objectives of individual studies within the project, and all the null hypotheses.

1.1 Overview of Life-stage and Species-specific Differences in the Sensitivity of Fish to Metals

Metals are naturally present in the earth's crust and they can neither be created nor destroyed. A lack of degradation after being released into the environment is the reason why metal contamination has become a matter of significant concern. Major sources of anthropogenic release of metals into the environment are mining and smelting operations, electronic wastes, and industrial production (Wood, 2012).

Susceptibility of an organism to a metal depends upon the exposure conditions as well as inherent physiology and biochemistry of the organism (Smith et al., 2015). Exposure conditions influence the toxic effect of metals because the bioavailability of a metal is governed by water quality variables such as dissolved organic carbon, hardness, alkalinity, pH, cations, and anions. The Biotic Ligand Model (BLM) was developed to account for the variability introduced by water quality parameters in the toxicity of metals. Considerable differences still exist among fish species in terms of their sensitivities to metals, even after adjusting the toxicity values (such as LC_{50}: the lethal concentration required to kill 50% of the population) using the BLM (USEPA, 2007), which is likely due to the inherent differences in the physiology and biochemistry among different fish species. Sensitivity of fishes to metal exposure also differs among different life-stages of the same species. The importance of species-specific differences in the sensitivity to metal exposure is reflected in the methodology for deriving ambient water quality criteria (AWQC) or water quality guidelines (WQG) across different jurisdictions. For example, WQG for metals in Canada are generally derived from species sensitivity distributions (SSDs) (CCME, 2014). SSDs for acute toxicity involve plotting toxicity data (e.g. LC_{50}) from various species on the Y axis and percentage rank of respective species on the X axis. Statistical techniques are applied thereafter to calculate the desired estimates. This methodology takes species-specific differences in sensitivity into consideration during the derivation of water quality criteria for metals (Wood, 2012). Similarly, selection of the most sensitive life-stage of a sensitive species is important for establishing water quality criteria that are universally protective for most of the species. For example, USEPA guidelines such as 'Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms' recommends the use of early life-stages due to their assumed higher susceptibility to toxicants (US Environmental Protection Agency, 2002).

It is generally believed that the sensitivity of an organism including fish to metals and other contaminants decrease with the age. In other words, larval fish are believed to be more sensitive than fish at other life-stages such as swim-up and juvenile (Mohammed, 2013). However, experimental evidence indicate that this may not always be true. For example, it was reported recently that swim-up cutthroat and rainbow trout fry tend to be more vulnerable to lead (Pb) and zinc (Zn) as compared to other life-stages (Mebane, 2012). Another study assessing the toxicity of copper (Cu) in white sturgeon (*Acipenser transmontanus*) showed that swim-up larvae (15 days post-hatch (dph)) and early juveniles (40–45 dph) were more sensitive to Cu than yolksac larvae (8 dph) and the later juvenile life stage (100 dph) (Vardy et al., 2013). Cadmium (Cd) also showed life-stage specific patterns of toxicity in developing sturgeon, with the highest sensitivity at 48 dph but lower sensitivity at 15 dph as well as 139 dph (Tang et al., 2016). Other examples include increased sensitivity of coho salmon to Cd, Cu, Pb, and Zn from 14 dph to 49 dph (Chapman, 1978) and increased sensitivity of rainbow trout and bull trout to Cd and Zn with increasing age (Hansen et al., 2002). Similarly, rainbow trout was more sensitive to Cd and Cu during the 46 dph and 95 dph life-stage than the early larval life stage (Calfee et al., 2014). Zebrafish (*Danio rerio*) adults were also more sensitive than larvae to chromium (Domingues et al., 2010). The 96-h LC_{50} for adults was 39.4 mg/L, whereas LC_{50} for larvae was 145.7 mg/L. These studies suggest that the relationship between sensitivity to metals and life-stage is complex and does not simply follow a linear inverse trend.

Similar to any other class of chemicals, species-specific differences in the sensitivity to metals have been widely reported in the literature (Table 1.1). Several species are more sensitive than standard fish testing species such as rainbow trout and fathead minnow. For example, white sturgeon are more sensitive to Cu than rainbow trout and fathead minnow at all comparable life stages tested (Vardy et al., 2013). In another 96h comparative study with Cu, bonytail chub (*Gila elegans*) and razorback sucker (*Xyrauchen texanus*) were more sensitive than fathead minnow but not rainbow trout (Dwyer et al., 2005). Brown trout fry were more sensitive than rainbow trout fry to metals (Zn, Cu, Pb and Cd) during 8 hours of exposure (Marr et al., 1995). In another study, toxicity of Zn, Cu and Cd to mottled sculpin (*Cottus bairdii*) from two different populations and rainbow trout was compared over 96 hours in early life-stage chronic (21- or 28-d) laboratory toxicity tests. Species average LC_{50} values indicated that for Cu and Zn, mottled sculpin was more sensitive than rainbow trout but both species were equally sensitive to Cd (Besser et al., 2007).

For Cd, alevin arctic grayling (*Thymallus arcticus*) and coho salmon (*Oncorhynchus kisutch*) were shown to be more sensitive than alevin rainbow trout. However, juvenile rainbow trout were equally sensitive as juvenile arctic grayling and slightly more sensitive than juvenile coho salmon. For silver (Ag), both alevin as well as juvenile arctic grayling were more sensitive than alevin rainbow trout. However, alevin coho salmon were equally sensitive as alevin rainbow trout, and juvenile coho salmon were slightly more sensitive than juvenile rainbow trout (Buhl and Hamilton, 1991). Yellow perch (*Perca flavescens*) was reported to be >400 times more tolerant to acute waterborne Cd challenge than rainbow trout (Niyogi and Wood, 2004a). All of these observations suggest that significant differences in sensitivity to metals exist across fish species.

Table 1. 1. Examples of species-specific differences in the sensitivity to Cd and Cu, reported in some freshwater fish species.

Species	Life-stage	Metal	96 h LC_{50} (µg/L)	Water chemistry		Reference
				Hardness (mg/L)	Alkalinity (mg/L)	
Rainbow trout	Juvenile	Cu	80	160 – 180	110 – 120	(Dwyer et al., 1995)
Rainbow trout	Juvenile	Cu	22	45	31	(Vardy et al., 2013)
White sturgeon	Juvenile	Cu	9	45	31	(Vardy et al., 2013)
Fathead minnow	Juvenile	Cu	470	160 – 180	110 – 120	(Dwyer et al., 1995)
Bonytail chub	Juvenile	Cu	220	160 – 180	110 – 120	(Dwyer et al., 1995)
Razorback sucker	Juvenile	Cu	270	160 – 180	110 – 120	(Dwyer et al., 1995)
Mottled sculpin	Juvenile	Cd	23	100	92	(Besser et al., 2007)
Rainbow trout	Juvenile	Cd	5.2	100	92	(Besser et al., 2007)
Arctic grayling	Juvenile	Cd	4	41	31	(Buhl and Hamilton, 1991)
Coho salmon	Juvenile	Cd	3.4	41	31	(Buhl and Hamilton, 1991)
Rainbow trout	Juvenile	Cd	1.5	41	31	(Buhl and Hamilton, 1991)
Yellow perch	Juvenile	Cd	8140	120	95	(Niyogi and Wood, 2004a)

1.2 Mechanistic Basis of Life-stage and Species-specific Differences in the Sensitivity to Metals

The most common presumptions about life-stage- and species-specific differences in sensitivity to metals are related to differences in surface area to volume ratio among organisms. In general, it is believed that species with smaller body size are generally more sensitive because of the higher metal uptake and ionic turnover owing to their higher surface area to volume ratio compared to species with larger body size (Grosell et al., 2002; Mohammed, 2013). For the same reasons, early life-stage fishes and other aquatic organisms are considered to be more sensitive compared to their juvenile or adult counterparts. Although true in many cases, the sensitivity to metals is not always inversely related to the size of an organism, as discussed in section 1.1.

Waterborne metals are known to cause toxicity, particularly during acute exposures, by disrupting branchial ion uptake. For example, uptake of Na and Ca is inhibited by Cu and Cd, respectively (Wood, 2012). Therefore, species-specific differences in metal-ion interaction at the gill and skin surface could be an important factor that may influence the differences in sensitivity of diverse fishes to metals. For example, yellow perch, which is less sensitive to Cd, was also found to be less sensitive to the Cd-induced inhibition of branchial Ca uptake relative to that in rainbow trout (Niyogi and Wood, 2004a). Similarly, the differences between bull trout and rainbow trout with regard to their tolerance to Cd and Zn have been suggested to be due to the different physiological strategies for Ca regulation, although a mechanistic understanding of this phenomenon remains unclear (Hansen et al., 2002).

As mentioned earlier, inhibition of Na uptake is the primary mechanism of acute Cu toxicity. Hence, by using available data on baseline whole body sodium uptake rate, body mass and acute toxicity data of Cu, smaller animals were shown to be more sensitive to Cu than larger animals because of their higher baseline sodium turnover rate. In other words, the same relative inhibition of sodium uptake in two organisms with different sodium turnover rates would result in faster depletion of the internal sodium pool in animals with a higher turnover (Grosell et al., 2002). The reason for higher sodium turnover in smaller animals has been suggested to be due to their greater surface area to volume ratio. A significant and positive correlation (r^2= 0.54) was found between 24 data points of body mass and LC_{50} (96 h) for Cu taken from several studies, which indicates decreasing sensitivity with increasing body mass (Grosell et al., 2002). It is noteworthy

that considerable variation still exists around this correlation, which indicates that factors other than body weight might also have a notable influence on the sensitivity of fishes to metals. Because the mechanism through which Cd influences Ca uptake is analogous to the mechanism of Cu on Na uptake, similar relationships between whole body baseline Ca uptake rate and sensitivity to Cd may also exist, although no experimental evidence of any such relationship has been reported to date.

Underdeveloped detoxification mechanisms for metals, especially metallothionein (MT), has also been suggested to be an important factor in the often-reported higher sensitivity of early life-stage fish. In a multi life-stage experiment with turbot (*Scophthalmus maximus*), lack of MT mRNA induction that was correlated with the highest sensitivity to Cd was reported at the larval life-stage. However, at all later stages of development, the sensitivity to Cd was an order of magnitude less, which was also associated with 3- to 5-fold inductions in MT mRNA levels (George et al., 1996). Another recent study found an inverse relationship between induction of MT and sensitivity to Cu in white sturgeon (Tang et al., 2016). Thus, species-specific differences in MT response to metal exposure could also be an important factor in understanding the species-specific differences in metal sensitivity. However, research in this area is scarce. A recent *in-vitro* study has demonstrated that although MT of white sturgeon is capable of binding metals, the magnitude of response following Cu and Cd exposure was lesser than that observed in other fish species (Doering et al., 2015).

Oxidative stress and antioxidant enzymes have also been studied as factors that may drive life-stage- and species-specific differences in metal sensitivity. In an acute waterborne Cu exposure study in rainbow trout, common carp and gibel carp, it was demonstrated that rainbow trout were three-times more sensitive to Cu exposure than common carp, and almost seven-times more sensitive than gibel carp (De Boeck et al., 2004). To understand these differences in sensitivity to Cu, the authors conducted another complementary study, and demonstrated that gibel carp and common carp reacted more efficiently and faster to Cu induced oxidative stress, and hence showed greater tolerance than rainbow trout (Eyckmans et al., 2011). The same research group conducted more studies to understand the role of differences in MT induction, and tissue-specific Cu bioaccumulation patterns in the same three fish species. After 24, 72 and 168 h of Cu exposure, gibel carp, which was the most Cu tolerant species among the three species tested,

exhibited the strongest correlation between Cu accumulation and MT induction in all tissues, indicating that MT induction plays a significant role in the detoxification of Cu. In contrast, common carp, which was intermediately tolerant to Cu, showed a significant correlation between Cu accumulation and MT induction only in liver and muscle. Rainbow trout, the most sensitive species, showed no correlation at all (De Boeck et al., 2003).

Metal that is accumulated in organs is further distributed in variable proportions to different subcellular fractions, which significantly influences its distribution to target sites, and thus, toxicity outcome of metal accumulation. The subcellular fractions that are considered as metal sensitive include organelles and heat denatured proteins, while other compartments such as metal rich granules and heat stable fractions are predominantly associated with metal detoxification (Wood, 2012). A few studies have indicated that differences in subcellular distribution of metals among different species may contribute in the sensitivity to metals. For example, a study with Cu (50 µg/L for 30 days) was conducted in rainbow trout, common carp and gibel carp to understand the differences in the dynamics of subcellular fractionation patterns of Cu in the gill and liver of these species (Eyckmans et al., 2012). Gibel carp and common carp, the more tolerant species, demonstrated a better capacity to handle excess Cu, as a high percentage of Cu was observed in the metal detoxified fraction. Conversely, rainbow trout distributed its additional Cu exclusively in the metal sensitive fraction. Overall, differences in the subcellular distribution of Cu in the gill and liver of these species seem to contribute to their differences in Cu sensitivity (Eyckmans et al., 2012).

In conclusion, evidence from diverse studies found in the literature suggest the involvement of a variety of toxicokinetic and toxicodynamic factors that may contribute to species-specific differences in the sensitivity to metals. However, the comparison of toxicological, physiological and biochemical effects of metals from different studies is challenging because of the profound impact of water chemistry on metal speciation and uptake in fish. Moreover, the majority of comparative toxicology studies are usually conducted in only one life-stage and address only one of the multiple mechanisms of metal toxicity. Due to these shortcomings, a complete understanding of the specific mechanistic drivers of species-specific differences in the sensitivity to metals are still lacking. Hence, to fully understand the mechanistic basis of species-specific differences in metal sensitivity in fish, it is critical to conduct a comprehensive comparative

analysis by including multiple life-stages and all the major toxicity mechanisms of metals. Moreover, comparative exposure studies must be conducted under identical water chemistry conditions.

1.3 Toxicity of Metals in Rainbow Trout and White Sturgeon

Rainbow trout have been widely used in aquatic toxicity assessment of contaminants because of their widespread availability, proven sensitivity and commercial importance (Environment Canada, 1990). Therefore, the magnitude of toxicity data available for rainbow trout is extensive. For example, searching USEPA's ECOTOX database for toxicity data for Cu alone produced 1299 records related to mortality, reproduction, growth and behavioural endpoints in rainbow trout (search date March 1, 2019). Similarly, a search for Cd produced 875 records. Salmonids are generally considered to be highly sensitive to metals. For example, based on available toxicity data for several metals, rainbow trout was significantly more sensitive than fathead minnow and yellow perch (Niyogi and Wood, 2004a; Teather and Parrott, 2006). Similarly, rainbow trout were more sensitive than some endangered species such as bonytail chub (*Gila elegans*) and razorback sucker (*Xyrauchen texanus*), to short term waterborne Cu exposure (Dwyer et al., 1995). On the other hand, several species are more sensitive to metals than rainbow trout, including white sturgeon (Besser et al., 2007; Marr et al., 1995; Vardy et al., 2013).

White sturgeon have shown a peculiar pattern of sensitivity to metals in a series of recent studies. For Cu, it was demonstrated to be more sensitive than rainbow trout, whereas for Cd and Zn, it was found to be less sensitive than rainbow trout (Calfee et al., 2014; Vardy et al., 2014, 2013). Toxicity data for metals with white sturgeon are not as extensively available as with rainbow trout, partly because its geographical distribution is limited to a few countries only. White sturgeon are the largest North-American fish species, which primarily lives in freshwater, but individuals have been known to enter marine and brackish waters (Fisheries and Oceans Canada, 2016). Several populations of white sturgeon are currently listed as endangered in Canada and the United States of America. Recruitment failure has been indicated as the primary cause of population decline in sturgeon (UCWCR, 2019). Metal contamination was hypothesised to be one possible reason that could have significant negative impact on sturgeon populations. Therefore, several studies were conducted recently with metals in order to estimate the relative sensitivity of sturgeon (Calfee et al., 2014; Tang et al., 2016; Vardy et al., 2014, 2013). These studies have

generated very useful information for site-specific risk estimates for sturgeon to metals. In a recent study, early juveniles sturgeon (45-60 dph) were the most sensitive life-stage to acute Cd exposure, whereas the larval life-stage was the most sensitive life-stage to Cu (Calfee et al., 2014).

1.4 Cadmium

Cd is a hazardous, naturally occurring ubiquitous element, which is mainly found in association with zinc sulfide-based ores. Cd is released into the environment through both natural as well as anthropogenic sources. Major natural sources include weathering of rock and volcanic eruptions, whereas mining, smelting, agricultural activities and application of sewage sludge are the major anthropogenic sources of Cd in the environment. The high potential of Cd to cause adverse environmental effects is reflected by its very low water quality criteria in many jurisdictions as compared to other metals of environmental concern (McGeer et al., 2012).

Cd exists in different chemical forms in natural waters. The chemistry of water has a strong influence on the toxicity of Cd because different species of Cd demonstrate different levels of toxicity in aquatic organisms. In general, the ionic form of Cd (Cd^{+2}) is considered to be the most toxic form. Since hardness, alkalinity, pH, inorganic and organic ligands can influence the concentration of free Cd^{+2} in water, all these factors can have significant influence on the overall toxicity of Cd in aquatic organisms (McGeer et al., 2012).

1.4.1 Cadmium Uptake Pathways

The major route of waterborne Cd uptake in freshwater fish is through the gill. The ionic forms of Cd and Ca have similar size and electronic charge. Therefore, Cd acts as an analogue of Ca on many biological targets including several transporters and channels involved in Ca uptake. The apical uptake of Cd occurs through the epithelial calcium channel (ECaC) located in Mitochondria Rich Cells (MRCs) (Figure 1.1). More recently, divalent metal transporter (DMT-1) and zinc importer (ZIP-8) have also been implicated in the apical uptake of Cd in fish. Two isoforms of DMT-1 are known in teleosts: Nramp-β and Nramp-γ, of which, only Nramp-β was shown to be involved in apical Cd uptake. Once Cd is inside the cell, it gains access to the bloodstream through two major basolateral transporters: plasma membrane Ca-ATPase (PMCA) and Na/Ca-exchanger (NCE) (McGeer et al., 2012).

Other possible routes of Cd uptake are through the gut and skin. Fish in freshwater have a very low drinking rate; therefore, uptake through gut is not considered critical for waterborne Cd exposure. Cd uptake mechanisms in fish guts during dietary exposures is not very well characterized. Available evidence suggests the presence of a Ca sensitive mechanism in the stomach and anterior intestine (McGeer et al., 2012). DMT-1 has also been shown to be involved in Cd uptake through the stomach and intestine. On the other hand, basolateral transport mechanisms in the gut and intestine are completely unknown (McGeer et al., 2012). Small amounts of Cd are transported through skin (Wicklund Glynn, 2001). Although the mechanism of Cd uptake through skin has not been studied to date, the presence of MRCs in skin suggests that the mechanism of Cd uptake could be similar to the gill (Hsiao et al., 2007).

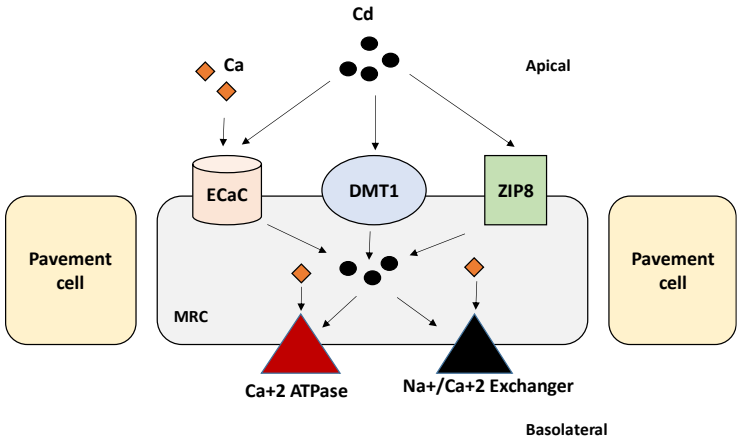

Figure 1. 1. Diagrammatic representation of the uptake routes for Cd in a Mitochondria Rich Cell (MRC) in fish gills. ECaC: epithelial calcium channel; DMT1: divalent metal transporter; ZIP8: Zrt- and Irt-like protein

1.4.2 Mechanism of Cd Toxicity in Fish

Acute Toxicity

During short term exposure, the major mechanism of Cd toxicity is the inhibition of Ca uptake through the gills. In fishes, MRCs are considered to be the major sites for Ca uptake. Uptake of Ca involves an apical channel known as ECaC and two basolateral transporters known as PMCA and NCE (Figure 1.1). According to the currently accepted Ca uptake model, a subset of MRC co-localizes ECaC, PMCA, and NCE; and this subset is responsible for majority of Ca uptake in fish

(Flik et al., 1995; Liao et al., 2007). The best characterized target of Cd action is the PMCA, which is inhibited in a competitive manner by Cd at environmentally relevant concentrations (Verbost et al., 1988). Ca is an essential ion which plays an important role in many homeostatic functions such as nerve conduction, cardiac rhythm, muscle contraction, and metabolism. Therefore, inhibition of Ca uptake leads to its deficiency, which causes adverse effects in organisms including death.

Chronic Toxicity

Mechanism of Cd toxicity during chronic exposure is much more complex and less specific as compared to acute exposure. During chronic exposure, Cd can accumulate in high concentrations in critical organs such as liver and kidney, causing their impairment through several mechanisms. Most commonly reported impairments are related to ionoregulatory processes, reproductive function, embryonic development and the immune system. The most common mechanisms that are involved in chronic Cd toxicity are disruption of ion homeostasis and oxidative stress (McGeer et al., 2012). Moreover, Cd has also been implicated as an endocrine disruptor because of its adverse effects on plasma cortisol and thyroxine levels; however, it is not clear whether endocrine effects are simply a physiological extension of some other effect of Cd or the result of direct interaction of Cd with the endocrine system (Ricard et al., 1998).

1.5 Copper

Cu is an essential metal for all aerobic organisms mainly because its redox potential is utilized by a large number of enzymes. However, because of its redox potential, Cu may also lead to the production of reactive oxygen species (ROS) at elevated concentrations which in turn may lead to adverse health effects in organisms. Hence, organisms must maintain a balance between a deficiency and excess of Cu (Grosell, 2012). Naturally occurring Cu concentrations in surface waters range from 0.2 to 30 µg/L. However, in mining areas, concentrations as high as 200 mg/L have been reported. Mining activities are the major anthropogenic source for Cu contamination. The world production of Cu was estimated at 15.8 million tonnes in 2009 (Grosell, 2012). Cu is one of the most frequently reported metals causing impaired water quality (Reiley, 2007).

As opposed to Cd, there is no general consensus on whether the free ionic form of Cu (Cu^{+2}) is the major toxic form or not. Nonetheless, in the risk assessment of Cu using the BLM, Cu^{+2} and $CuOH^{-}$, both are considered as toxic species. Similar to Cd, temperature and water

chemistry parameters such as hardness, alkalinity, salinity, pH, and dissolved organic matter can influence the speciation of Cu to varying degrees, and thus can influence its uptake and toxicity as well (Grosell, 2012).

1.5.1 Cu Uptake Pathways

Gill epithelium is the most important route of uptake for Cu in fishes during waterborne exposures in freshwater ecosystems. Available evidence suggests the existence of two types of Cu uptake sites on the apical surface of the gills (Figure 1.2): Na insensitive and Na sensitive (Grosell and Wood, 2002). Na sensitive sites are those sites where uptake of Cu is affected by the presence of Na in water: these sites are suspected to be amiloride sensitive epithelial Na channels (ENaC) (Handy et al., 2002); however, full identity of this channel is still elusive. On the other hand, two major candidates are currently believed to participate in Na insensitive apical Cu uptake: Cu transporter (CTR1) and divalent metal transporter (DMT1). On the basolateral surface of gills, Cu is transported through the membrane mainly via two Cu P-type ATPases: ATP7A and ATP7B. P-type ATPases normally reside in the trans-Golgi network but when faced with higher Cu intake, the cell directs these transporters to the basolateral membrane, which promotes the extrusion of excess Cu. ATP7A is considered as the predominant form of P-type ATPase in fish gills (Grosell, 2012).

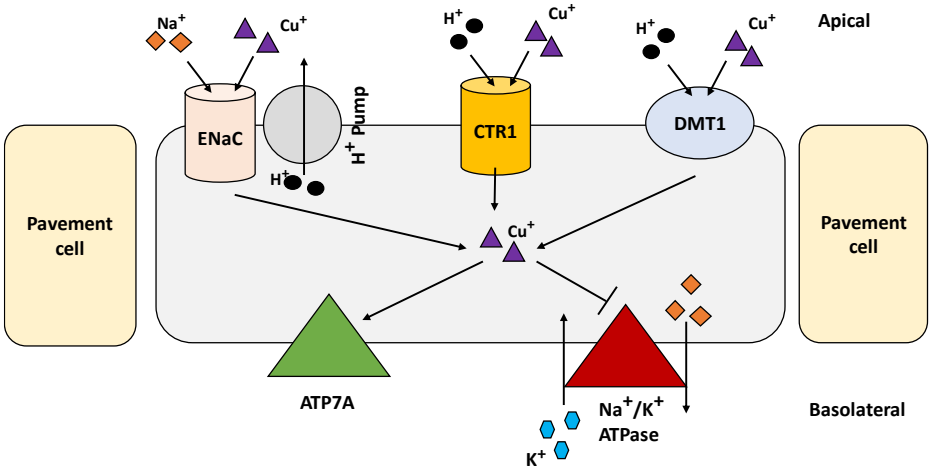

Figure 1. 2. Diagrammatic representation of the uptake routes for Cu in a mitochondria rich cell (MRC) in fish gills. ENaC: epithelial sodium channel; CTR1: copper transporter; DMT1: divalent metal transporter; ATP7A: Cu P-type ATPase type 7A

1.5.2 Mechanism of Cu Toxicity in Fish

Acute toxicity

Acute exposure to Cu has been shown to affect many systems in fish. Some of the most important effects are discussed below:

The most important mechanism of Cu induced mortality during short-term exposure is a reduction in plasma Na levels. Reduction in plasma Na levels could be due to reduced Na uptake, increased Na loss, or both. The most important mechanistic studies for Cu toxicity were performed by Lauren and McDonald (Lauren and McDonald, 1985). These studies established the basis of Cu induced mortality in rainbow trout by demonstrating Cu induced inhibition of Na influx and increased Na efflux, resulting in a pronounced net loss of Na. Na uptake has been shown to be a more sensitive parameter than Na efflux. Although basolateral Na^+/K^+-ATPase (NKA) has been hypothesized as a major site of action for Cu, experimental evidence for any such interaction has been scant. Moreover, an apical Na channel known as acid sensing ion channel (ASIC), whose activity is governed by a proton gradient established by apical V-type H^+-ATPase (VHA), is also thought to be a potential site of action for Cu. A recent study has confirmed the inhibitory effect

of Cu on NKA and VHA, thus confirming them to be the main targets for Cu induced inhibition of Na influx (Chowdhury et al., 2016). Regardless of the mechanism, reduced plasma Na levels induce a long chain of physiological effects leading to cardiovascular collapse (Figure 2).

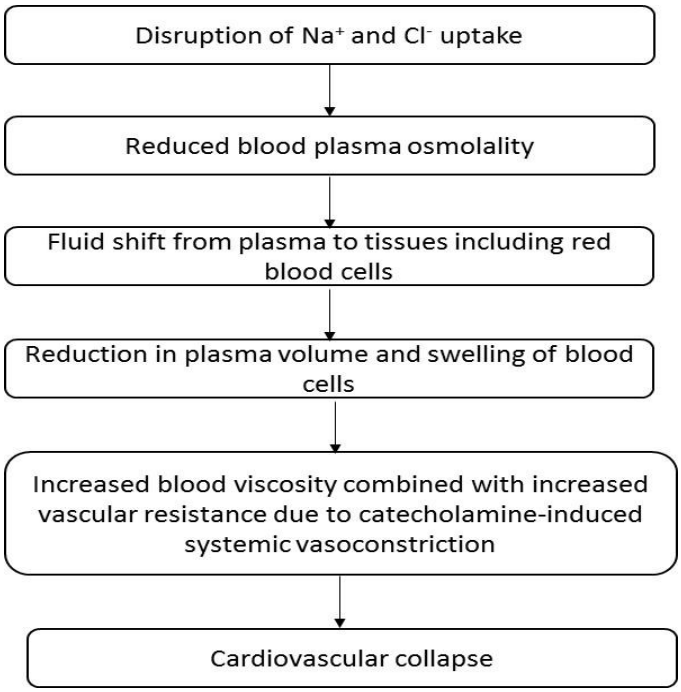

Figure 1. 3. Schematic representation of the steps involved in the manifestation of acute toxicity of Cu (Grosell, 2012).

Elevated plasma ammonia has been another consistent observation in acute Cu exposures that has been demonstrated by many studies (Beaumont et al., 2000; Lauren and McDonald, 1985). Impairment of ammonia excretion could contribute to elevated plasma ammonia during Cu exposure, which may lead to adverse effects including death of the organism (Grosell, 2012). Some recent studies have pointed out gill carbonic anhydrase as a major target for Cu, which explains impairment of ammonia excretion by Cu (Lim et al., 2015).

Cu is also a frequent cause of acid-base disbalance in aquatic organisms. Sublethal Cu exposure causes an elevation in extracellular pH, which is a result of elevated plasma bicarbonate. The mechanism of this effect is unknown but the inactivation of carbonic anhydrase by Cu has

been proposed as a potential pathway (Grosell, 2012). At very high Cu concentrations, respiratory acidosis occurs in combination with metabolic alkalosis (Grosell, 2012).

Short term Cu exposure at environmentally relevant concentrations causes a strong oxidative stress response in fish. Cu causes oxidative stress through a variety of mechanisms such as catalysis of Fenton's reaction to generate reactive oxygen species, depletion of glutathione, and inhibition of antioxidant enzymes (Grosell, 2012). Persistent oxidative stress may lead to DNA damage and peroxidation of membrane lipids and proteins. Apart from antioxidant enzymes, organisms have a few other defense mechanisms against oxidative stress such as metallothionein, reduced glutathione and ascorbate, all of which contribute to quenching metals and/or reactive oxygen species (Eyckmans et al., 2011). Induction of heat shock proteins (HSPs) is another defense mechanism routinely deployed by organisms to protect essential proteins from being damaged by reactive oxygen species (Wood, 2012).

The effect of Cu on olfactory systems has been demonstrated in many studies conducted with environmentally relevant concentrations (Baldwin et al., 2003; Beyers and Farmer, 2001; Pyle and Mirza, 2007). The effect of Cu on the olfactory system originates from the accumulation of Cu in the olfactory epithelium. Finally, Cu is also known to affect mechanoreception by lateral line hair cells. This effect can have detrimental effect on the swimming performance of fish. An important point to note here, and which demands further research, is that water chemistry parameters, with the exception of dissolved organic carbon, offer minor to no protection against Cu toxicity on olfactory and mechanoreception endpoints (Grosell, 2012).

Chronic Toxicity

Reports of chronic toxicity studies with Cu in adult fish are relatively few. The most common long-term studies reported in fish are generally 4-6 weeks duration. Development of acclimation to prolonged Cu exposure has been reported in many studies with compensatory counterbalancing of many parameters such as restoration of Na homeostasis, proliferation of MRCs, and a compensatory increase in NKA activity (Grosell, 2012). Despite the evidence of acclimation to Cu induced disturbances, chronic Cu exposure causes many adverse effects such as reproductive impairment, olfactory disturbances, decreased immune function, and reduced stress responsiveness. All of these adverse effects may have significant impact on fish populations (Grosell, 2012). Similar to the acute exposure scenarios, evidence of oxidative stress and MT

induction has been reported in many prolonged aquatic and dietary Cu exposures (Baker et al., 1998; Eyckmans et al., 2011; Lauren and Mcdonald, 1987; Wang et al., 2014).

1.6 Importance of Understanding the Mechanistic Basis of Life-stage and Species-specific Differences in the Sensitivity to Metals

Characterizing the toxicity of a contaminant in the most sensitive life-stage within the most sensitive species will enable the development of water quality criteria that will be universally protective for all species. Currently, North American water quality criteria are based on methods such as species sensitivity distribution (Canada) or cumulative probability of genus mean acute values (USA). The reason for the popularity of these methods is the fact that they include species of differing sensitivity, and hence provide a certain level of robustness to the derived water quality criteria against species-specific differences in sensitivity. However, current methods only incorporate toxicity data which are already available and there are no ways to predict the relative sensitivity of a previously untested species to a contaminant, partly because the mechanisms of such differences are not fully understood. In North America alone, ~1200 freshwater fish species are currently known, which only represent 8.9% of the diversity of freshwater fishes on earth. The high diversity among fish species makes it logistically challenging to test every combination of toxicant and fish to estimate differences in sensitivity. Therefore, alternative approaches for more efficiently estimating differences in sensitivity among fishes to toxic chemicals are needed. Well defined mechanistic targets for species- and life-stage-specific sensitivity can be utilized to build models for predicting life-stage- and species-specific sensitivities, which could play an important role in improving regulatory decision-making.

1.7 Research Objectives

The overall goal of the thesis was to investigate the mechanistic underpinnings of the species-specific differences in the sensitivity of rainbow trout and white sturgeon to waterborne Cd and Cu over multiple early life-stages. Physiological, biochemical and molecular drivers of species-specific differences in their sensitivity to metals, which were identified in this study, can be exploited in designing novel cross-species ecological assessment methods based on quantitative adverse outcome pathways (detailed description in Section 7.5). After further characterization in

more species, identified targets can be used to create *in-vitro* assays for predicting the dose response curves for Cd and Cu in previously untested species.

The specific research objectives and the experimental design employed for studies included in this thesis are outlined below:

Objective 1: Characterize the acute (96 h) toxicity of Cd in three different early life-stages of rainbow trout and white sturgeon, and investigate the Cd induced ionoregulatory disturbance as a potential driver of the observed species-specific difference in the sensitivity to Cd (Chapter 2)

In this study, I first conducted 96 h acute toxicity tests with Cd in three different life-stages of rainbow trout and white sturgeon: larval, swim-up, and juvenile. The purpose of this experiment was to confirm the previous observations that these two species show different levels of sensitivities to Cd. Ionoregulatory parameters such as whole-body Ca uptake, whole body Ca level, and Cd uptake were then compared between the species to understand their contribution in the apical sensitivity to Cd in the two species.

Objective 2: Characterize the acute (96 h) toxicity of Cu in three different early life-stages of rainbow trout and white sturgeon, and investigate the Cu induced ionoregulatory disturbance as a potential driver of the observed species-specific difference in the sensitivity to Cu (Chapter 3)

In this study, I first conducted 96-hour acute toxicity tests with Cu in three different life-stages of rainbow trout and white sturgeon: larval, swim-up, and juvenile. The purpose of this experiment was to confirm the previous observations that these two species show different levels of sensitivities to Cu. Thereafter, whole body Na uptake, whole body Na level, and whole-body Cu accumulation were studied and compared across species. Additionally, I also conducted a species-specific comparison of NKA activity following exposure to Cu. The purpose of this study was to understand the role of ionoregulatory processes in species-specific differences in the sensitivity to Cu.

Objective 3: Investigate oxidative stress, antioxidant response and detoxification mechanisms as potential drivers of the observed species-specific difference in the sensitivity of rainbow trout and white sturgeon to Cd (Chapter 5)

The objective of this study was to understand the role of oxidative stress, antioxidant defense mechanisms, metallothionein and heat shock protein in the species-specific differences in the sensitivity of sturgeon and trout to Cd across multiple early life-stages. Both species from three different life-stages were exposed to equitoxic concentrations of Cd for 96 hours and then the molecular and biochemical responses were compared across species. Oxidative damage was estimated by measuring lipid hydroperoxide (LPO), and expression of MT, HSP and antioxidant genes were analysed in both species using reverse transcription quantitative polymerase chain reaction (qPCR).

Objective 4: Investigate oxidative stress, antioxidant response and detoxification mechanisms as potential drivers of the observed species-specific difference in the sensitivity of rainbow trout and white sturgeon to Cu (Chapter 6)

The objective of this study was to understand the role of oxidative stress, antioxidant defense mechanisms, metallothionein and heat shock protein in the species-specific differences in the sensitivity of sturgeon and trout to Cu across multiple early life-stages. Both species from three different life-stages were exposed to equitoxic concentrations of Cu for 96 hours and then the molecular and biochemical responses were compared across species and life-stages. Oxidative damage was estimated by measuring lipid hydroperoxide (LPO), and gene expression of MT and HSP were analysed in both species using qPCR. The approach for measuring antioxidant enzymes was slightly different than the approach used in Objective 3. For Cd (Objective 3), I relied on gene expression of the antioxidant enzymes, whereas, for Cu (Objective 4), I used biochemical assays for measuring enzyme activities directly. The Cu study was further complemented by analysing glutathione and ascorbate levels.

Objective 5: Characterize suitable reference genes for gene expression analysis in support of toxicogenomics studies of metals in rainbow trout and white sturgeon (Chapter 4)

Although Chapter 4 does not contribute directly to the overall objective of the thesis, it is deliberately positioned in the middle of the thesis because the objective of this chapter was to identify and select the most suitable reference genes for conducting all the gene expression analysis in the research. Gene expression analysis was employed extensively for the objectives in chapter 5 and 6; therefore, it was of utmost importance that the reference genes, which were to be used in this research, are stable across life-stages in both species and are not affected by either Cd or Cu

exposure. To identify the reference genes that meet these criteria, I assessed the stability of the expression of 8 candidate reference genes in the gills and skin of three different early life-stages of rainbow trout after acute exposure (24 h) to Cd and Cu using qPCR. Parameters of the best reference genes identified for rainbow trout were then applied to candidate reference genes for white sturgeon to confirm their suitability. Reference genes which were identified in this chapter as the most stable were used for all the gene expression analysis described in subsequent chapters.

Hypotheses

For all the major endpoints (dependant variable) measured in this study, there were three independent variables: species, life-stage, and exposure to Cd or Cu. Therefore, these parameters were analysed using three-way ANOVA in statistical analyses. The primary focus of the research was to investigate the species-specific difference in all the parameters across different life-stages after exposure with Cd or Cu. Therefore, the following null hypotheses were tested for all the major parameters analysed in this research:

a) H_0: There is no statistical difference in the parameters of interest across the levels of exposure to Cd or Cu

b) H_0: There is no life-stage and species-specific difference in the parameters of interest in rainbow trout and white sturgeon

d) H_0: There is no significant interaction between species (in corresponding life-stages) and exposure for all the parameters when measured across the levels of exposure to Cd or Cu. In other words, Cd or Cu exposure do not affect the species differently

2 CHAPTER 2: SENSITIVITY OF WHITE STURGEON AND RAINBOW TROUT TO WATERBORNE CADMIUM EXPOSURE: A COMPARATIVE STUDY OF CADMIUM-INDUCED DISRUPTION OF CALCIUM HOMEOSTASIS

PREFACE

The aim of chapter 2 was to compare the effect of Cd on Ca uptake and whole-body Ca levels between rainbow trout and white sturgeon across multiple early life-stages. Whole-body Cd uptake was also compared between the two species. Species-specific Ca uptake and whole-body Ca levels were compared in this study because disruption of Ca homeostasis is one of the most important mechanisms of Cd toxicity during acute waterborne exposure. Hence, disturbances in Ca homeostasis could play an important role in life-stage and species-specific differences in the sensitivity to Cd. Additionally, Cd uptake experiments allowed us to compare the binding affinity of Cd between species.

The content of Chapter 2 was reprinted (adapted) from Environmental Science and Technology, (10.1021/acs.est.8b04828). Shekh, K., Tang, S., Hecker, M., Niyogi, S., "Investigating the Role of Ionoregulatory Processes in the Species- and Life-Stage-Specific Differences in Sensitivity of Rainbow Trout and White Sturgeon to Cadmium" 52, 12868–12876.

Author contributions:

Kamran Shekh (University of Saskatchewan) designed and conducted the experiment, analysed and visualised the data, and drafted the manuscript.

Dr. Song Tang (University of Saskatchewan) provided the necessary training, helped with the experiments, and commented on the manuscript.

Drs. Markus Hecker and Som Niyogi (both University of Saskatchewan) provided inspiration, guidance, and scientific input, commented on the manuscript, and provided funding for the research.

2.1 Abstract

There are huge variations in life-stage- and species-specific sensitivities among the fishes to the exposure with metals; however, the physiological mechanisms underlying these differences are not well understood to date. This study revealed significant life-stage (larval, swim up and juvenile) and species-specific differences between two evolutionarily distant species of fishes, rainbow trout (*Oncorhynchus mykiss*) and white sturgeon (*Acipenser transmontanus*) following acute exposures to cadmium (Cd). Although the 96h LC_{50} of Cd was similar in both species at the larval stage, trout demonstrated an increased sensitivity to Cd at later life-stages as compared to sturgeon. Moreover, exposure to Cd disrupted calcium (Ca) uptake and whole-body Ca levels in trout by a greater degree relative to that in sturgeon regardless of life-stage. Finally, white sturgeon demonstrated a lower affinity for Cd uptake relative to the more sensitive rainbow trout. This infers a differential nature of the interaction between Cd and Ca transport pathways in the two species and partially explains the differences in Cd sensitivity between rainbow trout and white sturgeon described previously. Overall, our results suggest that species- and life-stage-specific differences in sensitivity to waterborne Cd in fish are likely a function of the interplay between Cd uptake and Cd-induced disruption of Ca homeostasis.

2.2 Introduction

Fishes are the most diverse group of vertebrates. In North America alone, approximately 1,200 freshwater fish species are currently known, which only represent 8.9% of the diversity of freshwater fishes on our planet (Burkhead, 2012). This diversity is reflected by huge differences in their sensitivity to environmental pollutants, including metals. The high diversity among fish species makes it logistically challenging to test every combination of toxicant and fish in order to estimate differences in sensitivity. Therefore, alternative approaches for more efficiently estimating differences in sensitivity among fishes to toxic chemicals are needed. Evaluating the physiological and biochemical traits in a species of unknown sensitivity to a chemical and then linking its phylogenetic relationship to other species of known sensitivity could be a useful predictive approach for sensitivity estimation (Guénard et al., 2011). Hence, studies addressing the physiological and/or molecular basis of the species-specific differences in the sensitivity to contaminant exposure can be helpful in designing predictive models for evaluating differences in sensitivity to metals among fishes. However, such studies are scarce to date.

Different species demonstrate significant life-stage-specific differences in the sensitivity to metals such as cadmium (Cd) (Buhl and Hamilton, 1991; Calfee et al., 2014; Tang et al., 2016; Vardy et al., 2014). Hence, the most sensitive life-stage in one species may not be the most sensitive life-stage in another species. These life-stage-specific differences in the sensitivity to metals complicate the interspecific comparison of toxicity data and pose a challenge for cross-species extrapolation of sensitivities to metals. For example, it has been suggested previously that juvenile white sturgeon are more sensitive to Cd than larval rainbow trout, whereas larval sturgeon are less sensitive to Cd than juvenile rainbow trout (Calfee et al., 2014). Based on this and similar observations in other fish species (Buhl and Hamilton, 1991), it appears that comparing the toxicity data of a life-stage in one fish species to a different life-stage of another fish species might result in misleading conclusions about the most sensitive species for any particular metal. It is generally believed that the earlier life-stages of fishes are more sensitive to the exposure with metals than juveniles or adults, which has been attributed to the higher surface area to volume ratio and underdeveloped detoxification mechanisms in larval fish (Anderson and Weber, 1975; Grosell et al., 2002; Mohammed, 2013). Although these principles explain the differences in sensitivity among many fish species, they do not seem to apply universally, as several previous studies

reported that later early life-stages (late larvae/early juveniles) are more sensitive than embryonic or early larval life stages (Calfee et al., 2014; Tang et al., 2016; Vardy et al., 2014). The reasons for these conflicting data remain largely unexplained.

Overall, there is a lack of comprehensive understanding of the mechanistic basis of the species and life-stage-specific differences in metal sensitivity in fishes. Understanding these differences in sensitivity and underlying mechanisms are of critical importance for making informed regulatory decisions and to enable the conduct of objective ecological risk assessments, since it helps in the identification and selection of the most sensitive life-stages and species for further testing.

Cd is a ubiquitous metal of environmental concern because of its presence at elevated concentrations in many ecosystems worldwide and its low threshold to cause adverse effects in aquatic organisms (McGeer et al., 2012). The primary mechanism of its acute toxicity is the disruption of ion homeostasis, particularly calcium (Ca) regulation (McGeer et al., 2012; Niyogi and Wood, 2004a). Several recent studies also proposed other mechanisms leading to toxicity of Cd such as oxidative stress, cell death and immunotoxicity (Wang et al., 2013; Xu et al., 2016; Zheng et al., 2016). Although information on the mechanisms of species and life-stage-specific differences in the sensitivity of fishes to metals are scarce, some recent studies indicated that the molecular pathways driving metal and ion homeostasis and processes such as metal uptake, ionoregulatory disruption, metallothionein induction and oxidative stress response are likely among the most important contributing factors (Doering et al., 2015; Eyckmans et al., 2012, 2011, 2010; Niyogi and Wood, 2004a; Tang et al., 2016).

The aim of this study was to investigate the mechanistic basis of the species and life-stage-specific differences in the sensitivity of two evolutionarily distinct fish species, rainbow trout (*Oncorhynchus mykiss*) and white sturgeon (*Acipenser transmontanus*), to Cd. These two species were selected because white sturgeon and rainbow trout have well-demonstrated significant species and life-stage-specific differences in the sensitivity to Cd (Calfee et al., 2014; Tang et al., 2016; Vardy et al., 2014). In addition, white sturgeon is an endangered species (Fisheries and Oceans Canada, 2016) and information regarding its sensitivity to Cd in comparison to rainbow trout (a species most commonly used in environmental regulatory testing in North America) has important implications for the conservation of this species. We performed a comparative analysis

of Cd uptake across different life-stages of rainbow trout and white sturgeon (larval, swim up, early juvenile) during acute exposure to waterborne Cd. Furthermore, the effect of different Cd concentrations on waterborne Ca uptake and whole-body Ca levels were compared across life-stages and species. Finally, life-stage and species-specific responses of these physiological parameters were analyzed in relation to conventional acute toxicity endpoints for Cd (96h LC_{50}).

2.3 Materials and Methods

2.3.1 Experimental Organisms, Test Chemicals and Exposure Media

Embryos of white sturgeon and rainbow trout were obtained from the Nechako White Sturgeon Conservation Centre (BC, Canada) and Troutlodge (WA, USA), respectively, and were reared in the Aquatic Toxicology Research Facility at the University of Saskatchewan in a flow-through system maintained at approximately 13°C. Sturgeon were fed a diet of commercial frozen bloodworms (San Francisco Bay Brand, Newark, CA, USA) and rainbow trout were fed commercial trout food (Bio Vita Starter #0 Crumble, Bio-Oregon, British Columbia, Canada). Food was first introduced at 10 - 12 days post hatch (dph). Proper permits were obtained before research commenced (Fisheries and Oceans Canada SARA Permit XRSF 20 2013/SARA 305), and all procedures involving animals were approved by the University of Saskatchewan's University Council on Animal Care and Supply (Protocol 20140079).

Cadmium chloride hemi-pentahydrate (CAS 7790-78-5; purity 99.999%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All exposure waters were prepared by mixing reverse osmosis water and dechlorinated city of Saskatoon municipal tap water in the ratio of 3:1 in order to bring the hardness and alkalinity to a moderate level (~60 and 40 mg/L as $CaCO_3$, respectively). Stock solution of Cd was prepared in this water and stored at 13°C for at least 24 hours before exposure.

2.3.2 Acute Cd Toxicity Assessment

Acute toxicity of Cd to early life stages of white sturgeon and rainbow trout was assessed by exposing larval (5dph), swim-up (15 dph), early juvenile (45 dph) and juvenile (75 dph) fish to increasing concentrations of Cd for 96h. Exposures were conducted in triplicate using high density polyethylene (HDPE) tanks of varying capacity based on fish size at different life-stages (5 or 25 L) with 10 individuals per replicate tank (n = 3 with 10 fish per replicate). Fish were acclimated

in normal exposure water (no added Cd) for 5 days prior to the initiation of Cd exposures for acute toxicity assays as well as for all other experimental exposures described below. No food was given during the exposure. Exposures were conducted at a temperature of 13±1°C under static renewal conditions and 16:8 h light/dark cycle. Half of the exposure media was replaced every day and fish were observed for mortality every 12 hours. Trout were exposed to 0, 0.625, 1.25, 2.5, 5, 10 and 20 µg/L (0, 5.6, 11.1, 22.2, 44.5, 89, 177.9 nM) and sturgeon were exposed to 0, 5, 10, 20, 40, 80 and 160 µg/L (0, 44.5, 89, 177.9, 355.8, 711.7, 1423.5 nM) of nominal Cd exposure concentrations (160 µg/L concentration was excluded in 75 dph). Water samples were collected at the beginning and the end of the exposure, filtered, acidified with nitric acid and stored at -20ºC until the analysis of Cd concentrations as described later.

2.3.3 Effect of Cd on Whole Body Ca

The effects of Cd on whole body Ca levels were estimated after 24 h exposure to Cd in the above listed life-stages of both fish species. For each of the life-stages, six fish (n=6) were exposed individually to 0, 1.25 and 5 µg/L (11.1 and 44.5 nM) of Cd concentrations in 500 mL HDPE cups. Half of the exposure media was replaced after 12 hours. The volume of exposure media in each cup was 250 mL (for 5 and 15 dph fish) or 500 mL (for 45 and 75 dph fish). Other experimental conditions such as water chemistry and temperature were the same as described for the acute toxicity assessment. At the termination of exposure, each fish was euthanised with Aquacalm (Metomidate hydrochloride) (Syndel Laboratories Ltd., BC, Canada), washed twice in deionised water, blotted dry, weighed and stored at -80°C until further analysis. For whole body Ca analysis, each fish was digested for 48 h in 5 volumes of 2N trace metal grade Nitric acid (EMD Millipore, Billerica, MA, USA) at 60 ºC. After digestion, samples were centrifuged at 15,000g and whole body Ca was measured in the supernatants using flame atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer, USA) after appropriate dilution in 1% nitric acid. One water sample was collected at random from one of the replicates in each exposure group at the end of the exposure, filtered through a 0.45 µm polycarbonate filter, acidified with trace metal grade nitric acid and stored at -20°C until the analysis of Cd concentrations, as described later.

2.3.4 Whole Body Cd Uptake and the Effect of Cd on Whole Body Ca Uptake

Short-term (3h) concentration-dependent whole body Cd uptake at the above described life-stages of each fish species was determined based on the methods described elsewhere (Niyogi

and Wood, 2004a). For each life-stage of either species, 42 HDPE cups of 120 or 500 mL capacity (7 sets of 6 cups, each set representing one Cd concentration tested at 6 replicates) were filled with 100 or 400 mL of the same water as described in acute toxicity assessment. Each set of cups was spiked with cadmium chloride hemi-pentahydrate solution to achieve approximate nominal Cd concentrations of 0.625, 1.25, 2.5, 5.0, 10, 20, and 40 µg/L (5.6, 11.1, 22.2, 44.5, 89, 177.9, 355.8 nM) for rainbow trout. For white sturgeon, the nominal Cd exposure concentrations were 5, 10, 20, 40, 80 and 160 µg/L (89, 177.9, 355.8, 711.7, 1423.5 nM). Subsequently, each set of exposure cups was spiked with 3 µCi/L ^{109}Cd (as $CdCl_2$, specific activity=3.45 mCi mg/L, Perkin-Elmer, USA). Individual fish were added to each cup (n = 6) and exposed to radiolabeled Cd for 3 h. Due to small size of 5 dph and 15 dph fish, 5-7 larvae per replicate were exposed together and pooled as individual replicate.

At the end of exposure, fish were euthanised by an overdose of Aquacalm, rinsed separately in deionized water (Nanopure, Sybron Barnstead, Boston, USA) for 30 seconds, blotted dry and placed in polyethylene vials. Water samples (5 mL) were collected in duplicate at the beginning and end of the 3h exposure period and acidified with 50 µL concentrated trace metal grade nitric acid. Fish (whole body) and one set of water samples were analysed for radioactivity counts (CPM) using a Wallac Wizard 1480 automatic gamma counter (Perkin-Elmer, Shelton, Connecticut, USA). The other set of water was analysed for total Cd by graphite furnace atomic absorption spectroscopy as described later. The inward whole body influx (J_{in}) for Cd was calculated by an equation described previously (Eq. 1) (Hogstrand et al., 1994):

$$J_{in} = CPM_{wb} / (SA_w * t) \qquad \text{……………………………………………………………………….(2.1)}$$

Where CPM_{wb} is the Cd^{109} radioactivity counts in whole body samples (CPM/g wet weight whole body), SA_w is the mean specific activity of Cd^{109} in water (CPM L^{-1} water/total Cd in water) and t is the time of exposure.

A similar set-up was used to analyse the life-stage- and species-specific differences in the effect of Cd on whole body Ca uptake. For each life-stage, three sets of cups were spiked with cadmium chloride to achieve nominal Cd concentrations of 0, 1.25, and 40 µg/L, and then spiked with 7 µCi/L ^{45}Ca (as $CaCl_2$, specific activity=3.45 mCi mg/L, Perkin-Elmer, USA). Six cups were used for each Cd exposure concentration and individual fish were added to each cup (n = 6) and exposed for 3 h. After the exposure period, fish were euthanized, rinsed with deionized water and

stored at -20ºC. Later, fish were digested in 5 times volume of 2N HNO_3 at 60^0C for 48h and then centrifuged at 15,000g for 5 minutes. Supernatant was collected and diluted in the ratio of 1:5 with scintillation cocktail (Ultima Gold, PerkinElmer, ON, Canada) and counted for radioactivity using a Tricarb 2810TR beta scintillation counter (Perkin-Elmer, ON, Canada). One set of water samples was analysed for Ca^{45} radioactivity using aqueous scintillation fluor (500 µL water diluted 10x in scintillation fluor) (Optiphase, PerkinElmer, ON, Canada), and the other set of water samples was analyzed for total Ca content in water using flame atomic absorption spectroscopy. The inward whole-body influx (J_{in}) for Ca was calculated as described above for whole body Cd influx (Eq. 1). Both fish and water samples were kept overnight after adding scintillation fluid to reduce chemiluminescence. Quenching of Ca^{45} was taken into account by utilizing an external standard ratio method, as described previously (Franklin et al., 2005; McRae et al., 2018). The quench curve was generated by adding known amounts of Ca^{45} to whole-body tissue samples in the same counting cocktail.

2.3.5 Water Chemistry

The same exposure media was used across all studies. Detailed chemistry (Cl, DOC, Ca, Mg, K, Na, SO_4) was analysed for water samples collected at the beginning and end of the project by the analytical laboratory of the Saskatchewan Research Council, Saskatoon, SK, Canada. Parameters such as hardness, alkalinity, pH, dissolved oxygen (DO), ammonia and temperature were measured at the beginning of the exposure and every 24 h thereafter. Hardness, ammonia and alkalinity were determined by using Nutrafin Test kits (Hagen, Canada) and pH, temperature and DO were measured by using YSI Quatro Multi-Parameter probe (Yellow Springs, OH, USA). Dissolved Cd concentrations were measured in the water samples collected as described in the respective method sections using graphite furnace atomic absorbance spectroscopy (AAnalyst 800, Perkin Elmer, USA). The detection limit of the atomic absorption method was 0.005 µg/L. Quality control and quality assurance of Cd measurement was maintained by using appropriate method blanks and a certified Cd standard (Perkin Elmer, USA). In addition, a certified reference material (SLRS-6; National Research Council, Canada) was also analysed to validate the efficiency of the applied method, which yielded a 96% recovery rate of Cd.

2.3.6 Statistical Analysis

The LC_{50}s and concentration dependent Cd uptake parameters (affinity (K_m) and maximum uptake rate (J_{max})) were calculated by fitting the data using the two-parameter log-logistic function (LL.2) and Michaelis-Menten model (MM.2) functions, respectively, in the 'drc' package of R software version 3.1.2 (R Core team, 2015; Ritz et al., 2005). In cases where 50% mortality was not achieved at the greatest concentration tested, the LC_{50} was reported as being greater than the greatest concentration tested. Concentration dependent whole-body Cd uptake at different waterborne Cd concentrations were calculated on the basis of freely available waterborne Cd (as Cd^{2+}), which was estimated using Visual MINTEQ (version 3, Royal Institute of Technology, Stockholm, Sweden).

The effect of Cd on whole body Ca uptake rate and whole-body Ca levels were analysed by using 3-way ANOVA, where the three independent variables were life-stage, species and exposure concentration, and the dependent variable was either whole body Ca level or whole-body Ca uptake rate. After initially analyzing the data by three-way ANOVA, data from individual life-stages were isolated from both species and analyzed with two-way ANOVA, with species and exposure concentrations as independent variables. If the application of two-way ANOVA in a given life-stage revealed statistically significant two-way interactions between species and exposure concentration, it indicated the presence of species-specific difference in the effect of Cd. In such cases, multiple comparison among groups were performed by using Tukey's test and these multiple comparisons allowed us to assess differences among all possible sets of groups, for example: control *versus* control across species or treatment *versus* treatment across species or control *versus* treatment within species for both species. The purpose of multiple comparison approach was to explain the origins of interactions determined by two-way ANOVA and to confirm the presence or absence of the exposure induced species-specific difference in a given life-stage. Uptake parameters K_m and J_{max} among life-stages and species were analysed using 2-way ANOVA (life-stage x species) using the same approach as described for other parameters above. All dependent variables were tested for normality and homogeneity of variance using the Shapiro-Wilk test and Bartlett's test, respectively, and square root transformed if required. An adjusted p-value of <0.05 was considered to be significant while comparing different treatments.

2.4 Results

2.4.1 Exposure Verification and Water Quality

Measured concentrations of Cd in water were comparable to nominal concentrations in all experiments (Table C2.S2). Hardness and alkalinity of the exposure water used in this study were ~60 and ~40 mg/L of $CaCO_3$, respectively. The average temperature, pH and DO were 13.6±0.21, 7.4±0.12, and 91.4±5.12%, respectively (n = 210 water samples). Measured concentrations of anions, cations, and DOC have been provided in the Appendix (Table C2.S1).

2.4.2 Species and Life-Stage-Specific Differences in the Sensitivity to Cd

Rainbow trout showed lesser sensitivity to Cd at 5 dph with a 96h-LC50 of 12.4 µg/L (95% CI 11.66−13.13), relative to the other life stages, which did not differ significantly in sensitivity to exposure with Cd (96-h LC50: 1.93 – 2.93 µg/L) (Figure 2.1). White sturgeon showed greatest sensitivity at 45 dph with a 96h-LC50 of 13.4 µg/L (95% CI 12.4−14.3) compared to 16.7, 75.3, and >80 µg/L at 5 dph, 15 dph and 75 dph, respectively. White sturgeon demonstrated lesser sensitivity to Cd as compared to rainbow trout at all life-stages. The difference in sensitivity was greatest at 15 dph and 75 dph (35.5 and >27.3 times, respectively) as compared to 5 dph and 45 dph (1.35 and 6.92 times, respectively) (Figure 2.1).

2.4.3 Species and Life-stage Specific Differences in the Effects of Cd on Whole Body Ca

All two and three-way interactions for the parameter of whole-body Ca were statistically significant, which indicated the presence of life-stage and species-specific effects of Cd.

In 5 dph life-stage, the two-way interaction between species and exposure was not statistically significant ($F = 2.26$, $p = 0.12$). Furthermore, during 5 dph life-stage, there was no significant difference between species ($F = 0.53$, $p = 0.59$) and the exposure to Cd did not cause any significant reduction in whole-body Ca ($F < 0.001$, $p = 0.99$) (Figure 2.2). In all other life-stages, there were significant two-way interactions between species and exposure, indicating that Cd exposure has a species-specific effect on whole body Ca levels. Rainbow trout exposed at 15 dph, 45 dph, and 75 dph to 1.25 and 5.0 µg/L Cd showed large reductions in whole body Ca levels (44.3 – 61.3% reduction compared to controls) (Figure 2.2). These reductions were statistically significant (Tukey test, adjusted $p < 0.001$ in all life-stages, degrees of freedom (DF) = 30).

However, in contrast to rainbow trout, Cd did not show any significant reduction in whole body Ca levels of white sturgeon, regardless of the life-stages.

The baseline (control) whole body calcium level in larval (5 dph) trout was lowest among all life stages (9.58 µmol/g tissue) (Figure 2.2), which increased up to 114.41 µmol/g tissue by the 75 dph life-stage. In white sturgeon, the baseline Ca levels increased gradually from 7.85 – 39.7 µmol/g tissue between 5 dph to 75 dph life-stage. Across all life-stages except 5 dph, baseline whole body Ca levels were significantly lower in white sturgeon as compared to rainbow trout (Figure 2.2).

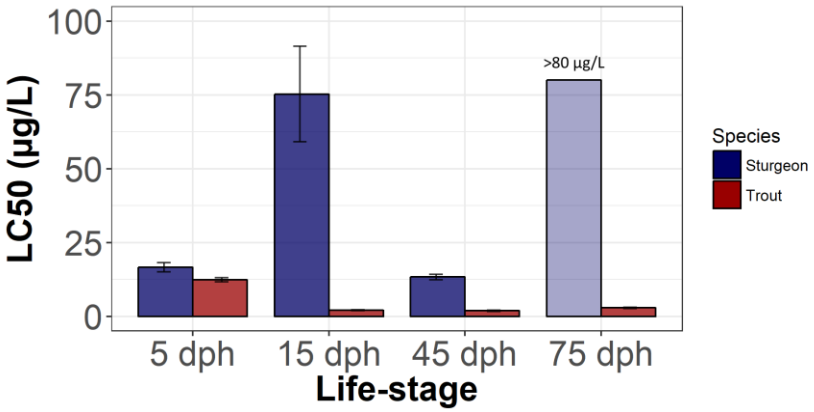

Figure 2. 1. Acute toxicity (96 h LC_{50}) of Cd in different life-stages of white sturgeon (blue) and rainbow trout (brown). Error bars represent 95% confidence interval (n = 3 tanks with 10 fish per replicate). When an LC_{50} value could not be calculated because mortality was less than 50% at the greatest concentration tested, it is represented as light coloured bar.

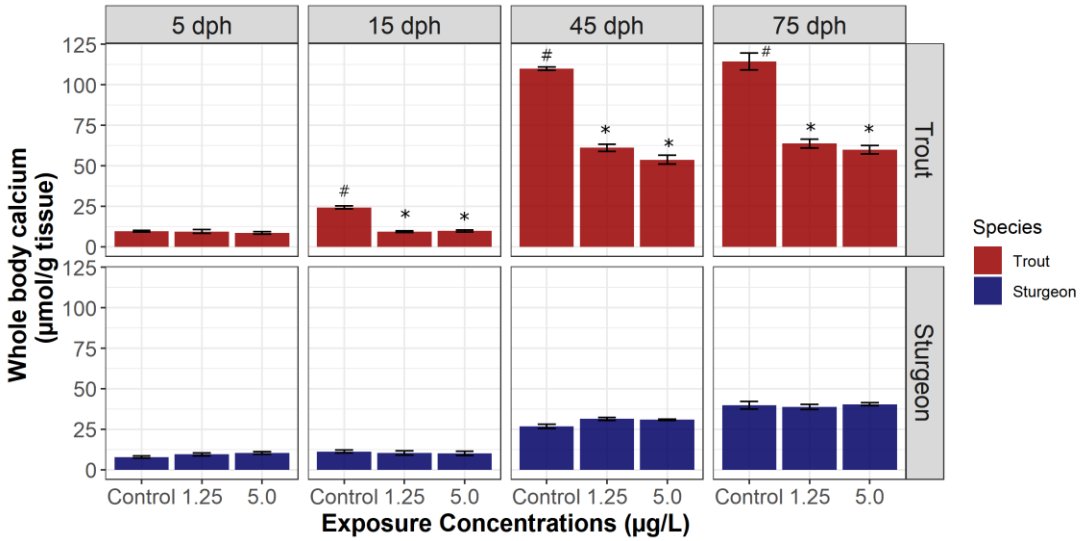

Figure 2. 2. Effect of 24 h exposure to waterborne Cd concentration on whole body Ca levels in rainbow trout and white sturgeon. Data are presented as mean ± SEM values of whole-body Ca (n = 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). # symbol on rainbow trout control group shows that the baseline levels of Ca between species are significantly different ($p < 0.05$).

2.4.4 Effect of Cd on Whole Body Ca Uptake

Application of three-way ANOVA on the effect of Cd on whole body Ca uptake rate revealed significant three and two-way interactions between Cd exposure and species as well as Cd exposure and life-stage, which indicates that Cd exposure has species- and life-stage-specific effects on Ca uptake (two way ANOVA, $F = 4.24, 30.34, 6.09$ and $p = 0.02, <0.001, 0.006$, for 5 dph, 15 dph, and 45 dph, respectively).

In all life-stages, there were significant two-way interactions between species and exposure, indicating that Cd exposure has a species-specific effect on whole-body Ca uptake. The post-hoc t-tests demonstrated that with the exception of 5 dph life-stage, the control levels of Ca uptake were significantly higher in rainbow trout than white sturgeon. Cd exposure (40 µg/L) caused a significant reduction in Ca uptake in all life-stages of rainbow trout, however the magnitude of the effect was lower at 5dph (46.9% reduction) relative to that at 15 and 45 dph (63.2 – 82.9% reduction) (adjusted p values < 0.001 for all life-stages in Tukey tests with DF of 15 for each test) (Figure 2.3). Similarly, in white sturgeon, more sensitive life-stages, 5 dph and 45 dph, exhibited a higher magnitude of effect of Cd on Ca uptake (67.8% and 69.2% reduction, respectively) relative to that in the less sensitive life-stage, 15 dph at 40 µg/L Cd exposure. Between the species, exposure to the same Cd concentration (40 µg/L) led to a greater average reduction in Ca uptake in the more sensitive rainbow trout relative to white sturgeon at 15 and 45 dph, which explained the two-way interactions between species and exposure observed in these life-stages and hence, confirms the species-specific differences in the response to Cd (Figure 2.3). Overall, the effect of Cd on whole body Ca uptake was more prominent in those life-stages and species that were more sensitive to Cd which suggested a possible link between the species or life-stage-specific sensitivity to Cd and Cd-induced inhibition of Ca uptake.

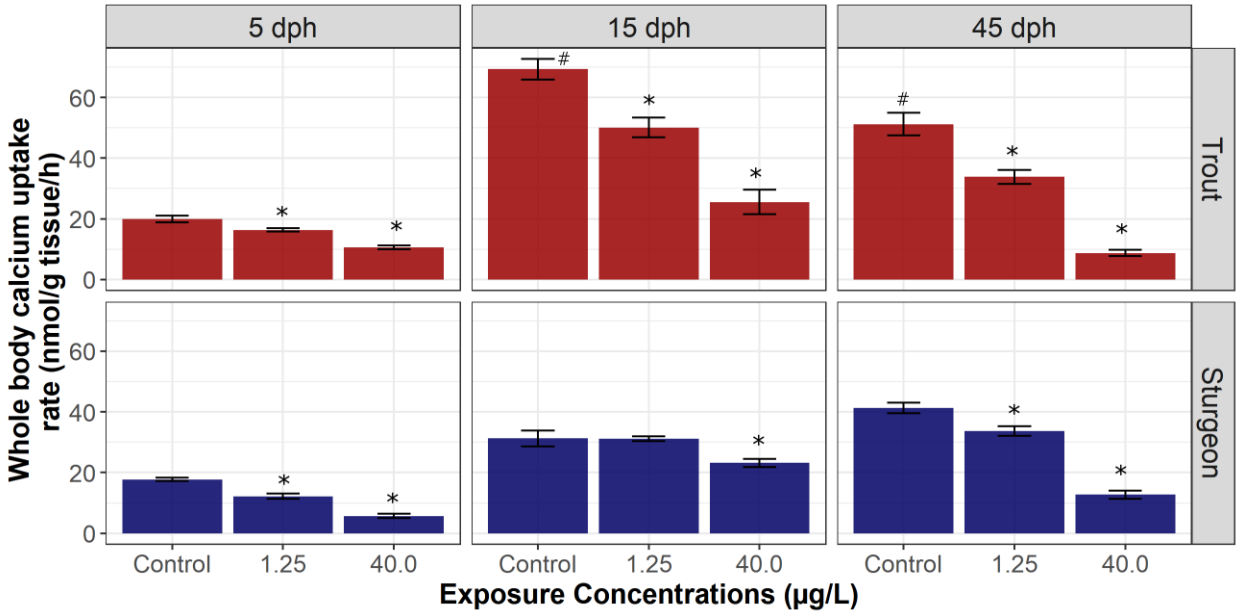

Figure 2. 3. Effect of short-term exposure (3 h) to waterborne Cd concentration on whole body Ca uptake influx in rainbow trout and white sturgeon across different life-stages. Data are presented as mean ± SEM values of whole-body Ca uptake rate (n = 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). # symbol on rainbow trout control group shows that the baseline levels of Ca uptake rate between species are significantly different ($p < 0.05$).

Table 2. 1. Unidirectional concentration dependent Cd uptake parameters (K_m and J_{max}) ± standard error in rainbow trout and white sturgeon from different life-stages. Asterisks on rainbow trout K_m and J_{max} values indicate the statistically significant difference between two species at the corresponding life-stage and different letters in J_{max} indicate life-stage specific differences. No statistically significant life-stage specific differences were found in K_m within a species (tested by two-way ANOVA, n = 6).

		5 dph	**15 dph**	**45 dph**
trout	**K_m (nmol)**	117.9 ± 36.3*	118.9 ± 27.7*	78.94 ± 11.1*
Sturgeon	**K_m (nmol)**	197.34 ± 35.8	282.8 ± 38.8	252.3 ± 37.1
trout	**J_{max} (nmol/g wet wt/h)**	0.04 ± 0.006^{A}*	0.07 ± 0.007^{B}*	0.12 ± 0.006^{C}*
Sturgeon	**J_{max} (nmol/g wet wt/h)**	0.31 ± 0.022^{A}	0.46 ± 0.023^{B}	0.483 ± 0.025^{B}

2.4.4 Species and Life-stage Specific Differences in Cd Uptake

The short-term (3h) rate of Cd uptake in all life-stages exhibited saturation at higher range of Cd exposure concentrations in both species (Figure C2.S1). Michaelis-Menten analysis demonstrated no significant difference in K_m across different life-stages in both species (Table 2.1). The values of K_m ranged between 78.94 – 118.9 nM in rainbow trout and 197.3 – 282.8 nM in white sturgeon across three life-stages. In contrast, both species demonstrated a trend of increased J_{max} values from 5 dph to 45 dph (Table 2.1). The J_{max} value of waterborne Cd uptake in 5 dph rainbow trout was 0.04 (±0.0055) nmol/g wet wt./h, which increased to 0.12 (±0.0061) nmol/g wet wt./h in 45 dph rainbow trout (Tukey test, adjusted p = <0.001, DF= 30). On the other hand, the J_{max} value of 5 dph white sturgeon was 0.31 (±0.022) nmol/g wet wt./h, which increased to 0.48 (±0.025) nmol/g wet wt./h in 45 dph white sturgeon (Tukey test, adjusted $p < 0.001$, DF = 30).

For J_{max} values of Cd uptake between species, there was no significant two-way interaction between species and life-stage ($F = 2.2$, $p = 0.128$) and a significant main species effect ($F = 845.71$, $p<0.001$), which indicated that J_{max} of Cd uptake was significantly higher in white sturgeon as compared to rainbow trout, among all life-stages (Table 2.1). Similarly, for K_m of Cd uptake between species, there was no significant two-way interaction between species and life-stage ($F= 1.26$, $p = 0.299$) and a significant main species effect ($F = 27.24$, $p<0.001$), which indicated that K_m of Cd uptake was significantly higher in white sturgeon as compared to rainbow trout, among all life-stages.

2.5 Discussion

The potential influence of exposure water on species-specific effects of Cd was ruled out by the fact that the water of same chemistry was used across all species and life-stage experiments (Table C2.S1). Therefore, it can be concluded that water chemistry had no influence on the biological endpoints assessed for white sturgeon and rainbow trout in this study.

Our study showed that in rainbow trout and white sturgeon, the earliest life-stage(s) are less sensitive than later life-stages, as opposed to observations in several other aquatic species with a variety of toxicants including metals (Mohammed, 2013). Moreover, in this study, rainbow trout were found to be several-times more sensitive to Cd as compared to sturgeon during later life-stages. Comparative evaluation in previous studies also demonstrated that rainbow trout and

several other salmonids are significantly more sensitive to waterborne Cd than other model fish species (CCME, 2014). The life-stage and species-specific differences in the sensitivity of rainbow trout and white sturgeon to Cd observed in this study followed similar patterns noted in other studies (Calfee et al., 2014; Tang et al., 2016; Vardy et al., 2014). It has been suggested in these previous studies that sensitivity to metals such as Cd can change considerably in fishes during development over a very short duration (e.g. from 5 to 15 dph). Therefore, testing multiple early life-stages could be a better approach to estimate the true sensitivity of the early life-stage of a fish species.

The effect of Cd on whole body Ca levels across different life-stages of rainbow trout followed a similar pattern as observed with the acute toxicity of Cd, indicating a possible link between these two parameters. Loss of Ca from the body (disruption of Ca homeostasis) is considered to be the major lethal mechanism of Cd toxicity in fish (McGeer et al., 2012). While most studies investigating the effect of Cd on Ca levels in fishes have been conducted on plasma (Roch and Maly, 1979), whole body Ca levels have also been successfully used for studying the toxic effects of metals such as Cd in organisms such as fish, midge larvae and sea urchin (Gillis and Wood, 2008; McGeer et al., 2000; Tellis et al., 2014). The baseline (control) whole body calcium level in larval (5 dph) trout was lowest among all life stages and no significant effect of Cd on Ca levels occurred at this life-stage at 1.25 as well as 5.0 µg/L exposure concentrations (Figure 2.2). As the baseline Ca levels increased in later life-stages, the effect of Cd and its toxicity also became more prominent. A lower baseline of whole-body Ca content in larval life-stages might indicate a lower requirement for Ca absorption from the external environment, which could result in a lesser whole-body Ca uptake rate, and thus, lower ability for Cd to produce toxic effects assuming that Cd-induced loss of Ca is the main driver of Cd toxicity. The greater need for Ca during later life-stages of fish is expected due to skeletal growth. A number of studies with rainbow trout and other teleost species have also demonstrated a comparable trend in whole body baseline Ca concentrations with increasing age in growing fry (Chen et al., 2003; Fontagné et al., 2009). The greater need for Ca in later life-stages can potentially make them more susceptible to Cd toxicity. In white sturgeon, the baseline Ca levels increased gradually from 7.85 – 39.7 µmol/g tissue between 5 dph to 75 dph life-stage (Figure 2.2). However, in contrast to rainbow trout, Cd did not show any significant reduction in whole body Ca levels regardless of the life-stages. Based

on these results, it can be suggested that the whole-body Ca levels are an indicator of life-stage-specific sensitivity to Cd in rainbow trout but not in white sturgeon.

Across all life-stages except larval, baseline whole body Ca levels were lower in white sturgeon as compared to rainbow trout (Figure 2.2). These findings are in agreement with previous observations of lower plasma and whole body Ca levels in sturgeon species relative to other fish species (Fuentes et al., 2007; McEnroe and Cech, 1994). The lower Ca levels of sturgeons compared to other fish species has been suggested to be due to their cartilaginous skeleton (McEnroe and Cech, 1994). White sturgeon showed no significant reduction in whole body Ca levels in presence of Cd (1.25 and 5 µg/L), which might explain the lesser sensitivity of white sturgeon than rainbow trout. Higher baseline whole body Ca levels (reported here) and uptake rates (see below) in rainbow trout suggest a higher demand of exogenous Ca and a resultant higher vulnerability to Cd in this species relative to early life-stages of white sturgeon. Considering the significantly lower toxicity (high LC_{50}) of Cd in sturgeon than trout, 5 µg/L of Cd exposure concentration might not have been high enough to produce Ca disbalance in sturgeon. Nonetheless, significant decrease in Ca levels induced by the exposure of 5 µg/L Cd in the more sensitive species used in this study and the lack of similar effect in the less sensitive species at identical Cd exposure level further indicate the linkage between the disruption of Ca balance in the body and species-specific differences in Cd sensitivity. It is to be noted here that exposure to a higher Cd concentration of 40 µg/L resulted in a reduction of Ca uptake in white sturgeon (Figure 2.3). Although we did not measure the effect of 40 µg/L Cd on the whole-body Ca levels in white sturgeon, the decrease in the Ca uptake rate indicates that it might have resulted in the depletion of whole-body Ca as well.

It has been reported previously that Cd elicits differential effects on Ca uptake at different life-stages in the same fish species (Chang et al., 1997). Similar to these previous findings, we also demonstrated here that the inhibitory effect of Cd on Ca uptake was variable between different species and among life-stages. Cd exposure (40 µg/L) caused a significant reduction in Ca uptake in all life-stages of rainbow trout, however the magnitude of the effect was lower at 5dph relative to that at 15 and 45 dph (Figure 2.3). Similarly, in white sturgeon, more sensitive life-stages, 5 dph and 45 dph, exhibited a higher magnitude of effect of Cd on Ca uptake relative to that in the less sensitive life-stage, 15 dph at 40 µg/L Cd exposure. Between the species, exposure to the same Cd concentration (40 µg/L) led to a greater average reduction in Ca uptake in the more sensitive

rainbow trout relative to white sturgeon at 15 and 45 dph. Overall, the effect of Cd on Ca uptake indicated that quantitative differences in the capacity to affect waterborne Ca uptake might be a potential contributor in rendering species and life-stage specific differences in the sensitivity to waterborne Cd. Observations in the Ca uptake study are in agreement with some of the findings of previous studies. Studies with other fish species demonstrated that Ca uptake varies with species as well as life-stages (Chang et al., 1997; Chen et al., 2003; Niyogi and Wood, 2004a). Baseline Ca uptake rate in 15 and 45 dph rainbow trout in our study was in a similar range as previously reported in juvenile rainbow trout (Niyogi and Wood, 2004a). For white sturgeon, the uptake rate of Ca has not been reported in any previous study.

It is also notable that the effect of Cd on Ca uptake seemed to be dependent on baseline Ca uptake rate. For example, 15 dph rainbow trout demonstrated a significantly higher baseline Ca uptake rate than white sturgeon, and the effect of Cd on Ca uptake was more pronounced in trout than sturgeon at this life-stage. At 5 dph, the baseline Ca uptake rate was not significantly different between species and the Cd-induced reduction in Ca uptake was also comparatively similar in both species (Figure 2.3). Hence, our data provided evidence of a relationship between the baseline Ca uptake rate and the toxicity of Cd in both species. A similar relationship between Na uptake rate and Ag toxicity has been suggested in juvenile rainbow trout, juvenile and adult crayfish, and neonate and adult daphnids (Bianchini et al., 2002). Turnover of ions such as Ca is governed by their uptake (influx) and efflux. Sensitivity to Cd is expected to be highly dependent on Ca turnover. Therefore, species-specific differences in Ca efflux could be another important factor in determining the differences in sensitivity to Cd. However, Ca efflux was not measured in this study. Further research linking sensitivity to metals, baseline ion uptake and efflux rates and how these processes are influenced by metals using a variety of species and life-stages are required to gain a more comprehensive understanding of the differences in metal sensitivity in fish.

The stable K_m values across all life-stages in both species indicated that similar pathways (e.g., transporters/channels) of Cd uptake are likely involved in waterborne Cd uptake at each life-stage (Table 2.1). A gradual increase in J_{max} values from larval to later life-stages in both species could be attributed to the gradual increase in the expression of epithelial transporters involved in Cd uptake (e.g., epithelial calcium channel (ECaC), and plasma membrane Ca-ATPase) as suggested previously (Niyogi et al., 2008). A comparison of species-specific waterborne Cd uptake

parameters revealed that generally white sturgeon had a significantly lower affinity (higher K_m) of Cd uptake than rainbow trout across all corresponding life-stages (Table 2.1). The lower affinity of Cd uptake in white sturgeon might also explain its lower sensitivity to Cd compared to rainbow trout, at least in part. However, as discussed earlier, the affinity of waterborne Cd (K_m) did not change significantly across life-stages in both species, indicating that the affinity of waterborne Cd uptake was not a contributing factor for life-stage specific differences in Cd sensitivity in both species. The uptake sites for Cd that showed saturation at higher Cd exposure concentrations are most likely represented by ECaCs and Ca-ATPases, which are known to have a high affinity for Cd, and are thus characterized as the main sites of toxic action for Cd in fish (Niyogi et al., 2008). An examination of life-stage and species-specific differences in gene and protein level response of ECaC and Ca-ATPase during waterborne Cd exposure may provide further insights into the mechanisms underlying the differences in life-stage and species-specific Cd sensitivity. The life-stage specific whole-body K_m values of waterborne Cd uptake in rainbow trout (78.94 – 118.7 nM) were comparable to the K_m values (31.3 – 86.7 nM) previously reported in juvenile rainbow trout in exposure water that contained similar Ca levels (100 - 500 µM) as the water used in present study (300 µM) (Niyogi and Wood, 2004a). We used the negative logarithm of K_m to obtain the affinity constant of whole body Cd uptake (log $K_{Cd\text{-}wb}$) (Wood, 2012). This conversion was useful in directly comparing the affinity of waterborne Cd uptake observed in this study with the waterborne Cd uptake affinity reported previously in fish gills. The whole-body K_m values across different life stages of rainbow trout derived in the present study translate into log $K_{cd\text{-}wb}$ values of 6.9-7.1. This is comparable to the reported affinity constant of gill Cd uptake (log $K_{Cd\text{-}gill}$ =7.1) in rainbow trout exposed to Cd under similar water chemistry conditions as used in the present study (Hollis et al., 2000). For white sturgeon, log $K_{cd\text{-}wb}$ values were in the range of 6.5 – 6.7 for all life stages.

2.6 Conclusion

In conclusion, physiological processes related to acute metal sensitivity among fishes have not been well characterized to date. In this study, significant species and life-stage specific differences were found in Cd toxicity, an observation which was in agreement with previous studies. The two evolutionary distant fish species, white sturgeon and rainbow trout, were shown to present a high degree of variation in species and life-stage specific response to waterborne Cd

exposure: mortality, Cd uptake kinetics and Cd-induced disruption of Ca homeostasis. The 96h LC_{50} of Cd was similar in both species at the larval stage; however, trout demonstrated a greater sensitivity to Cd in later life-stages than sturgeon. We also observed that Cd disrupted Ca uptake and whole-body Ca levels in trout by a greater degree relative to that in sturgeon, especially at those life-stages where trout were significantly more sensitive than sturgeon. Finally, the evaluation of waterborne Cd uptake across life-stages and species demonstrated that the less sensitive species white sturgeon had a relatively lower affinity for Cd uptake compared to the more sensitive species rainbow trout. Interspecific difference in Cd uptake affinity between sturgeon and trout infers differential nature of interaction between waterborne Cd and Ca transport pathways in the two species, which at least partly explain the differences in their Cd sensitivity. Overall, our findings suggest that species and life-stage specific differences in Cd sensitivity depends on the interplay among Cd uptake and the pathways that regulate Ca uptake and Ca levels in the body. Future studies with more species and metals are needed for generating additional data that can be useful in further establishing the correlation between the physiological variables and acute metal sensitivity, as characterized in this study. Well-defined mechanisms for species specific differences in sensitivity to metals can be helpful in building phylogenetically based predictive models for evaluating the sensitivity to metals among different fish species.

3 CHAPTER 3: SENSITIVITY OF WHITE STURGEON AND RAINBOW TROUT TO WATERBORNE COPPER EXPOSURE: A COMPARATIVE STUDY OF COPPER-INDUCED DISRUPTION OF SODIUM HOMEOSTASIS

PREFACE

The aim of chapter 3 was to compare the effect of Cu on Na uptake and whole-body Na levels between rainbow trout and white sturgeon across multiple early life-stages. Species-specific Na uptake and whole-body Na levels were compared in this study because disruption of Na homeostasis is one of the key mechanisms of Cu toxicity during acute waterborne exposure. Hence, disturbances in Na homeostasis could play an important role in life-stage and species-specific differences in the sensitivity to Cu. Na^+/K^+-ATPase is the major target of Cu binding, through which Cu produces its effect on Na uptake process; hence, we also performed a cross species comparison of the effect of Cu on Na^+/K^+-ATPase. Finally, a species and life-stage specific comparison of whole-body Cu accumulation was also performed to evaluate its role in life-stage and species-specific differences in the sensitivity to Cu.

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Author contributions:

Kamran Shekh (University of Saskatchewan) designed and conducted the experiment, analysed and visualised the data, and drafted the manuscript.

Alper James Alcaraz (University of Saskatchewan) helped with the experiment and commented on the manuscript.

Drs. Markus Hecker and Som Niyogi (both University of Saskatchewan) provided inspiration, guidance, and scientific input, commented on the manuscript, and provided funding for the research.

3.1 Abstract

Recent studies have demonstrated that white sturgeon are more sensitive to acute exposure to copper (Cu) than rainbow trout (*Oncorhynchus mykiss*), especially during early life-stages. However, the physiological mechanisms underlying this difference in sensitivity to Cu is not known. In the present study, we first confirmed the higher sensitivity (lower 96h LC_{50} values) of white sturgeon to Cu at three different life stages (larva, swim-up, and juvenile) relative to their counterparts in rainbow trout. We also demonstrated that acute exposure to Cu (50 µg/L for 4.5h) causes a significantly greater reduction in the rate of waterborne Na uptake in white sturgeon relative to that in rainbow trout across all three life-stages. In agreement with this observation, we also found that acute exposure to Cu (20 µg/L for 48h) elicits a significantly greater decrease in whole-body Na level in all of the life stages of white sturgeon compared to rainbow trout. In contrast, white sturgeon demonstrated a higher or similar level of Cu body burden relative to rainbow trout during acute Cu exposure (20 µg/L for 24h), thereby indicating that Cu bioaccumulation is not a good indicator of its toxicity in these species. Overall, our study demonstrated that the differences in sensitivity to acute Cu exposure between white sturgeon and rainbow trout can be explained on the basis of differential effects of Cu on Na homeostasis.

3.2 Introduction

Copper (Cu) is an essential element which plays an important role in maintaining homeostasis in organisms. Nonetheless, Cu is known to be extremely toxic to aquatic organisms beyond its required essential concentrations (Grosell, 2012). Therefore, soil and surface waters with elevated Cu levels are situations of significant environmental concern. Although Cu is present naturally in the environment, anthropogenic activities such as mining, fossil fuel combustion and industry operations in leather, electric, and paint sectors have contributed significantly to the elevation of Cu levels in aquatic and terrestrial ecosystems globally. Due to its ubiquitous release and ability to cause toxic effects, Cu is a very frequent cause of impaired water quality (Grosell, 2012).

While in the majority of cases, acute ambient water quality criteria (AWQC) are governed by data from invertebrates because of their higher sensitivity to toxicants than fish species, it is not always the case. For example, species sensitivity distribution (SSD) used for deriving Canadian AWQC for cadmium (Cd) demonstrated that among the fish species for which reliable toxicity data on Cd are available, rainbow trout (*Oncorhynchus mykiss*) is the most sensitive species among fishes and invertebrates (CCME, 2014). Toxicity testing is usually performed on well characterised model species and hence, data on native non-model fish species are often not available for a majority of contaminants including metals. In such cases, data from surrogate model species such as rainbow trout are routinely used for risk assessment purposes. Therefore, characterizing species-specific differences in sensitivity to metal exposure is of critical importance before making any extrapolation of toxicity data across species. One species of particular concern with regard to its sensitivity to Cu is white sturgeon (*Acipenser transmontanus*). White sturgeon is a North American fish species of commercial, aboriginal and recreational importance. Several populations of white sturgeon are currently endangered or in decline (Fisheries and Oceans Canada, 2016). Recent studies have demonstrated that white sturgeon are more sensitive to acute Cu exposure as compared to rainbow trout during early life-stages. In fact, two recent studies have shown that current AWQCs in many jurisdictions such as those defined by USEPA and Washington State WQS may not be protective of early life-stage white sturgeon from exposure to Cu (Calfee et al., 2014; Vardy et al., 2013). Moreover, white sturgeon have also demonstrated significant life-stage specific differences in their sensitivity to aqueous exposure to Cu, with larval and swim-up life-

stages being several times more sensitive than the juvenile life-stage (Calfee et al., 2014). Life-stage and species-specific differences in the sensitivity to Cu is not only limited to white sturgeon and rainbow trout but also apply to many other fish species studied to date (USEPA, 2007). Similar to other metals, toxicity of Cu is governed by many factors, which include external factors such as ambient concentration and water chemistry as well as internal factors such toxicokinetic and toxicodynamic properties of Cu. However, even when applying normalizing factors based on water chemistry (e.g. the biotic ligand model) to the available acute toxicity data, there remain significant life-stage and species-specific differences in the sensitivity to Cu across fish species (USEPA, 2007). Mechanistic underpinnings of such life-stage and species-specific differences are currently not well understood. Understanding the mechanism of these differences is of vital importance because the knowledge of these mechanisms can be helpful in conducting better informed risk assessments of metals and aid in building predictive models for hazard assessment.

Emerging evidence suggests that the sensitivity of an organism to metals could be determined by toxicokinetic factors such as uptake and elimination and/or toxicodynamic factors such as iono-regulatory disruption, oxidative stress and metallothionein homeostasis (Buchwalter et al., 2008; Eyckmans et al., 2011; Shekh et al., 2018; Tang et al., 2016). Baseline uptake rates of essential ions such as calcium (Ca) and sodium (Na) have been shown to influence the sensitivity of several aquatic species to exposure to Cadmium (Cd) and silver (Ag), respectively (Bianchini et al., 2002; Shekh et al., 2018). Exposure to Cu in three different fish species of differing sensitivity (rainbow trout, common carp and gibel carp) demonstrated species-specific effects on hormonal and ionoregulatory parameters (Eyckmans et al., 2010). Rainbow trout, which was the most sensitive species to Cu, showed significant reduction in plasma Na and the effects occurred more rapidly in this species relative to the two carp species. This could explain, at least partially, the higher sensitivity of rainbow trout to acute Cu exposure than that of the other species (Eyckmans et al., 2010). To date, no experimental evidence exists that could explain the higher sensitivity of white sturgeon to Cu. Based on the current knowledge of the mechanisms of Cu toxicity, it could be hypothesized that higher sensitivity of white sturgeon to Cu is due to their greater susceptibility to the loss of Na balance during exposure to Cu.

Therefore, the aim of the current study was to investigate the underlying physiological basis of the greater sensitivity of white sturgeon to Cu relative to rainbow trout, across several

early life-stages. Since the mechanisms of acute Cu toxicity in fish are well characterized, we conducted a comparative analysis of some of the main acute Cu toxicity endpoints across different life-stages of rainbow trout and white sturgeon. Such an approach could be successful in characterizing the mechanistic basis of sensitivity to Cu and other metals that have similar mechanisms of toxicity. One of the most important mechanisms through which Cu is known to cause acute toxicity in fish is the inhibition of branchial Na uptake and the subsequent disruption of Na homeostasis and the ensuing cascade of events leading to the cardiovascular collapse (Grosell, 2012). In addition to inhibiting Na influx, Cu exposure has also been shown to increase the efflux of Na, possibly through paracellular pathways (Grosell, 2012).

In the present study, we performed a comparative analysis of Cu accumulation and the effect of Cu on Na uptake and whole-body Na levels during acute Cu exposure in three different early life-stages (larval, swim-up and juvenile) of rainbow trout and white sturgeon. Moreover, we also performed a cross-species comparative analysis of the effect of Cu on Na^+/K^+-ATPase (NKA) and H^+-ATPase (HAT), which are important enzymes regulating branchial Na uptake, and hence represent important targets of Cu toxicity in fish. To understand the mechanisms of species and life-stage specific differences in the sensitivity to Cu, these physiological and biochemical parameters were compared and discussed in relation to the most common acute apical indicator of sensitivity (96 h LC_{50}).

3.3 Materials and Methods

3.3.1 Experimental Organisms

The experiments were performed using early life-stages of rainbow trout and white sturgeon. White sturgeon embryos were provided by Nechako White Sturgeon Conservation Centre (BC, Canada), whereas rainbow trout embryos were obtained from Troutlodge (WA, USA). Embryos were reared at a temperature of ~13 °C under a flow through system located at the Aquatic Toxicology Research Facility (ATRF), University of Saskatchewan, Canada. Food was first introduced to fish at the beginning of the swim-up life-stage which was 10-12 days post hatch (dph) for both species. All required permits and approvals were obtained prior to the commencement of experiments (Fisheries and Oceans Canada SARA Permit XRSF 20 2013/SARA 305 and Protocol # 20140079 approved by University of Saskatchewan's University Council on Animal Care and Supply).

3.3.2 Test Chemical and Exposure Media

All exposures were performed with Copper (II) sulfate pentahydrate (CAS# 7758-99-8; purity, 99.995%, Sigma-Aldrich (St. Louis, MO, USA)). Exposures were conducted in a water of moderate hardness and alkalinity (~65 and 40 mg/L as $CaCO_3$, respectively), which was achieved by mixing dechlorinated tap water with reverse osmosis water in the ratio of 1:3. Stock solution of Cu was prepared in this water and stored for at least 24 hours prior to exposure at the temperature of 13 °C.

3.3.3 Assessment of Acute Cu Toxicity

Assessment of acute Cu toxicity was carried out at three analogous early life-stages in rainbow trout and white sturgeon: larval (5 dph), swim-up (28 dph) and early juvenile (65 dph). Fish were exposed to increasing waterborne concentrations of Cu for 96 hours in triplicate high-density polyethylene (HDPE) tanks with 10 individuals per replicate. The nominal Cu concentrations used for the exposures were 0, 2.5, 5, 10, 20, and 40 µg/L for white sturgeon and 0, 5, 10, 20, 40, and 80 µg/L for rainbow trout. Fish were acclimated to exposure water (without added Cu) for 5 days prior to initiation of the experiments. Throughout the acclimation and exposure periods, the average temperature was maintained at 13±1 °C and 16:8 hours light and dark cycle. All exposures were conducted under static renewal conditions, and no food was provided to fish during the exposure. Cu concentrations were measured in water samples that were collected at the beginning and end of the exposure. All water samples were filtered through 0.45 µm syringe filter (VWR, PA, USA), acidified with nitric acid and stored at 4° C until the measurement of dissolved Cu concentrations.

3.3.4 Assessment of the Effect of Waterborne Cu on Na Uptake

Effect of Cu on whole body Na uptake was measured by exposing both species to Cu over a period of 6 hours as described in detail previously (Zimmer et al., 2014). Briefly, for each life-stage, a set of 6 HDPE cups (120 mL) were assigned to each exposure group: control, 5 and 50 µg/L (n = 6). The highest concentration (50 µg/L) for this experiment was chosen on the basis of a previous study which showed that this concentration inhibits Na uptake in early life-stage rainbow trout (Zimmer et al., 2014). After achieving nominal concentrations of Cu in the respective groups, individual fish were added into each cup and exposed under these conditions

for 3 hours. Following the pre-exposure period, each cup was spiked with 7 μCi/L ^{22}Na (PerkinElmer, USA). After allowing a period of 5 minutes for mixing, water samples were collected for the measurement of radioactivity counts (CPM) and total dissolved concentrations of Na and Cu. The Na^{22} flux was measured for a period of 1.5 h. At the end of the flux period, another set of water samples was collected, and fish were euthanized with Aquacalm (metomidate hydrochloride) (Syndel Laboratories Ltd., BC, Canada), rinsed twice in 5 mM NaCl for 10 seconds, blotted dry, weighed and collected in polyethylene vials. CPM counts were measured using a Wallac Wizard 1480 automatic gamma counter (PerkinElmer, Shelton, CT), and total Na and Cu concentrations in water samples were measured using flame and furnace atomic absorption spectroscopy, respectively (AAnalyst 800, Perkin Elmer, USA). Finally, CPM counts in whole fish were also measured and inward whole-body influx (J_{in}) for Na was calculated for each exposure group by the following equation (Eq. 1):

$$J_{in} = CPM_{wb} /(SA_w t) \quad \text{.....(3.1)}$$

Where, CPM_{wb} represents the ^{22}Na radioactivity counts in whole body (CPM/g wet weight whole body), SA_w is the mean specific activity of ^{22}Na in water (CPM L^{-1} water/total Na in water), and t is the time of exposure, which was 1.5 h in this experiment.

3.3.5 Whole Body Accumulation of Cu and the Effects of Waterborne Cu on Whole Body Na Levels

Accumulation of Cu was measured in whole bodies of fish after the exposure of both species to 0 and 20 μg/L Cu at three life-stages: larval, swim-up and juvenile. For both control and exposure groups, five individual HDPE cups were used as replicates (n = 5). Because of the small size of the fish in the larval and swim-up life-stages, six fish were pooled for each replicate cup; whereas, for juvenile life-stage, individual fish were added in each cup. Exposure duration was 24 and 48-hours for Cu accumulation and whole-body Na levels, respectively, after which, the fish were euthanized with Aquacalm (metomidate hydrochloride; Syndel Laboratories Ltd., BC, Canada), washed twice in deionized water for 30 seconds, blotted dried and weighed. Fish were then digested in 2N trace metal grade nitric acid (EMD Millipore, Billerica, MA, USA) for 48 hours at 60 °C. Finally, samples were centrifuged, and Cu and Na levels were measured in supernatants after appropriate dilution in 0.1% nitric acid. Cu and Na in fish were measured using graphite furnace and flame atomic absorption spectroscopy, respectively. Certified Cu standard

(Fisher Scientific, Canada), appropriate method blanks, and a reference material (DOLT-4; National Research Council of Canada) were utilized to ensure the quality control of the measurements. The recovery percent of Cu in the reference material was 104%. One water sample was collected randomly from each exposure group at the beginning of exposure and filtered through a 0.45 μm syringe filter (VWR, PA, USA). All water samples were acidified and stored in 4°C until the analysis of dissolved Cu concentrations (described later).

3.3.6 Assessment of Cu Induced Inhibition of Na+/K+-ATPase (NKA) and H^+-ATPase (HAT)

A comparative analysis of Cu induced inhibition of NKA and HAT was performed between juvenile rainbow trout and white sturgeon by exposing them to 0 and 40 μg/L of Cu over an exposure period of 24 hours. After the exposure period, the fish were euthanized and the gill tissues from both species were dissected immediately, rinsed with water, blotted dried and stored at -80 °C until the assay for NKA and HAT. The assays were performed as described in detail previously (Chowdhury et al., 2016). Plates were read in a multimode plate reader (Verioskan Flash,Thermo Scientific, ON, Canada) immediately after the assay and the readings were taken at 340 nm wavelength for 20 minutes at 10 seconds interval and at a constant temperature of 25 °C. A standard curve was prepared from 0 to 20 nmol ADP/well.

NKA and HAT enzyme activities were expressed as ADP/mg protein/h which was calculated using the slope of linear disappearance of the reduced form of nicotinamide adenine dinucleotide (NADH) from the reaction over time. All enzyme activities were normalized to the total protein content in the respective tissue. Protein concentrations in homogenates were measured using the Bradford reagent (Sigma-Aldrich, MO, USA).

3.3.7 Water Chemistry

Water samples were collected at the beginning and the end of the exposures and concentrations of Cl, DOC, Ca, Mg, K, Na, and SO_4 were analyzed. Water hardness, alkalinity, temperature, pH, dissolved oxygen (DO) and ammonia were measured at the beginning as well as every 24-h during the exposures. Moreover, dissolved Cu concentrations were measured in the water samples collected as described in respective method sections, to ensure the accuracy of the nominal concentrations. Dissolved Cu concentrations were measured in the water samples using a graphite furnace atomic absorbance spectrometer (AAnalyst 800, PerkinElmer, USA), which had

a detection limit of 0.01 μg/L. Quality control for the Cu measurement was established by using appropriate method blanks, certified Cu standard (PerkinElmer, USA), and a certified reference material (SLRS-6; National Research Council, Canada). A 96% recovery rate of Cu was achieved in the reference material.

3.3.8 Statistical Analysis

LC_{50} values were calculated using the "drc" package of R software version 3.1.2 by fitting the mortality and measured exposure concentration data to a two-parameter log-logistic function (LL.2). Three-way ANOVA was employed to analyze whole-body Cu accumulation, effect of Cu on whole body Na levels and whole-body Na uptake rates, where the independent variables were species, life-stage and Cu exposure concentrations, and the dependent variable was either whole body Cu, whole body Na level or whole-body Na uptake rate. After initially analyzing the data by three-way ANOVA, data from individual life-stages were isolated from both species and analyzed with two-way ANOVA, with species and exposure concentrations as independent variables. If the application of two-way ANOVA in a given life-stage revealed statistically significant interaction between species and exposure concentration, it indicated the presence of species-specific difference in the effect of Cu. In such cases, multiple comparison among groups were performed by using Tukey's test and these multiple comparisons allowed us to assess differences among all possible sets of groups, for example: control *versus* control across species or treatment *versus* treatment across species or control *versus* treatment within species for both species. The purpose of multiple comparison approach was to explain the origins of interactions determined by two-way ANOVA and to confirm the presence or absence of the exposure induced species-specific difference in a given life-stage. Effect of Cu on gill NKA and HAT enzyme activities were analysed only in juvenile rainbow trout and white sturgeon by using two-way ANOVA with species and Cu exposure concentrations as the independent variables and enzyme activity as the dependent variable. All dependent variables were tested for homogeneity of variance and normality using Shapiro−Wilk test and Bartlett's test, respectively, and square root transformed if required. A p-value of <0.05 was considered to be significant while comparing different treatments.

3.4 Results

3.4.1 Exposure Verification and Water Quality - Toxicity Assessment Experiment

Measured concentrations of major cations, anions and DOC are present in Table 1. Nominal and measured concentrations of Cu were comparable in all exposure groups (Table S3). The average pH, temperature, and DO of water used in this study were 7.3±0.11, 13.8±0.26 and 93.7±4.66%, respectively Chemistry of all the exposure water was kept consistent throughout the study by mixing dechlorinated tap water with reverse osmosis water in the ratio of 1:3; therefore, it can be concluded that there was no measurable influence of water chemistry on the biological endpoints assessed in this study.

3.4.2 Water Quality - Na Uptake Experiment

Measured Cu concentrations were comparable to the nominal concentration used in this study (Table C3.S1). The average temperature during the exposures was 13.1 ± 0.29 ºC. The average pH and DO were 7.2 ± 0.14 and 94.17 ± 3.57%, respectively. Measured concentrations of anions, cations, and DOC have been provided in the Appendix (Table C2.S1).

3.4.3 Waterborne Acute Cu Toxicity

The 96-hour LC_{50} values for Cu ranged from 24.15 to 36.36 µg/L in white sturgeon, whereas for rainbow trout, LC_{50} values were 60.55 – 83.05 µg/L (Figure 3.1). The highest difference in the sensitivity to Cu between rainbow trout and white sturgeon was at swim-up life stage, followed by juvenile and larval life-stage (Figure 3.1).

3.4.4 Effect of Cu on the Whole Body Sodium Level

Application of three-way ANOVA revealed no three-way interaction (species x life-stage x exposure; $F = 1.63$, $p = 0.21$). Moreover, interactions between life-stage and exposure ($F = 0.43$, $p = 0.65$) as well as species and life-stage ($F = 2.73$, $p = 0.08$) were statistically non-significant. Finally, interaction between species and exposure were statistically significant ($F = 10.29$, $p = 0.002$). These observations suggest that there could be a species-specific difference in the effects of Cu on whole body Na levels. To understand species-specific differences more closely, we separated each life-stage and applied a two-way ANOVA between species within each life-stage, keeping exposure and species as two independent variables. During the larval life-stage, there was

no significant two-way interaction between species and exposure ($F = 0.73$, $p = 0.41$), suggesting that there were no species-specific effects of Cu on whole body Na levels. Indeed, the effects of Cu on whole body Na levels were very similar in rainbow trout and white sturgeon (11.6% and 16.2% reduction, respectively) (Figure 3.2). On the other hand, in both swim-up and juvenile life-stage, there was significant species-specific differences between rainbow trout and white sturgeon. Exposure to 20 µg/L Cu for 48 hours resulted in a 26.2% (adjusted $p = 0.01$, $DF = 20$) and 39.7 % (adjusted $p = 0.02$, $DF = 20$) reduction in whole body sodium in swim-up and juvenile white sturgeon, respectively, whereas the same Cu exposure regime caused only a 12.7% reduction in swim-up (adjusted $p = 0.71$, $DF = 20$) and no reduction in juvenile rainbow trout (adjusted $p = 0.9$, $DF = 20$) (Figure 3.2). Exposure to 20 µg/L Cu for 24 h also caused a minor reduction in whole body Na levels in both species, but the species-specific differences were not significant (Table C3.S2). It is to be noted that we also exposed both species to 40 µg Cu /L for 48 hours, which showed a significant reduction in whole body Na levels in rainbow trout, but the effects in rainbow trout could not be compared with white sturgeon because of the high number of mortalities in the latter species during Cu exposure.

3.4.5 Effect of Cu on Whole Body Sodium Uptake Rate

Analysis of Na uptake rate with three-way ANOVA revealed a significant interaction among species, life-stages and exposure ($F = 3.93$, $p = 0.03$). To fully understand the species-specific difference, we analyzed each life-stage individually using two-way ANOVA, with species and exposure as independent variables.

During the larval life-stage, exposure to Cu (50 µg/L) resulted in a higher % reduction in whole body Na uptake rate in white sturgeon (76.7%) (adjusted $p < 0.001$, $DF = 30$) compared to rainbow trout (27.8%) (adjusted $p = 0.04$, $DF = 30$) (Figure 3.3), which explains the two-way interaction observed between species and exposure during this life stage ($p < 0.001$). Furthermore, it also explains the higher sensitivity of white sturgeon to Cu during this life-stage. Similarly, at the swim-up life stage, exposure to Cu caused a significantly greater reduction in Na uptake rate in sturgeon relative to trout (70.9% and 11.8%, respectively) (adjusted p values = 0.004 and 0.95, respectively, $DF = 30$). Finally, at the juvenile stage, exposure to Cu (50 µg/L) resulted in a significant reduction in Na uptake rate in both white sturgeon and rainbow trout; however, the magnitude of the effect was again significantly greater in white sturgeon (68.4%, adjusted p

<0.001, DF = 30) than that in rainbow trout (32.1%, adjusted p = 0.005, DF = 30), which explains the two-way interaction observed between species and exposure during this life stage ($p < 0.001$) and also explains the higher sensitivity of sturgeon to Cu at this life-stage (Figure 3.3).

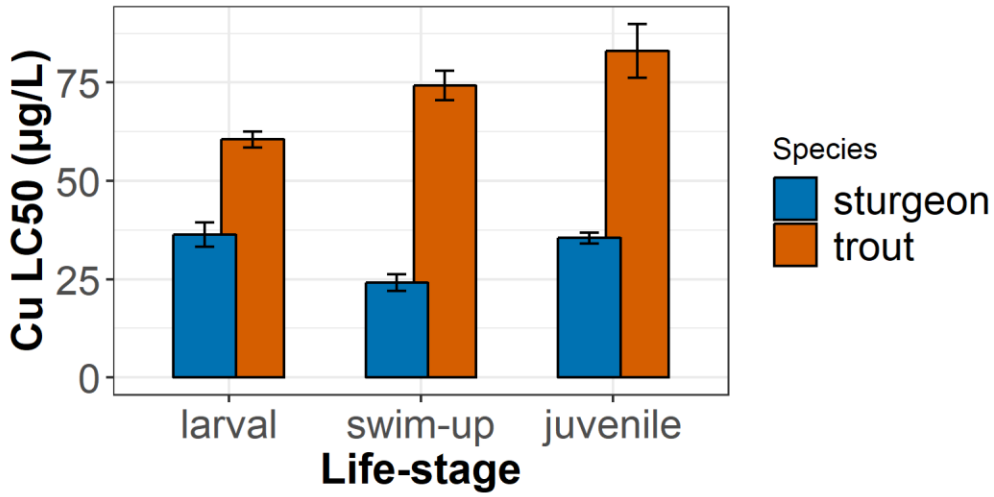

Figure 3. 1. Acute toxicity (96 h LC_{50}) of Cu in different life-stages of white sturgeon (blue) and rainbow trout (yellow). Error bars represent 95% confidence interval (n = 3 tanks with 10 fish per replicate).

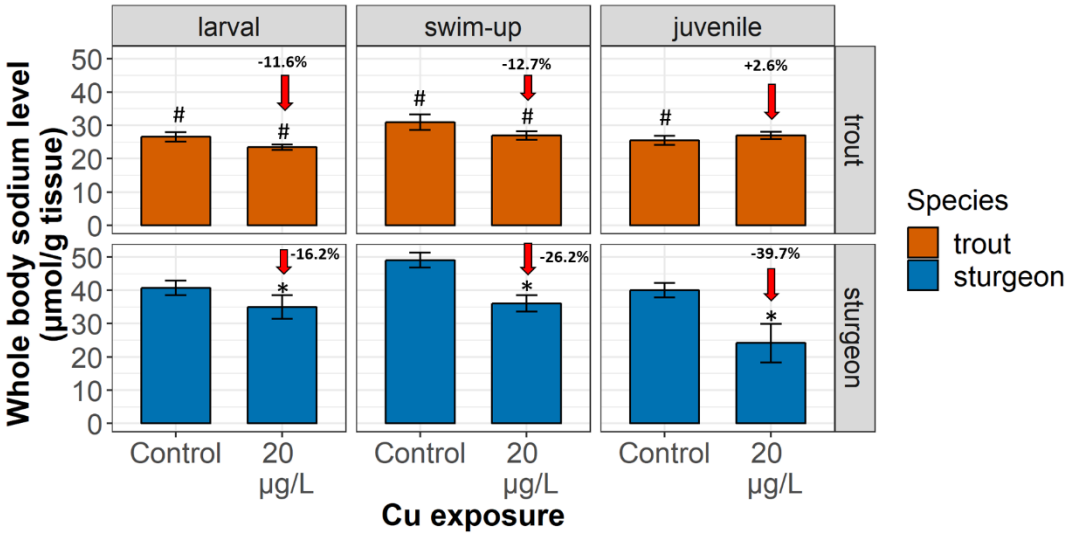

Figure 3. 2. Effect of 48 h exposure to different waterborne Cu concentrations on whole body Na levels in rainbow trout and white sturgeon. Data are presented as mean ± SEM values of whole-body Na (n = 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). A number symbol (#) on rainbow trout bars represents a significant difference between rainbow trout and white sturgeon for the corresponding exposure group.

3.4.6 Effect of Cu on Na+/K+-ATPase (NKA) and H^+-ATPase (HAT) Activity

The effect of Cu on NKA and HAT was only measured in the gills of fish at the juvenile life-stage, because the gills were not fully developed in the earlier life-stages, and thus, difficult to isolate. Applying the two-way ANOVA revealed no statistically significant two-way interactions between species and Cu exposure in either NKA or HAT ($F = 5.1e^{-4}$ and 0.15, and $p = 0.98$ and 0.71, respectively). Moreover, the main effect in species and exposure was also not statistically significant (Figure 3.4). Overall, these findings suggest that there were no species-specific differences in the activities of these enzymes as a result of Cu exposure.

3.4.7 Whole Body Cu Accumulation

Statistical analysis of whole-body Cu accumulation showed significant three-way interactions ($F = 29.32$, $p < 0.001$), which suggested the possibility of species and life-stage specific differences in whole-body Cu accumulation. In the larval life-stage, the two-way interaction between species and Cu exposure was statistically significant ($F = 11.15$, $p = 0.004$), which indicates a species-specific difference in whole body Cu accumulation at this life-stage. Post-hoc Tukey test revealed no significant difference between the control groups of white sturgeon and rainbow trout (adjusted $p = 0.93$, DF = 20), whereas exposure to Cu resulted in a significant increase in whole body Cu burden in sturgeon relative to trout (adjusted $p < 0.001$) (Figure 3.5). Thus, at the larval life-stage, whole body Cu accumulation was significantly higher in white sturgeon compared to that in rainbow trout. In contrast, during swim-up, exposure to Cu resulted in statistically significant higher Cu accumulation in rainbow trout relative to that in white sturgeon (adjusted $p < 0.001$, DF = 13). For juvenile trout and sturgeon, the two-way interaction between species and exposure was not statistically significant ($p = 0.51$), which indicated that there was no species-specific difference in the whole-body Cu accumulation as a result of acute exposure to Cu. As Figure 3.5 indicates, there was a 1.9-times increase in whole body Cu following exposure to Cu in juvenile rainbow trout, which was very similar to a 2.1-times increase in white sturgeon exposed to the same waterborne Cu concentration.

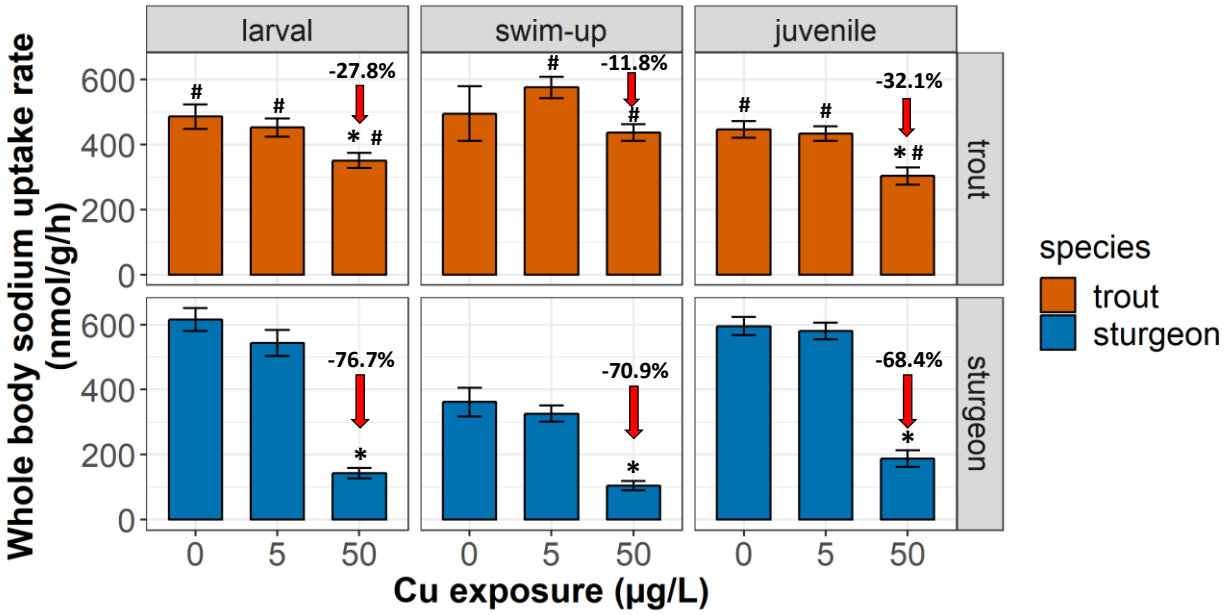

Figure 3. 3. Effect of short-term exposure (4.5 hours) to different waterborne Cu concentrations on whole-body Na uptake influx in rainbow trout and white sturgeon across different life-stages. Data are presented as mean ± SEM values of whole-body Na uptake rate (n = 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). A number symbol (#) on rainbow trout bars represents a significant difference between rainbow trout and white sturgeon for the corresponding exposure group.

A) B)

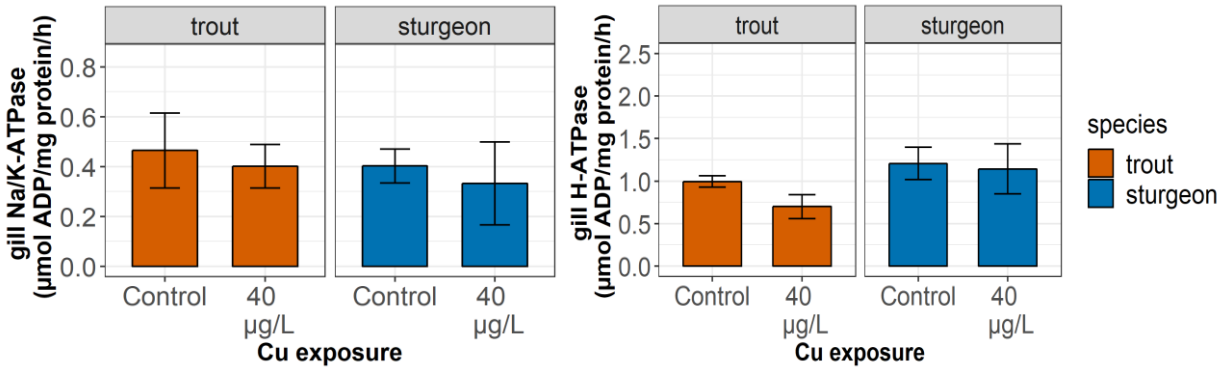

Figure 3. 4. Enzymatic activities of (A) Na^+/K^+-ATPase (NKA) and (B) H^+-ATPase (HAT) in the gills of control and 40 µg/L Cu group of juvenile rainbow trout and white sturgeon after an exposure duration of 24 hours. Data are presented as mean ± SEM of enzyme activities (n = 6). Data were analysed using two-way ANOVA followed by analysis of two-way interaction between species and exposure. No statistically significant effect of Cu was observed in either NKA or HAT.

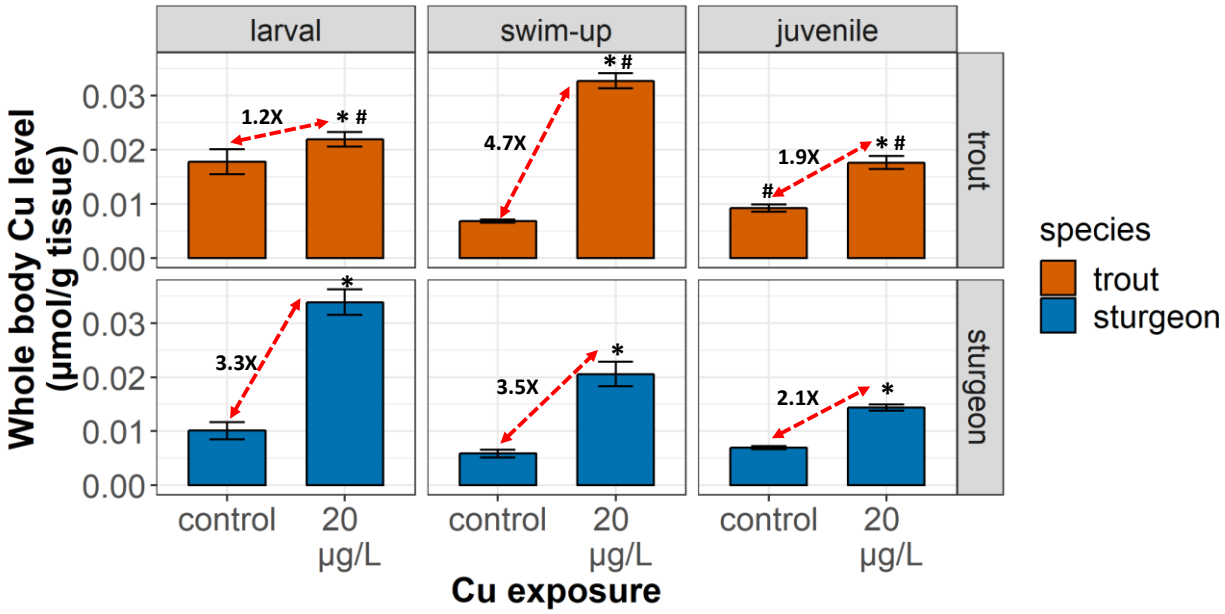

Figure 3. 5. Whole body Cu accumulation as a result of 24 h exposure to different waterborne Cu concentrations in rainbow trout and white sturgeon across different life-stages. Within each life-stage, data are presented as mean ± SEM of absolute values of Cu accumulation (n = 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). A number symbol (#) on rainbow trout bars represents a significant difference between rainbow trout and white sturgeon for the corresponding exposure group ($p < 0.05$).

3.5 Discussion

In all life-stages examined in this study, white sturgeon were consistently more sensitive to the exposure with Cu than rainbow trout. These findings confirmed several previous observations that reported greater sensitivity of white sturgeon to Cu relative to rainbow trout (Calfee et al., 2014; Vardy et al., 2014). Higher sensitivity to Cu for a non-model species such as white sturgeon could be a matter of significant concern. Due to the lack of toxicity data for many non-model species, the majority of water quality criteria are governed by data rich surrogate species such as rainbow trout or fathead minnow. Therefore, it is of critical importance that the water quality criteria values are lower than the toxicity values of species not represented in the risk assessment procedures. In agreement with the findings of this study, a few previous studies suggested that rainbow trout might not be a suitable surrogate species for the protection of all white sturgeon populations in case of Cu exposure (Calfee et al., 2014; Vardy et al., 2013).

Reduction in plasma Na levels is the most important mechanism of Cu-induced mortality in aquatic organisms (Grosell, 2012). Reduced plasma Na level induces a chain of physiological events which eventually result in the collapse of the cardio-vascular system. In this study, the pattern observed in mortality data over different life-stages was explained well by whole body Na data. For example, the species-specific difference in LC_{50} values of Cu was lowest in larvae and the corresponding effect of Cu on whole body Na level was also very similar between trout and sturgeon during this life-stage. In contrast, at swim-up and juvenile life-stages, the species-specific differences in LC_{50} values were greater and the corresponding species-specific differences in the effect of Cu on whole body Na was also much higher at these life-stages (Figure 3.1 and 3.2). Similar to the present study, it has been shown previously that the relative potency of metals towards whole body or plasma ion levels could be an important predictor of species-specific differences in the sensitivity to metals. For example, in a comparative study with Cu, rainbow trout, which was the most sensitive species in that study, demonstrated reduced plasma Na levels within 24 hours, whereas more tolerant species such as common carp and gibel carp did not show significant reduction in plasma Na levels (Eyckmans et al., 2010). However, the researchers noted that plasma Na levels in trout recovered after a week and a month of continuous Cu exposure. Regardless, the study still explained the higher sensitivity of rainbow trout during short term Cu exposure (Eyckmans et al., 2010). Similarly, in another recent study, the higher sensitivity of

rainbow trout to cadmium was explained by the greater effect of cadmium on whole body calcium levels in rainbow trout in comparison to white sturgeon (Shekh et al., 2018). Thus, based on the observations in present study, the higher sensitivity of white sturgeon in swim-up and juvenile life-stages to acute Cu exposure can be explained by the greater Cu-induced reduction of whole-body Na in white sturgeon than in rainbow trout.

Similar to whole body Na levels, the data from Cu induced whole-body Na uptake reduction also explained the higher sensitivity of white sturgeon very well. It has been reported previously that a higher baseline uptake rate for ions in certain species could lead to a higher sensitivity to the exposure with metals such as Cd, Cu or silver (Ag) (Bianchini et al., 2002; Grosell et al., 2002; Shekh et al., 2018). It is believed that the baseline uptake rate of ions such as Na and Ca are governed by the homeostatic ion requirements in the body of the organism. Species with inherently higher diffusive loss of ions tend to counterbalance plasma and whole-body ions with a higher baseline ion uptake rate (Grosell et al., 2002). Hence, such species are assumed to be more sensitive to metal exposure because inhibition of ion uptake in these species is expected to have more rapid and potent effects on whole body ion levels relative to species with lower ion uptake rates. In our experiments, we also noted that the general baseline Na uptake rate was higher in white sturgeon than in rainbow trout, with the exception of the swim-up life-stage (Figure 3.3). Therefore, the greater susceptibility of Na uptake in sturgeon after exposure to Cu can be explained in larval and juvenile life-stage on the basis of their higher baseline Na uptake rate, but not in the swim-up life-stage. These observations suggest that additional factors other than baseline Na uptake rate might also contribute in the higher susceptibility of the Na uptake pathway in white sturgeon. One such factor is the ability of Cu to bind and inhibit the active site of the Na^+/K^+-ATPase (NKA) enzyme, which is considered to be a key mechanism behind the Cu induced Na uptake inhibition in fish (Chowdhury et al., 2016). It is possible that branchial NKA in white sturgeon has a greater affinity to Cu than rainbow trout, which may contribute to a greater degree of reduction of Na uptake in sturgeon. However, the data from NKA and HAT activities showed that there were no species-specific differences in the activities of these enzymes as a result of Cu exposure. In fact, our observations indicated that Cu did not reduce the activities of NKA and HAT in gills of both species at all (Figure 3.4). The evidence of Cu-induced inhibition of NKA and HAT are conflicting in the literature. Although there are several studies that showed Cu-dependent inhibition of the activity of NKA and HAT (Chowdhury et al., 2016; Lauren and Mcdonald, 1987),

the lack of statistically significant effects on the activities of these enzymes in our study are not surprising, because there are many similar examples in the literature, where Cu did not show significant effect on NKA and other enzymes involved in the Cu toxicity pathway, despite a significant Cu-induced reduction in Na transport (Grosell et al., 2004; Heath, 1987; Lim et al., 2015). It has been pointed out that dilution of Cu during the enzymatic assays leads to the reduction in binding of Cu to the enzymes causing the reversal of the inhibition (Grosell, 2012). Additionally, existence of other mechanisms for Cu-induced Na disruption cannot be ruled out. Recent studies on the mechanisms of Cu toxicity have indicated that the cytosolic enzyme carbonic anhydrase (CA) could be an important target of Cu toxicity (Zimmer et al., 2012). CA is one of the suppliers of hydrogen ion (H^+) in the cytoplasm of gill ionocytes, and the H^+ ions play a key role in Na uptake across the apical gill membrane by influencing the activities of H^+-ATPase and Na^+/H^+-exchanger. Hence, future studies should investigate CA further as a potential target for Cu toxicity as well as for understanding species specific differences in whole- body Na levels and uptake rate. In addition to inhibiting Na influx, Cu exposure has also been shown to increase the efflux of Na, possibly through paracellular pathways (Grosell, 2012). However, efflux of Na appears to be less sensitive to Cu exposure as compared to influx, as demonstrated in two separate studies with 6 h and 12 h exposure duration, respectively (Chowdhury et al., 2016; Lauren and McDonald, 1985). The potential role of Na efflux in creating species-specific differences in the sensitivity to Cu cannot be ruled out; however, Na efflux was not measured in this study.

Cu accumulation did not demonstrate a consistent species or life-stage specific pattern as was observed with other physiological and/or toxicological parameters examined in this study (e.g., 96h LC_{50}, and Cu-induced reduction in Na uptake and whole-body Na level). While during the larval life-stage, white sturgeon accumulated more Cu, rainbow trout accumulated more Cu during the swim-up life-stage (Figure 3.5). Moreover, juvenile fish of both species showed similar levels of whole-body Cu burden during exposure to Cu. The lack of relation between whole body Cu accumulation and sensitivity to Cu was not surprising because several studies and literature reviews with different metals have shown that the relationship between whole-body accumulation and the sensitivity to metals are often inconsistent across aquatic organisms (Adams et al., 2011; Shaukat and Javed, 2013; US EPA, 2016). The probable reason for poor correlation between metal accumulation and toxic effects of metals is that the whole-body accumulation does not account for the distribution of metals to toxicologically critical tissues and cellular compartments as well as

the inherent metal detoxification capacity in organisms (Rainbow, 2002). Metal accumulation during short term exposure to metals including Cu has been shown to cause impairment in antioxidant defense mechanisms resulting in oxidative stress (Qu et al., 2014; Ransberry et al., 2015). Since the greater whole-body Cu accumulation did not translate into higher mortality in this study, a comparative evaluation of the interrelationship between differential Cu accumulation and oxidative stress response as well as detoxification capacity across different life stages between white sturgeon and rainbow trout is warranted. Such comparative studies will greatly improve our understanding of the entire sequence of events that lead to the higher susceptibility of some species to metals.

3.6 Conclusion

In conclusion, the present study demonstrated that the species-specific differences in the sensitivity of two disparate fish species, rainbow trout and white sturgeon, to Cu can be explained by the relative differences in the susceptibility of their Na homeostasis towards Cu. We first showed that white sturgeon is significantly more sensitive than rainbow trout in all life-stages, which further confirmed the concerns raised in previous studies about the suitability of using rainbow trout as a surrogate species for white sturgeon in metal risk assessment. Furthermore, our study also revealed that the waterborne Na uptake pathway is more sensitive to disruption by Cu in white sturgeon than in rainbow trout, which elicited a greater magnitude of reduction in whole body Na levels in white sturgeon, thereby making this species more susceptible to the exposure with Cu relative to rainbow trout. This study showed that physiological parameters could explain the higher vulnerability of certain species to metals. The correlation between the physiological variables and acute metal sensitivity can be further cemented by conducting future studies with more species and metals, which in turn could establish physiological evaluation as a tool for rapid assessment of the sensitivity of non-model native fish species of regulatory concern. In the present study, in some life-stages such as the swim-up, the difference in the Cu induced reduction in whole-body Na levels between white sturgeon and rainbow trout was relatively moderate in comparison to the large difference in their sensitivity to Cu (96h LC_{50}). This suggests the potential involvement of additional mechanisms (e.g., oxidative stress response, detoxification capacity) driving the species-specific differences in Cu sensitivity, which need to be examined in future studies.

4 CHAPTER 4: EXPRESSION STABILITY AND SELECTION OF OPTIMAL REFERENCE GENES FOR GENE EXPRESSION NORMALIZATION IN EARLY LIFE STAGE RAINBOW TROUT EXPOSED TO CADMIUM AND COPPER

PREFACE

The objective of chapter 4 was to identify and select the most suitable reference genes for the gene expression analysis conducted in chapter 5 and 6. To identify the most suitable reference genes, we assessed the stability of the expression of 8 candidate reference genes in the gills and skin of three different early life-stages of rainbow trout after acute exposure (24 h) to Cd and Cu using reverse transcription quantitative real-time polymerase chain reaction (qPCR).

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Author contributions:

Kamran Shekh (University of Saskatchewan) designed and conducted the experiment, analysed and visualised the data, and drafted the manuscript.

Dr. Song Tang (University of Saskatchewan) provided the necessary training, helped with the experiment, and commented on the manuscript.

Drs. Som Niyogi and Markus Hecker (both University of Saskatchewan) provided inspiration, guidance, and scientific input, commented on the manuscript, and provided funding for the research.

4.1 Abstract

Gene expression analysis represents a powerful approach to characterize the specific mechanisms by which contaminants interact with organisms. One of the key considerations when conducting gene expression analyses using reverse transcription quantitative real-time polymerase chain reaction (qPCR) is the selection of appropriate reference genes, which is often overlooked. Specifically, to reach meaningful conclusions when using relative quantification approaches, expression levels of reference genes must be highly stable and cannot vary as a function of experimental conditions. However, to date, information on the stability of commonly used reference genes across developmental stages, tissues and after exposure to contaminants such as metals is lacking for many vertebrate species including teleost fish. Therefore, in this study, we assessed the stability of expression of 8 reference gene candidates in the gills and skin of three different early life-stages of rainbow trout after acute exposure (24 h) to two metals, cadmium (Cd) and copper (Cu) using qPCR. Candidate housekeeping genes were: beta actin (*b-actin*), DNA directed RNA polymerase II subunit I (*DRP2*), elongation factor-1 alpha (*EF1a*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), glucose-6-phosphate dehydrogenase (*G6PD*), hypoxanthine phosphoribosyltransferase (*HPRT*), ribosomal protein L8 (*RPL8*), and *18S* ribosomal RNA (*18S*). Four algorithms, geNorm, NormFinder, BestKeeper, and the comparative ΔC_t method were employed to systematically evaluate the expression stability of these candidate genes under control and exposed conditions as well as across three different life-stages. Finally, stability of genes was ranked by taking geometric means of the ranks established by the different methods. Stability of reference genes was ranked in the following order (from lower to higher stability): *HPRT* < *GAPDH* < *EF1a* < *G6PD* < *RPL8* < *DRP2* < *b-actin* in gills of fish exposed to Cd; *b-actin* < *GAPDH* < *G6PD* < *DRP2* < *RPL8* < *HPRT* < *EF1a* in gills of fish exposed to Cu; *RPL8* < *HPRT* < *GAPDH* < *G6PD* < *EF1a* < *DRP2* < *b-actin* in the skin of fish exposed to Cd; and *EF1a* < *GAPDH* < *RPL8* < *HPRT* < *G6PD* < *b-actin* < *DRP2* in the skin of fish exposed to Cu. The results demonstrated that the stability of reference genes depended on the metal, life-stage and/or organ in question. Thus, attention should be paid to these factors before selection of reference gene for relative quantification of gene expression.

4.2 Introduction

Gene expression analysis provides critical information regarding the molecular mode of toxic action of contaminants in organisms (Hahn and Stegeman, 2000). Reverse transcription quantitative real-time polymerase chain reaction (qPCR) is one of the most commonly used molecular biology techniques for measuring gene expression. The popularity of qPCR can be attributed to its simplicity, sensitivity, and specificity. The use of qPCR has increased tremendously in nearly all branches of biology including ecotoxicology. There are two major methods for the quantification of gene expression in a biological sample by qPCR: absolute and relative (Bustin, 2005). Absolute quantification is performed by generating a standard curve from serially diluted standards of known concentrations of genetic material. These standards are usually DNA fragments containing the target sequence (Wong and Medrano, 2005). Because of the standard curve, absolute quantification does not require normalisation of target gene against a reference gene. However, absolute quantification is far more labour intensive than relative quantification and it is particularly difficult when quantification of a large number of genes is required. In relative quantification, the result for the gene of interest (GOI) is normalised against a reference gene in the same sample (Livak and Schmittgen, 2001). The normalised numbers of GOIs are then compared among treatment groups and control in order to obtain a fold change difference in the expression of GOIs as a result of the treatment. Genes that are expressed constitutively in a given organism are usually used as reference genes (Li et al., 2010). In order to reach meaningful conclusions using relative quantification, expression levels of reference genes must not undergo changes in response to the stressor of interest and should be consistent among replicates (Gilliland et al., 1990). Because of the ease of performance and consistent results, relative quantification is more widely used method in qPCR experiments as compared to absolute quantification. However, there is increasing evidence that expression of assumed reference genes can vary significantly with experimental conditions such as developmental stage and chemical treatment, which can significantly affect the interpretation of relative quantification qPCR results (McCurley and Callard, 2008).

Metals are considered to be among the most toxic substances to aquatic organisms (Govind, 2014), and understanding the mechanisms that drive the sensitivity of aquatic organisms such as fish to metals is critical for conducting objective ecological risk assessments. Gene expression

analyses have played a substantial role in furthering our understanding of several aspects of metal toxicity, such as metal induced oxidative stress, toxic effects of metals to the nervous system, and identification of biomarkers of exposure for metals in fish and other organisms (Gonzalez et al., 2006; Lu et al., 2005; Misra et al., 1989; Morcillo et al., 2016; Price-Haughey et al., 1986). Gene expression analyses have also helped in advancing our knowledge of metal uptake pathways in fish (Komjarova and Bury, 2014). A large number of mechanistic studies with metals revolve around measuring expression of genes such as metallothionein (*MT*), oxidative stress pathways, metal transporters, etc. (Doering et al., 2015; Komjarova and Bury, 2014; Tang et al., 2016).

Fish are considered to be most sensitive to metals during their early life-stages (Calfee et al., 2014; Grosell et al., 2002; Tang et al., 2016). Hence, many studies use early life-stages of fish as model test organisms. However, it is important to note that significant differences in metal sensitivity have been reported among different early life-stages of selected fish species (Vardy et al., 2013). Moreover, large differences in sensitivity to metals are commonly observed among different fish species (Besser et al., 2007; Marr et al., 1995). At present, the mechanistic knowledge regarding species and life-stage specific differences in sensitivity of fish to metals is very limited at best. However, it is generally believed that differences in metal sensitivity occur due to differences in toxicokinetics and ionoregulatory physiology among different life-stages and species of fish, which in turn may arise due to the differences in the expression patterns of the genes encoding proteins involved in regulating metal and ion transport and homeostasis, as indicated recently by global transcriptome profiling of metal exposed populations of brown trout (*Salmo trutta*) (Uren Webster et al., 2013). Differences in oxidative stress and *MT* responses are also important candidates for possible roles in species and life-stage specific differences in metal sensitivity (Tang et al., 2016). Hence, there is an increasing focus on the quantification of the expression patterns of genes involved in metal/ion-homeostasis and other molecular pathways (Tang et al., 2016; Uren Webster et al., 2013).

As discussed above, meaningful and reliable interpretation of changes in gene expression as a function of exposure to metals or other contaminants relies on reference genes that show high stability among treatment and control conditions. However, there is a lack of information regarding the stability of reference genes in fish species after exposure to metals or as a function of developmental stages during early life-stages. Therefore, in this study, we have evaluated the most

suitable genes from a set of 8 most commonly used reference genes in three different life-stages of rainbow trout as a function of short term metal exposure by using four different mathematical algorithms: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and comparative ΔC_t method (Chen et al., 2011). Four different algorithms were used because each of them is based on slightly different principles and sometimes produce slightly different results. Results obtained with four algorithms were then used to prepare a final ranking of the most suitable reference genes as described previously (Chen et al., 2011). Rainbow trout was chosen as the model organism in this study because among the fish species for which toxicity data on metals are available, trout is known to be one of the most sensitive fish species and because it is widely used for regulatory testing procedures (Calfee et al., 2014). Copper (Cu) and Cadmium (Cd) were used as model metals as they are among the most studied metals in aquatic toxicology and are of high concern worldwide (Wood, 2012). Further, three distinct early life-stages viz. larval, swim up and juvenile were selected in order to determine the most suitable reference genes during critical developmental stages of rainbow trout. Skin and gills are the major ionoregulatory organs in fish, which play a critical role in metal uptake and elimination (Fu et al., 2010; Grosell and Wood, 2002). Hence, skin and gills were selected as model organs in this study.

4.3 Materials and Methods

4.3.1 Test Organisms

Eyed eggs of rainbow trout were obtained from Troutlodge Inc. (Washington, United States) and were reared in the Aquatic Toxicology Research Facility at the University of Saskatchewan in a flow through system maintained at approximately 12 - 13 °C. Fish were fed commercial trout food (Bio Vita Starter #0 Crumble, Bio-Oregon, British Columbia, Canada). Food was first introduced to fish at 12 - 13 days post hatch (dph). All procedures were approved by the University of Saskatchewan's University Council on Animal Care and Supply (Protocol 20140079).

4.3.2 Test Chemicals and Exposure Protocol

Copper (II) sulfate pentahydrate (Chemical Abstracts Service [CAS] 7758-99-8; purity 99.995%) and cadmium chloride hemi-pentahydrate (CAS 7790-78-5; purity 99.999%) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in laboratory reverse

osmosis water. All exposure waters were prepared by mixing reverse osmosis water and dechlorinated city of Saskatoon municipal tap water at a ratio of 3:1 in order to adjust hardness and alkalinity to a moderate level (~65 and 40 mg/L as $CaCO_3$, respectively). Stock solutions of Cu and Cd were prepared in this water and stored at 13 °C for at least 24 hours before exposure. Exposures were conducted for 24 hours with each of three different life-stages: larval (5 dph), swim-up (15 dph), and juvenile (45 dph). Fish were exposed to 40 µg/L Cu (625 nM) and 5 µg/L Cd (45 nM), in addition to the control group (water), under static renewal conditions. The concentrations were selected on the basis of previous studies which demonstrated that similar ranges of concentrations were capable of inducing specific biological effects without causing mortality (Lim et al., 2015; McGeer et al., 2000). Four fish were used per group based on other studies (Dos Santos et al., 2015; McCurley and Callard, 2008; Zhang et al., 2012). Individual 500 mL high density polyethylene cups were used for exposing each fish. Half of the exposure media was replaced after every 12 hours. The volume of exposure media in each cup was 250 mL (5 and 15 dph) or 500 mL (45 dph). Exposures were conducted under a 16:8 h light/dark cycle and at a temperature of 13 ± 1 °C. Fish were acclimated in exposure systems for 2 days prior to initiation of the exposure. No food was given during the study. At the termination of exposure, fish were euthanized by cervical dislocation and skin was immediately removed by surgical blade and forceps under a dissection microscope and stored in RNA-later (Sigma Aldrich, St. Louis, MO, USA). Because of the small size of the fish, gill baskets could not be isolated. Hence, head was used as a proxy of gill which was separated from body and stored in RNA later. Samples were kept at 4 °C overnight and then transferred to -20 °C until RNA isolation.

4.3.3 Water Quality Parameters

Hardness and alkalinity of the exposure water was measured at the beginning of exposure using Nutrafin Test kits (Hagen, Canada). Temperature and pH were measured at the beginning and end of exposure. Water samples (35 mL) were collected randomly from two cups of each exposure group at the beginning and end of exposure, filtered through a 0.45 µm polycarbonate filter, acidified with trace metal grade Nitric acid (EMD Millipore, Billerica, MA, USA) and stored at 4 °C until further analysis. Dissolved metal (Cu or Cd) concentrations were measured using an atomic absorption spectrometer (AAnalyst 800, Perkin Elmer, USA) to confirm exposure

concentrations. The quality control/assurance of metal analysis was ensured using appropriate method blanks and certified standards (Fisher Scientific, Canada).

4.3.4 Candidate Reference Genes

Eight commonly used reference genes were identified from 40 randomly selected publications on gene expression in rainbow trout from PubMed. These reference genes were: beta actin (*b-actin*), DNA directed RNA polymerase II subunit I (*DRP2*), elongation factor-1 alpha (*EF1a*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), glucose-6-phosphate dehydrogenase (*G6PD*), hypoxanthine phosphoribosyltransferase 1 (*HPRT*), Ribosomal protein L8 (*RPL8*), and *18S* ribosomal RNA (*18S*). Primer sequences for all candidate reference genes are listed in Table C4.S1.

4.3.5 Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from gill (head was used as proxy organ) and skin by using RNeasy Lipid Tissue Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. RNA concentration and purity (260/280 and 260/230 ratio) were quantified by a NanoDrop ND-1000 Micro-Volume UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All RNA samples were diluted to a concentration of 159.3 ng/µL. An amount of 0.95 µg total RNA from each sample was used for cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada) as per the protocol recommended by the manufacturer.

4.3.6 Reverse Transcription Quantitative Real-time Polymerase Chain Reaction (qPCR)

qPCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 96-well plates. Each cDNA sample and primer combination were subjected to the following steps: A 50 µL reaction mixture was prepared with 2x concentrated QuantiFast SYBR green master mix, 2.5 µL of cDNA, 10 pmol gene-specific primers, and nuclease free water. Each cDNA sample was analyzed in duplicate with 20 µL reaction volumes per well. The reaction mixture for qPCR was denatured at 95 ºC for 10 min followed by a thermal cycle profile consisting of denaturing at 95 ºC for 10 s and extension for 1 min at 60 ºC for a total of 40 PCR cycles. Melting curve analysis was also conducted after 40 cycles to verify the specificity of amplicons.

4.3.7 Data Analysis

Before analysis of gene stability, PCR reaction efficiency was determined for each primer set by generating standard curves of C_t values from serial dilutions of the cDNA samples and plotting the observed C_t values against the log transformed template concentration and determining the slope of the standard curve plot. Expression stability of the candidate reference genes was determined by four commonly used methods/algorithms. These methods are known as: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and comparative ΔC_t method (Chen et al., 2011). After establishing the ranks of genes according to each method, a final stability rank was established by taking all the algorithms into consideration, as described previously (Chen et al., 2011). The data was analysed in two different ways. Firstly, data of samples from all the metal and control groups from the three life-stages were analyzed together and the most suitable reference genes were identified (Tables 4.3-4.6). Since 4 fish were assigned to each group, 24 samples were available for analysis by each method (n = 24). The purpose of this analysis was to characterize the stability of the expression of these genes across different life-stages in fish exposed to Cd or Cu. Secondly, we analysed the data across three different life-stages in only non-exposed fish to characterize the effect of development on the stability of reference genes (Table 4.1 and 4.2).

GeNorm, NormFinder and BestKeeper are freely available Microsoft Excel® based programs. GeNorm and NormFinder programs require conversion of raw C_t values of each sample within each gene into relative expression values, which were calculated as follows (Eq. 1):

$$\text{Relative } C_t \text{ value for each sample} = E \wedge (C_{t\,(lowest)} - C_{t\,(sample)}) \quad \text{.....(4.1)}$$

Where, E = efficiency of the primer for each gene, $C_{t\,(lowest)}$ = lowest C_t value among all the samples.

geNorm calculates average pairwise variation of a single candidate reference gene relative to all other tested genes, which is defined as M value. A lower M value indicates higher stability (Vandesompele et al., 2002). In contrast, NormFinder considers variation among subgroups of samples in addition to the overall expression variation of the candidate reference genes (Andersen et al., 2004). BestKeeper utilizes raw C_t values from each sample within each candidate reference gene in order to calculate standard deviation (SD) and coefficient of variation (CV) for each

candidate reference gene. The candidate gene with the least SD is considered to be the most stable gene. Genes with SD greater than 1 are considered to be inconsistent and should not be used in downstream qPCR applications (Pfaffl et al., 2004). Finally, we used the comparative ΔC_t method. The comparative ΔC_t method is based on the identification of most stable reference genes by comparing relative expression of ''pairs of genes'' within each treatment. This method has been described in detail previously (Chen et al., 2011). ΔC_t and mean ΔC_t for each gene combination was calculated. Plotting a boxplot of ΔC_t values for each gene against every possible combination of genes, provides a good way to visually reveal the variability of each gene expression (Figures 4.3 & 4.4). For analysis by this method, first of all, SD for each set of ΔC_t values was determined. Finally, the mean of the SD for each gene was calculated and the gene with lowest mean SD was considered to be the most stable gene. All genes were then ranked for stability based on increasing mean SD.

Since the fundamental mathematical principle underneath each algorithm is different, final ranking was conducted using a multiple lines of evidence approach considering all sets of results together (Chen et al., 2011). Briefly, ranking of each candidate reference gene was determined by using the methods mentioned above. Then, the geometric mean of all the rank numbers for each gene was calculated. Finally, all the geometric means of candidate reference genes were ranked. All the graphs were created using either base R language and environment for statistical computing or "ggplot2" package in R (R Core team, 2015; Wickham, 2009).

4.3.8 Validation of Reference Genes

Based on the final rankings, candidate reference genes were validated in the gills of fish exposed to both Cd and Cu using *HSP70a* (heat shock protein 70a) gene expression because both Cd and Cu have been shown to induce its expression in several previous studies (Feng et al., 2003; Kwong et al., 2011; Nadeau et al., 2001). To perform this validation, *HSP70a* gene expression was normalised against the most stable and least stable reference genes for Cd and Cu exposures in early life-stage rainbow trout (*b-actin*, *HPRT* and *EF1a*, *b-actin* for Cd and Cu, respectively). The exposure concentrations of Cd and Cu for *HSP70a* gene expression measurement were same as reported in the reference gene stability experiment (5 and 40 µg/L, respectively). Expression level of *HSP70a* was calculated as fold difference in comparison to the control group using $\Delta\Delta C_t$ method.

4.4 Results

4.4.1 Exposure Verification and Water Quality

Measured concentrations of the metals used in this study were comparable to nominal concentrations. The average measured concentration of Cd in water samples across all exposures was 4.52 ± 0.29 µg/L and the mean concentration of Cu was 46.1 ± 0.54 µg/L. The average water temperature throughout the exposures was 13 ± 1.2 ºC and the average pH was 7.3 ± 0.3. Hardness and alkalinity were 65 and 40 mg/L, respectively.

4.4.2 Comparative Expression Levels of Candidate Reference Genes

Visualizing gene expression data as a function of life-stage revealed that all reference genes had more stable C_t values in the gills as compared to skin (Figures 4.1 A and B). Among different life-stages, median C_t values ranged between 16.6 and 28.2 across different genes in gills and between 18.2 and 27.8 in skin (Figures 4.1 A and B). Exposure to Cd and Cu resulted in tissue specific differences in the stability of reference genes. For example, exposure to Cd affected the C_t values of *HPRT* to a much greater degree in gill as compared to skin. In gills, median C_t values ranged between 16.8 and 28.1 and the range of median C_t values in skin was between 18.1 and 28.0 for both metals when all life-stages and metal exposures were considered together (Figures 4.2 A and B). Interquartile range of genes also varied widely in both organs. In gills of fish exposed to Cd, *b-actin*, *DRP2*, and *RPL8* had least variation, whereas *EF1a*, *HPRT*, and *RPL8* showed least variation after exposure to Cu (Figure 4.2 A). In skin, all genes were more variable than in gills. Among the 7 genes studied in skin, *b-actin* and *DRP2* showed least variation after exposure to Cd and Cu (Figure 4.2 B).

4.4.3 Evaluation of Stability of the Candidate Reference Genes

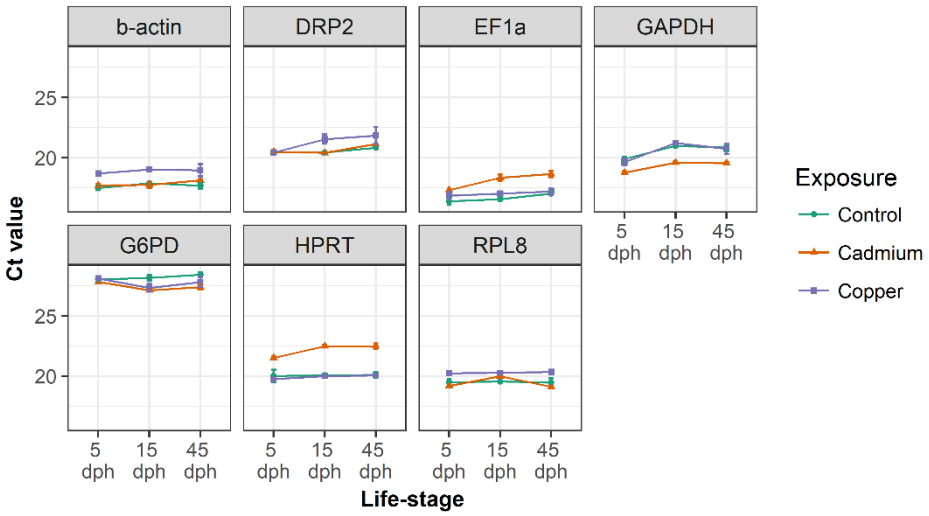
4.4.3.1 geNorm

When comparing stability of reference genes across different life-stages of unexposed fish, *DRP2*, *EF1a* and *b-actin* were the three most stable genes in gills and *b-actin*, *G6PD*, and *EF1a* were the most stable reference genes in skin (Tables 4.1 and 4.2).

As per geNorm analysis, *b-actin*, *DRP2*, and *RPL8* were the three most stable reference genes in the gills after exposure to Cd across different life-stages (Table 4.3). With Cu exposure

across different life-stages, the three most stable reference genes in gills were *RPL8*, *EF1a* and *HPRT* (Table 4.4). In the skin, *b-actin*, *EF1a*, and *DRP2* were the three most stable reference gene after exposure to Cd (Table 4.5), whereas *b-actin*, *DRP2* and *GAPDH* were the most stable reference genes after exposure to Cu (Table 4.6).

A)



B)

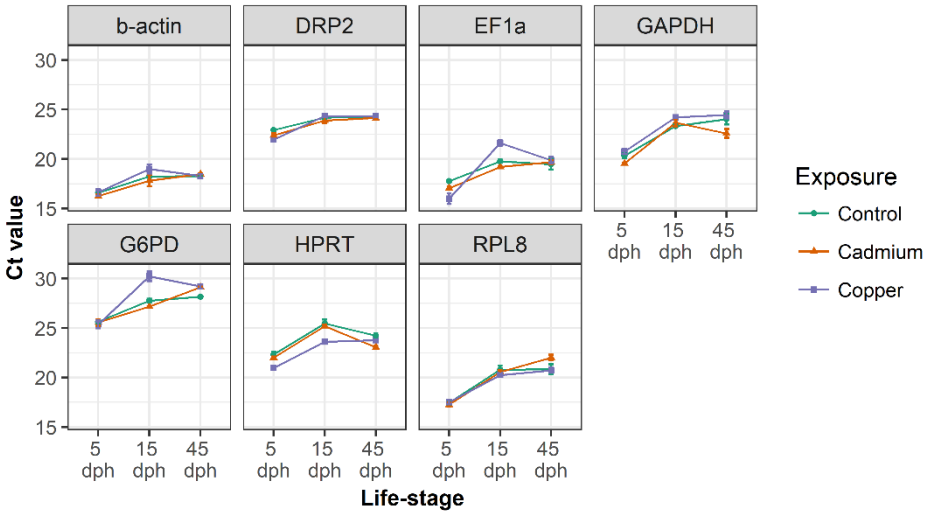
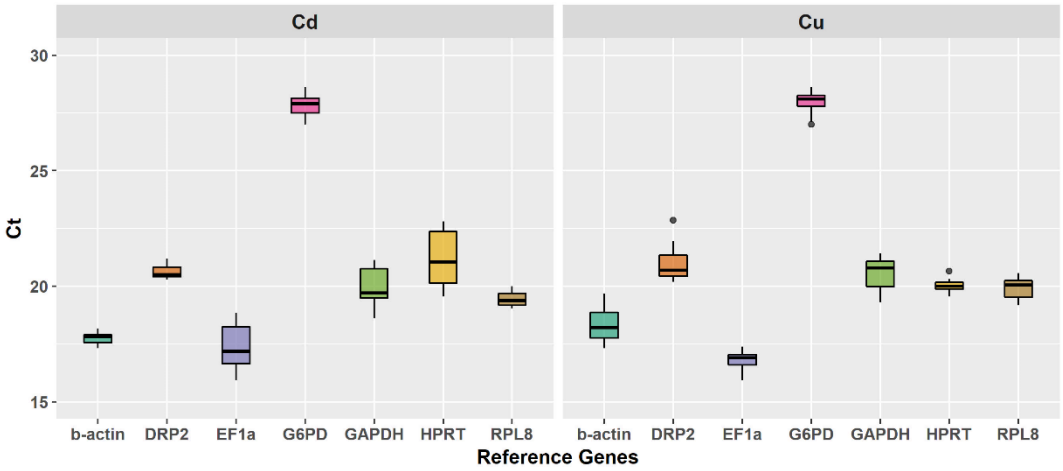


Figure 4. 1. Visualization of the changes in gene expression of candidate reference genes obtained by qPCR in A) gill B) skin as a function of life stage for each exposure group. Data is presented as mean ± SD of Ct values for each group with four fish in each group.

A)



B)

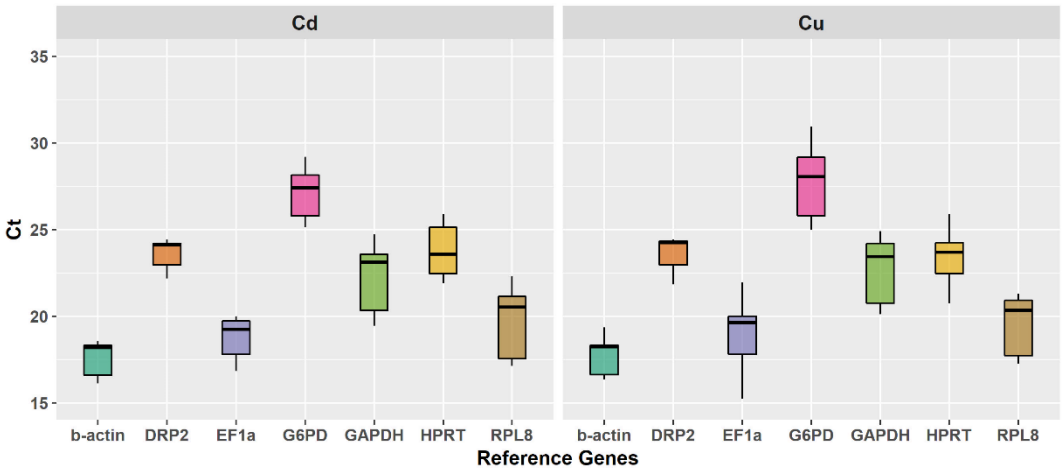


Figure 4. 2. Distribution of threshold cycle (C_t) value for candidate reference genes obtained by qPCR in A) gill B) skin of Cd and Cu exposed rainbow trout larvae of 5 days post hatch (dph), 15 dph and 45 dph age. Coloured boxes demonstrate range of C_t values and the black centre line indicates median C_t value. The upper and lower hinges of boxes indicate 75 and 25 percentiles and whiskers show the largest and smallest C_t values. Outliers are represented as dark circles. Each box represents pooled data from control and exposed group from the three life-stages (n = 24)

4.4.3.2 NormFinder

The most stable genes identified by NormFinder in both tissues and metal combinations were very similar to the results obtained with geNorm except for a few discrepancies (Tables 4.3 – 4.6). For example, after exposure to Cu over different life-stages, the most stable reference gene identified by NormFinder in skin was *G6PD* (Table 4.6). However, *G6PD* was ranked 5th by geNorm (Table 4.6).

Similarly, the NormFinder results for life-stage specific differences in gene expression in unexposed fish were comparable to those obtained by geNorm (Table 4.1).

4.4.3.3 BestKeeper

The three reference genes with the least variation identified by BestKeeper were mostly in agreement to those identified by the other methods with few exceptions. For example, after exposure to Cu over different life-stages, the reference gene with the 3rd least variation in skin identified by BestKeeper was *HPRT*, which was ranked 7th and 4th by geNorm and NormFinder, respectively (Table 4.6). Similarly, the results for life-stage specific differences in the gene expression in unexposed fish, as identified by BestKeeper, were very similar to those obtained by other algorithms with minor exceptions. For example, *EF1a* was identified as one of the three most stable genes in gills by geNorm and NormFinder. However, it was ranked 6th by BestKeeper method (Table 4.1).

Table 4. 1. Candidate reference genes ranked by different methods and final recommended ranking as a function of life-stage for gills of unexposed rainbow trout

Ranking	geNorm	BestKeeper	Comparative ΔC_t method	Normfinder	Recommended final ranking
1	DRP2 | EF1a	DRP2	DRP2	EF1a	DRP2
2		RPL8	RPL8	DRP2	EF1a
3	b-actin	b-actin	b-actin	b-actin	b-actin
4	G6PD	G6PD	G6PD	HPRT1	RPL8
5	HPRT	HPRT	HPRT	G6PD	G6PD
6	RPL8	EF1a	EF1a	RPL8	HPRT
7	GAPDH	GAPDH	GAPDH	GAPDH	GAPDH

Table 4. 2. Candidate reference genes ranked by different methods and final recommended ranking as a function of life-stage for skin of unexposed rainbow trout

Ranking	geNorm	BestKeeper	Comparative ΔC_t method	Normfinder	Recommended final ranking
1	b-actin | G6PD	DRP2	DRP2	b-actin	b-actin
2		b-actin	b-actin	G6PD	DRP2
3	EF1a	EF1a	EF1a	EF1a	G6PD
4	DRP2	G6PD	G6PD	DRP2	EF1a
5	HPRT	HPRT	HPRT	RPL8	HPRT
6	GAPDH	RPL8	RPL8	HPRT	RPL8
7	RPL8	GAPDH	GAPDH	GAPDH	GAPDH

Table 4. 3. Candidate reference genes ranked by different methods and final recommended ranking for gills of rainbow trout exposed to Cd over three life-stages

Ranking	geNorm	BestKeeper	Comparative ΔCt method	Normfinder	Recommended final ranking
1	b-actin | DRP2	b-actin	b-actin	b-actin	b-actin
2		DRP2	RPL8	DRP2	DRP2
3	RPL8	RPL8	DRP2	RPL8	RPL8
4	G6PD	G6PD	G6PD	EF1a	G6PD
5	EF1a	GAPDH	GAPDH	G6PD	EF1a
6	GAPDH	EF1a	EF1a	GAPDH	GAPDH
7	HPRT1	HPRT1	HPRT1	HPRT1	HPRT1

Table 4. 4. Candidate reference genes ranked by different methods and final recommended ranking for gills of rainbow trout exposed to Cu over three life-stages

Ranking	geNorm	BestKeeper	Comparative ΔC_t method	Normfinder	Recommended final ranking
1	RPL8 | EF1a	HPRT1	HPRT1	EF1a	EF1a
2		EF1a	EF1a	HPRT1	HPRT1
3	HPRT1	RPL8	G6PD	RPL8	RPL8
4	DRP2	G6PD	RPL8	DRP2	DRP2
5	b-actin	DRP2	GAPDH	b-actin	G6PD
6	GAPDH	GAPDH	DRP2	GAPDH	GAPDH
7	G6PD	b-actin	b-actin	G6PD	b-actin

Table 4. 5. Candidate reference genes ranked by different methods and final recommended ranking for skin of rainbow trout exposed to Cd over three life-stages

Ranking	geNorm	BestKeeper	Comparative ΔC_t method	Normfinder	Recommended final ranking
1	b-actin | EF1a	DRP2	DRP2	b-actin	b-actin
2		b-actin	b-actin	EF1a	DRP2
3	DRP2	EF1a	EF1a	GAPDH	EF1a
4	G6PD	G6PD	G6PD	DRP2	G6PD
5	GAPDH	HPRT1	HPRT1	G6PD	GAPDH
6	HPRT1	GAPDH	GAPDH	RPL8	HPRT1
7	RPL8	RPL8	RPL8	HPRT1	RPL8

Table 4. 6. Candidate reference genes ranked by different methods and final recommended ranking for skin of rainbow trout exposed to Cu over three life-stages

Ranking	geNorm	BestKeeper	Comparative ΔC_t method	Normfinder	Recommended final ranking
1	b-actin | DRP2	b-actin	DRP2	G6PD	DRP2
2		DRP2	b-actin	b-actin	b-actin
3	GAPDH	HPRT1	HPRT1	DRP2	G6PD
4	RPL8	RPL8	RPL8	HPRT1	HPRT1
5	G6PD	EF1a	GAPDH	RPL8	RPL8
6	EF1a	G6PD	G6PD	GAPDH	GAPDH
7	HPRT1	GAPDH	EF1a	EF1a	EF1a

4.4.3.4 Comparative ΔC_t Method

Analysis of box plots revealed that *b-actin*, *RPL8* and *DRP2* were the most stable genes in gills of fish exposed to Cd over different-life-stages (Figure 4.3). For gills of fish exposed to Cu, it was difficult to distinguish the most stable genes because all of the analyzed genes showed very similar stability (Figure 4.3). Similarly, in skin of the same fish, all genes showed similar levels of stability (Figure 4.4). Based on the mean of the SD of ΔC_t values, genes were ranked for stability (Tables 4.3 – 4.6). The results obtained by this method were largely in agreement with the other methods with a few exceptions. For example, after exposure to Cu over different life-stages, the 3rd most stable reference gene in gills identified by this method was *G6PD*, which was ranked 7th by NormFinder and geNorm (Table 4.4). Similarly, the results for life-stage specific differences in gene expression of unexposed fish were very similar to those obtained with the other algorithms with minor exceptions. For example, *DRP2* was identified as one of the three genes that showed least variation in skin by this method. However, it was ranked 4th by geNorm and NormFinder methods (Table 4.2).

4.4.3.5 Final Ranking

The final rankings of reference genes in the gills and skin of fish exposed to Cd and Cu over different life-stages revealed that the performance of reference genes varied based on life-stage, metal, and tissue in question (Table 4.7). In general, *b-actin* and/or *DRP2* ranked among the best reference genes in all the variables tested in this study except in the gills of fish exposed to Cu (Table 4.7). *b-actin* and *DRP2* were the two most stable reference genes in the gills and skin of early stage rainbow trout exposed to Cd as well as the skin exposed to Cu. In contrast, *EF1a* and *HPRT1* were the two best performing reference genes in the gills of early life-stage rainbow trout exposed to Cu (Table 4.7). The final rankings of reference genes over different life-stages in the unexposed fish revealed *DRP2* and *EF1a* as the most stable reference genes in gills and *b-actin* and *DRP2* in skin. The detailed stability rankings for all the genes calculated by all methods as well as final rankings are available in Table 4.1 – 4.6.

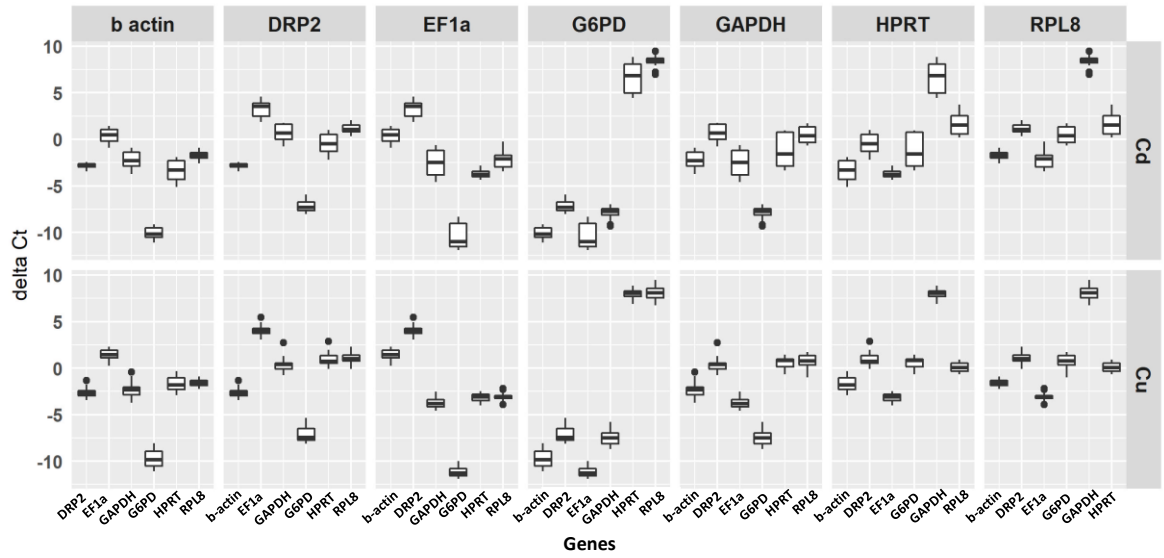


Figure 4. 3. Pairwise gene expression stability analysis of reference genes in the gills of Cd (upper panel) and Cu (lower panel) exposed rainbow trout larvae using comparative ΔC_t method. ΔC_t was calculated for each gene against each possible pair and plotted in vertical grids. Boxes demonstrate range of ΔC_t values and the black centre line indicates median ΔC_t value. The upper and lower hinges of boxes indicate 75 and 25 percentiles and whiskers show the largest and smallest C_t values. Outliers are represented as dark circles. Each box represents pooled data from control and exposed group from the three life-stages (n = 24)

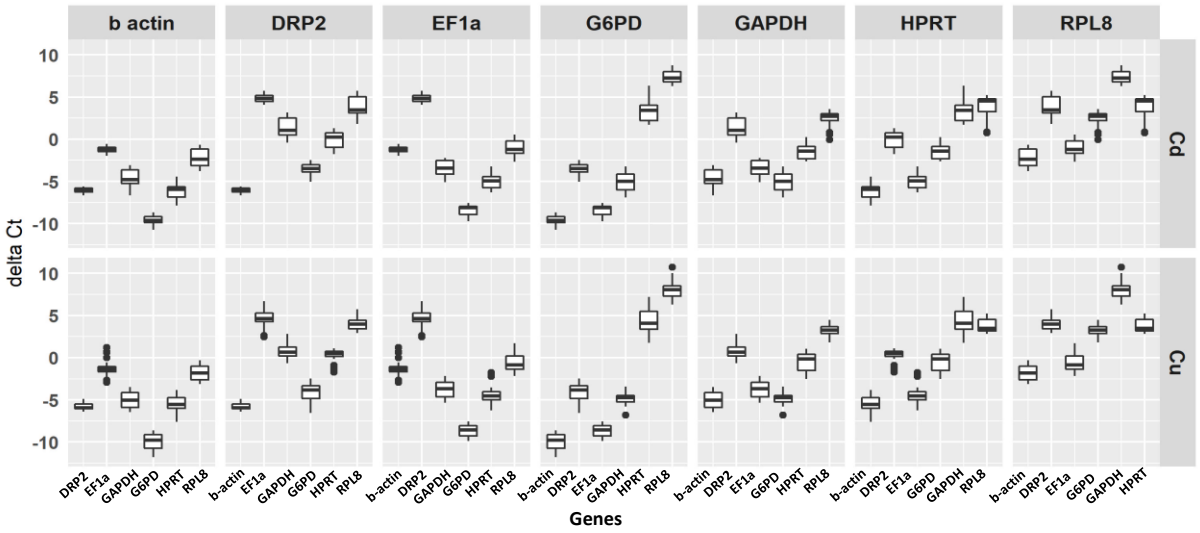


Figure 4. 4. Pairwise gene expression stability analysis of reference genes in the skin of Cd (upper panel) and Cu (lower panel) exposed rainbow trout larvae using comparative ΔC_t method.

ΔC_t was calculated for each gene against each possible pair and plotted in vertical grids. Boxes demonstrate range of ΔC_t values and the black centre line indicates median ΔC_t value. The upper and lower hinges of boxes indicate 75 and 25 percentiles and whiskers show the largest and smallest C_t values. Outliers are represented as dark circles. Each box represents pooled data from control and exposed group from the three life-stages (n = 24)

Table 4. 7. Three best candidate reference genes (final rankings determined by geometric means of rankings by all methods) under different conditions: A & B) Genes ranked in gills and skin as a function of life-stage only without any exposure; C & D) Genes ranked in gills and skin of fish exposed to Cd over different life-stages; E & F) Genes ranked in gills and skin of fish exposed to Cu over different life-stages

Final Ranking	**A) Gill (unexposed)**	**B) Skin (unexposed)**	**C) Gill (Cd exposed)**	**D) Skin (Cd exposed)**	**E) Gill (Cu exposed)**	**F) Skin (Cu exposed)**
1	DRP2	b-actin	b-actin	b-actin	EF1a	DRP2
2	EF1a	DRP2	DRP2	DRP2	HPRT1	b-actin
3	b-actin	G6PD	RPL8	EF1a	RPL8	G6PD

4.4.3.6 Validation of Reference Genes

Choice of reference genes between most stable and least stable genes demonstrated significant effect on *HSP70a* gene expression fold change. *B-actin*, which was the most stable reference gene for Cd exposure in the gills, resulted in an approximately 4-fold upregulation in *HSP70a*, whereas, *HPRT* (the least stable gene) resulted in a roughly 17-fold upregulation (Figure 4.5A). Similarly, Cu exposure resulted in an approximately 2 and 4-fold upregulation of *HSP70a* in gills when normalization was performed with *EF1a* and *b-actin*, respectively (Figure 4.5B).

4.5 Discussion

The comparative ΔC_t method, also known as $\Delta\Delta C_t$ method, is one of the most commonly used evaluation methods of gene expression data obtained by qPCR analysis. In this method, the gene of interest is normalised against a reference gene. One of the key assumptions of this approach is that the mRNA of reference gene must be stably expressed across all experimental conditions and the tissue in question. In this study, many of the reference genes commonly used with the standard teleost fish model rainbow trout showed significant variability across different experimental conditions including exposure to environmental contaminants such as metals, life-stages and tissues. Preferable reference genes appeared to vary under different conditions depending on the chemical, tissue or life-stages assessed.

Among the eight candidate housekeeping genes investigated in this study, *18S* showed very high expression in all exposure groups (C_t range 4-6). This was expected because *18S* is a part of ribosomal RNA (rRNA), and typically the abundance of rRNA is much higher than that of mRNA. Most of the specific target transcripts of interest, which are to be measured in experiments, are expressed at much lower levels (C_t levels usually 20 or greater). Hence, in a typical relative quantitative qPCR experiment, a difference of approximately 15 C_t values can be expected between *18S* and a 'hypothetical' target transcript, which corresponds to a difference of 10^4-10^5 fold in expression level. Currently, there is no information on the effect of such a high difference in the abundance of transcripts on the efficiency of processes involved in PCR such as reverse transcription (for cDNA synthesis). Such a massive difference in the expression of *18S* as a reference gene and the target gene of interest will reduce the resolution of the qPCR experiment, which in turn, will make it difficult to observe smaller changes in the gene expression. Furthermore, rRNA and mRNA transcription is linked with RNA polymerases 1 and 2,

respectively. Hence, the control of the synthesis of rRNA and mRNA are independent of each other and it is currently unknown if the variability introduced by the processes involved in PCR are similar for rRNA and mRNA (Kozera and Rapacz, 2013). Based on these arguments, *18S* transcript does not represent a preferable candidate reference gene for mRNA expression analysis. Hence, all the calculations were performed on data from seven reference genes instead of eight and data of *18S* were not included in the analysis.

In this study, expression of all reference genes generally showed greater stability in the gills as compared to skin, regardless of life-stage or metal (Figure 4.1 and 4.2, respectively). Although no data on the stability of the reference genes is available for fish skin epithelium, tissue specific differences in the stability of reference gene as a result of different independent variables including metal exposure have been demonstrated in many studies (Filby and Tyler, 2007; Lee and Nam, 2016; Zhang et al., 2016). Tissue dependent variation in the stability signifies the importance of pre-validation of reference genes in a tissue specific manner in qPCR experiments. In the skin, *GAPDH,* and *RPL8* apparently showed high variability across different conditions including metal exposure and life-stages (Figure 4.2B, 4.1A and 4.1B). Combined results from all the four algorithms used in this study confirmed this observation as these reference genes attained poor overall stability rankings with both Cd and Cu over different life-stages in skin (Tables 4.5 and 4.6). These reference genes also showed high variability in skin as a function of developmental stage (Figure 4.1B). *GAPDH* is one of the most commonly used reference genes in gene expression studies. However, several studies, including this study, have demonstrated the effect of metals and developmental stages on *GAPDH* expression, highlighting the limitation of this gene as a reference (Barber et al., 2005; Glare et al., 2002; Gunnarsson et al., 2007; Mitter et al., 2009). *G6PD*, which performed as one of three best reference genes when measured as a function of only life-stage in skin, showed marked variation as a function of exposure to Cd when all life-stages were taken into consideration together (Tables 4.2 and 4.5). Both Cd and Cu were previously shown to affect the expression of *G6PD* in different organisms (Chen et al., 2013; Hu et al., 2017; Man and Woo, 2008). Although the mechanisms underlying the effect of Cd and Cu on GAPDH and G6PD expressions are not known, it might be due to the ability of these metals to induce oxidative stress (Tang et al., 2016). Oxidative stress has been shown to modulate the expression of *GAPDH* and *G6PD* in several studies (Ito et al., 1996; Ursini et al., 1997), and as such these genes could have been affected by the oxidative stress induced by these metals. *G6PD* and *GAPDH* were ranked

similarly poorly (4th, 6th and 5th, 6th) in gills after exposure of trout to Cd and Cu, respectively, when the samples from all the life-stages were taken into consideration together (Tables 4.3 and 4.4). In contrast, *RPL8* demonstrated a different pattern in gills as compared to skin and was ranked among the three genes that performed best after exposure to both Cd and Cu. Similar to our observation, in another recent study, gene expression of *RPL8* was demonstrated to be highly stable in gills during metal exposure (Cu, Zn, and Cd) and was concluded to be the second best performing reference gene in gene expression studies investigating effects of metal exposures in the gills of *Haliotis discus* (Lee and Nam, 2016). No other studies are available that assessed the stability of the *RPL8* reference gene in skin of fish exposed to metals. Nevertheless, another study with Cd exposure in zebrafish demonstrated comparatively poor stability of closely related genes such as ribosomal protein L7 and Large P2 in other organs such as the spleen (Lang et al., 2016). Hence, the reason for the high variability in the expression of *RPL8* in skin as a function of developmental stages or metal exposure remains speculative and requires further investigation.

In general, the expression of *b-actin* and *DRP2* were very stable regardless of the life-stage, tissue or metals investigated. *B-actin* and *DRP2* consistently ranked among three genes that performed best in the gills and skin of trout exposed to Cd and in skin of trout exposed to Cu when samples from all the life-stages were taken into consideration together (Tables 4.3, 4.5 and 4.6). Even in gills of fish exposed to Cu, these two genes demonstrated little variation in their expression levels and yet, they ranked among the less performing genes due to comparatively better stability in the expression of other candidate genes (Table 4.4). Studies with different species have demonstrated that *b-actin* is developmentally regulated during early life-stages (J. M. O. Fernandes et al., 2008; Øvergård et al., 2010; Zhang et al., 2016). However, no such data was previously available for rainbow trout. In this study, mRNA abundance of *b-actin* decreased in skin as the fish grew (Figure 4.1B). While the specific reasons for these changes in mRNA abundance in the skin of fish are unknown, we speculate that this might be due to the rapid changes in skin morphology during the early life-stages (Ángeles Esteban and Cerezuela, 2015). Despite these changes in expression as a function of developmental stage, *b-actin* was comparatively more stable than all other genes (Table 4.2). In contrast, *b-actin* expression in gill remained very stable regardless of stage of development (Figure 4.1A). Although gills also go through marked morphophysiological changes during early life-stages such as the development of arches, filaments and secondary lamellae, as well as significant alterations in functional properties such as

ionoregulation and gas exchange (Glover et al., 2013; Hughes and Morgan, 1973), the effect of life-stages on the expression stability of *b-actin* and *DRP2* was found to be non-significant as demonstrated by their good stability rankings (1st and 3rd, respectively) (Table 4.1). It indicates that the processes affecting the expression stability of these reference genes could be skin specific such as collagen deposition and mineralization. The effect of Cd exposure on *b-actin* as well as *DRP2* was negligible, which resulted in their good ranking in general (Figures 4.1A and 4.2A). *B-actin* is an isoform of the actin family of globular proteins and it is the monomeric subunit of microfilaments, which are the major components of cytoskeleton (Khaitlina, 2001). Data on the effect of Cd or Cu on *b-actin* gene expression is scarce in aquatic organisms. However, similar to this study, *b-actin* was found to be one of the three best performing reference genes in the gills of the snail *Haliotis discus* exposed to Cd (Lee and Nam, 2016). Hence, the data presented in this study as well as data previously reported for *Haliotis discus* indicated that short term exposure to metals such as Cd did not affect the gene expression of *b-actin* to a significant extent. In contrast, some *in vitro* studies in mammalian cells have demonstrated that metals such as Cd and Cu can affect the gene expression of *b-actin* before causing effects on cell viability and paracellular permeability (Calabro et al., 2011). However, no such data is available for *in vivo* vertebrates. An *in vivo* study in rats demonstrated that Cd, at comparatively much higher dose range of 1,000 µg/kg administered intraperitoneally, caused disruption in basal microfilaments of testicular Sertoli cells (Hew et al., 1993). However, the effect of Cd on cytoskeleton has been suggested to be a direct action of Cd on the proteins related to cytoskeleton (Wan and Zhang, 2012). Thus, it is concluded that in teleost fish, which show much greater sensitivity to metals such as Cd compared to terrestrial vertebrates, *b-actin* represents a preferred reference gene for use in short-term metal toxicity studies.

HPRT demonstrated a metal-specific response in its expression stability. It ranked poorly as it was significantly altered in both gill and skin after exposure of trout to Cd (7th and 6th respectively) (Tables 4.3 and 4.5). In contrast, *HPRT* showed better stability in gill and skin of fish exposed to Cu. *HPRT* codes for an enzyme called hypoxanthine-guanine phosphoribosyltransferase (HPRT), which is one of the central enzymes in recycling of purine nucleotides, the building blocks of DNA and RNA (Torres and Puig, 2007). *HPRT* gene expression and protein levels have been shown to be induced by hypoxia inducible factor 1 (HIF1A), a transcription factor that is involved in adaptation to hypoxic conditions (Wu et al., 2015). Given

the fact that both Cd and Cu have been shown to induce HIF1A, it was not surprising to observe poor stability in *HPRT* expression after exposure to Cd (Heerden et al., 2004; Jing et al., 2012). Interestingly, *HPRT* showed a much greater stability after exposure to Cu, which was opposite to what was expected based on the effect of Cu on HIF1A. However, these differences in the effects of Cd and Cu on *HPRT* expression might be due to the quantitative differences in the effect levels of Cd and Cu on HIF1A mediated induction of *HPRT* or due to some additional unknown mechanism of the effects of Cd or Cu on the expression of *HPRT*.

In general, the effect of Cu on the expression of most of the reference genes studied was highly tissue specific. The stability rankings of reference genes in the skin of fish exposed to Cu over different life-stages followed similar patterns as unexposed fish (Tables 4.6 and 4.2). This pattern indicated that developmental stage was the driving factor for the changes in the expression of reference genes in fish skin. To date, no data is available on the effects that developmental stages may have on the expression of commonly used reference genes in the skin of teleost fish. However, it is known that skin undergoes rapid morphological and physiological changes during early life-stage development in fish (Ángeles Esteban, 2012; Glover et al., 2013). During the larval stages in trout, several important physiological processes such as ammonia excretion, ionoregulation, organic nutrient absorption, and respiration predominantly occur through skin until the swim-up stage (20-28 days post hatch), when gills become fully functional (Glover et al., 2013). These massive changes in skin physiology might be the reason for the observed impact of early developmental stages on the expression patterns of reference genes in this tissue. On the other hand, Cu exposure rather than life-stage had a greater impact on stability rankings of reference genes in the gills. For example, *b-actin* was one of the three best performing reference genes in the gills over the three life-stages investigated in unexposed fish (Table 4.7 and 4.1). However, in the presence of Cu, *b-actin* was ranked as the least stable reference gene (Table 4.4). Gills are the major target sites for Cu toxicity (Dang et al., 2000; Pandey et al., 2008; Tang et al., 2016). However, the molecular basis of the effects of Cu on the expression of reference genes has not been studied to date. As discussed above, although Cu affected the stability ranking in gills heavily, the absolute changes in the gene expression were small (Table 4.4 and Figure 4.1A). Hence, it can be safely concluded that Cu has negligible effects on the expression of reference genes in gills of rainbow trout.

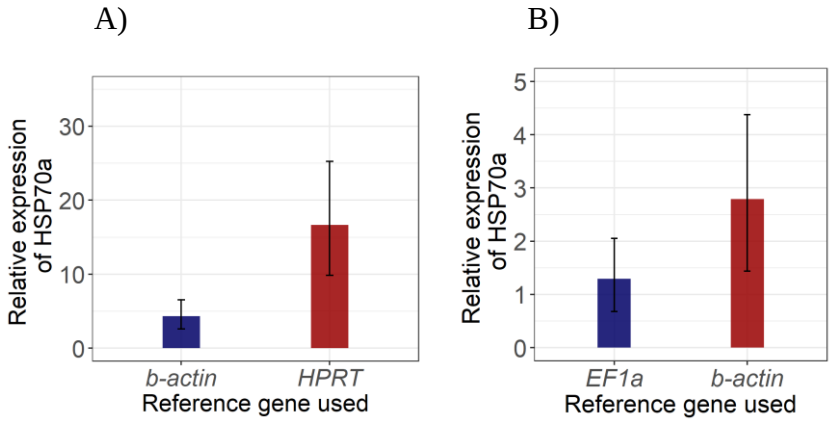


Figure 4. 5. Validation of reference gene with HSP70a (Heat Shock Protein 70a) gene expression in the gills of rainbow trout exposed to (A) Cd (5 µg/L), (B) Cu (40 µg/L), for 24 h. In both cases, HSP70a expression levels were normalized with most stable and least stable reference genes identified in this study. Results of HSP70a gene expression were analysed using ΔΔCt method and the results are presented as fold change (± SD) as compared to the control. Four fish were used for each group.

A) B)

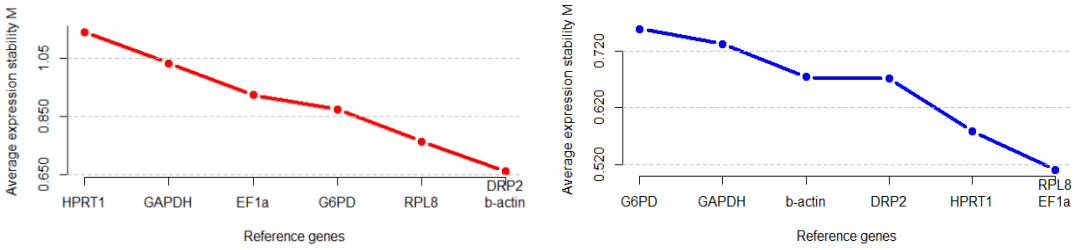


Figure 4. 6. The average expression stability values (M) of seven reference genes in the gills of rainbow trout larvae exposed to A) Cd and B) Cu, as analysed by GeNorm. The lower values represent better stability. M values for all the genes are less than the cut-off of 1.5, meeting the high expression stability criteria. Calculations were performed on pooled data from control and exposed group from the three life-stages (n = 24)

A) B)

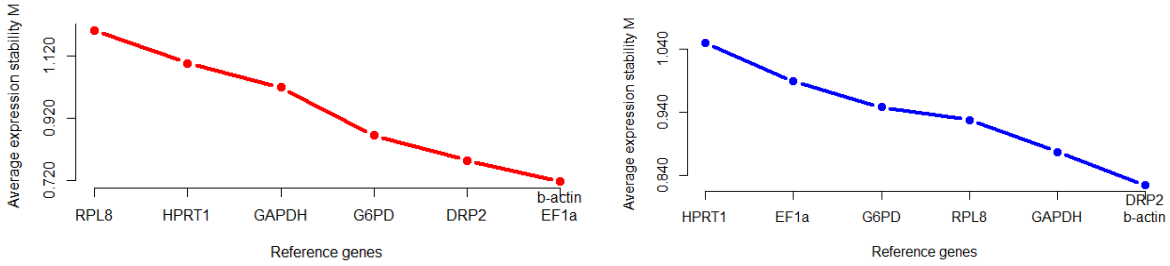


Figure 4. 7. The average expression stability values (M) of seven reference genes in the skin of rainbow trout larvae exposed to A) Cd and B) Cu, as analysed by GeNorm. The lower values represent better stability. M values for all the genes are less than the cut-off of 1.5, meeting the high expression stability criteria. Calculations were performed on pooled data from control and exposed group from the three life-stages (n = 24)

Overall, ranking of genes produced by the four algorithms applied in this study were very similar across the different tissue and metal combinations with few exceptions (Tables 4.1 – 4.6). Nonetheless, minor discrepancies among the ranks produced by these methods were not surprising because the mathematical basis for these methods are different. Because of these minor differences in the results, we took the geometric mean of the ranking numbers of each gene assigned by the four methods and produced the final ranking from lowest to highest mean (Tables 4.1 – 4.6). Based on this geometric mean, *b-actin* and *DRP2* were identified as the most stable reference genes in the gills and skin exposed to Cd and skin exposed to Cu over different life-stages. In the gills of fish exposed to Cu over different life-stages, *EF1a* and *HPRT* were the most stable reference genes. BestKeeper method dictates that the reference genes with standard deviation of greater than 1 must not be used for normalization. In this study, the two best genes in both tissues and across metal exposures as discussed above, had a SD less than 1 when BestKeeper method was applied. Hence, these reference genes seem to be acceptable for performing normalization of GOI in qPCR experiments. It is also notable that since all the calculation methods used in this study rank the reference genes on a relative scale, the least stable reference gene may not necessarily be a "bad" reference gene for use in qPCR quantification if the absolute effects of the contaminants are minor. Conversely, any threshold criteria such as M value cut-offs of >1.5 in geNorm method should also be applied carefully. M values of less than 1.5 are considered as a criteria for indicating high expression stability for any given gene (Amoako et al., 2013; Cicinnati et al., 2008; Galisa et al., 2012; Plusquin et al., 2012). In this study, the expression stability values (M values) for all 7 reference genes were less than 1.5 in the gills and skin for both metals (Figures 4.6 and 4.7). Based on this condition, all reference genes in our study met the high expression stability criteria (M < 1.5) (Tables 4.3 – 4.6). Yet, normalising expression of the model gene *HSP70a* using *b-actin* and *HPRT* (both with acceptable M value of <1.5) resulted in a difference of >12-fold in the interpretation of results (Figure 4.5A). Validation of the most stable and least stable reference genes using *HSP70a* gene expression as a biomarker of metal exposure showed that the interpretation of the effect of a contaminant on gene expression can vary widely based on the reference gene used.

4.6 Conclusion

Overall, our study demonstrated that exposure to metals such as Cd and Cu, life-stage during early development and tissue in question can have significant impacts on the stability of reference genes that are commonly used in gene expression studies with aquatic species such as rainbow trout. Further, comparing the data of this study to other studies with different species also demonstrated that comparative stability of reference genes can vary significantly among different species, developmental stages, and different experimental conditions (Lee and Nam, 2016; Mitter et al., 2009; Plusquin et al., 2012; Zhang et al., 2016). Hence, as pointed out by other authors, we strongly recommend that the most suitable reference genes under the given experimental conditions should be studied prior to actual qPCR study (Kozera and Rapacz, 2013). In conclusion, based on the observations in this study, *b-actin* and *DRP2* were the two most stable reference genes in the gills and skin of early stage rainbow trout exposed to Cd as well as the skin exposed to Cu. In contrast, *EF1a* and *HPRT* were the two best performing reference genes in the gills of early life-stage rainbow trout exposed to Cu.

5 CHAPTER 5: ROLE OF CADMIUM ACCUMULATION, OXIDATIVE STRESS, METALLOTHIONEIN AND HEAT SHOCK PROTEIN RESPONSES IN SPECIES AND LIFE-STAGE SPECIFIC DIFFERENCES IN THE SENSITIVITY OF WHITE STURGEON AND RAINBOW TROUT TO CADMIUM

PREFACE

Studies conducted in Chapter 2 showed that ion physiology parameters such as Ca uptake and whole-body Ca levels explain higher sensitivity of rainbow trout to Cd as compared to white sturgeon. However, in some life-stages, the species-specific differences in the sensitivity to Cd were several times higher than the differences observed in physiological parameters. This observation suggested that there are possibly additional mechanisms involved in the species-specific differences in the sensitivity to Cd. Hence, the aim of chapter 5 was to compare the differences in Cd accumulation and Cd induced oxidative stress, metallothionein and heat shock protein responses between rainbow trout and white sturgeon across multiple early life-stages. Species-specific differences in these parameters were evaluated because these parameters are known to be important pathways through which Cd causes toxicity. Hence, quantitative differences in Cd accumulation, oxidative stress, metallothionein and heat shock protein responses could be important contributing factors in life-stage and species-specific differences in the sensitivity to Cd.

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Author contributions:

Kamran Shekh (University of Saskatchewan) designed and conducted the experiment, analysed and visualised the data, and drafted the manuscript.

Dr. Song Tang (University of Saskatchewan) provided the necessary training, helped with the experiment, and commented on the manuscript.

Vladimir Kodzhahinchev (University of Saskatchewan) helped with the experiment and commented on the manuscript.

Drs. Som Niyogi and Markus Hecker (both University of Saskatchewan) provided inspiration, guidance, and scientific input, commented on the manuscript, and provided funding for the research.

5.1 Abstract

Understanding the mechanistic basis of differences in the sensitivity of fishes to metals is important for developing informed ecological risk assessment approaches for metals. Whole body metal accumulation, metallothionein induction, oxidative stress and associated antioxidant response, as well as heat shock proteins (mainly HSP70) are known to play important roles in determining the toxicity of metals in fish. Hence, in this study we have cross-evaluated these parameters as a function of cadmium (Cd) exposure across different life-stages of two evolutionarily distinct fish species, namely rainbow (*Oncorhynchus mykiss*) trout and white sturgeon (*Acipenser transmontanus*). These two species have been shown to differ significantly in their physiological and apical responses to Cd exposure. The findings of the present study suggest that species-specific differences in the sensitivity to Cd could partially be explained by HSP70 gene response and oxidative damage biomarkers. However, not all the parameters studied here could explain the life-stage specific differences universally and were limited to only some life-stages. Based on the observations in the present study and other recent studies, it is apparent that species- and life-stage specific differences in the sensitivity to Cd and possibly other metals is a complex phenomenon and could be driven by multiple toxicokinetic and toxicodynamic factors.

5.2 Introduction

Metals are aquatic contaminants of global concern because of their capacity to produce a wide variety of toxic effects. Among the metals, cadmium (Cd) is of particular concern because of its high level of toxicity to aquatic organisms, which is reflected by its much lower water quality guideline value relative to many other metals (CCME, 2014). Cd is released into the environment from diverse industrial and agricultural sources and its elevated concentrations have been reported for several aquatic ecosystems around the world (Järup and Åkesson, 2009).

Several studies have demonstrated significant life-stage and species-specific differences in the acute sensitivity of fishes to Cd (Calfee et al., 2014; CCME, 2014; Vardy et al., 2014). Life-stage and species-specific differences are crucial factors that need to be considered when conducting ecological risk assessment of metals. This is because toxicity reference values (TRVs) or water quality criteria should be based on the most sensitive life-stage of the most sensitive species to be protective of a given ecosystem. Sensitivity to metals is generally assumed to be inversely related to the age of the fish, mainly due to higher surface area to volume ratio of the smaller fishes (Mohammed, 2013). Although this assumption holds true in many cases (Grosell et al., 2002), several studies have demonstrated that in some cases later life-stages can be more sensitive than the early larval life-stage in fish (Calfee et al., 2014; Shekh et al., 2018; Tang et al., 2016; Vardy et al., 2014).

To date, some studies have attempted to explain species-specific differences in metal sensitivity with basal ionic uptake rate and its disruption by metals, or subcellular distribution of metals (Bianchini et al., 2002; Eyckmans et al., 2012; Niyogi and Wood, 2004a). Moreover, some recent studies have evaluated life-stage or species-specific differences in metal sensitivity on the basis of biochemical mechanisms of toxicity (e.g., oxidative stress) and/or detoxification mechanisms of metals (e.g., metallothionein induction) (Eyckmans et al., 2011; Tang et al., 2016). However, a comprehensive understanding of the physiological, biochemical, and molecular mechanisms underlying the species and life-stage specific differences in the sensitivity of fish to the exposure with metals such as Cd is still lacking.

Accumulation of Cd has been shown to induce oxidative stress in several organisms (see Cuypers et al., 2010 for review). Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) act as a first line of defense against the

oxidative damage in organisms (Di Giulio et al., 1989; Valavanidis et al., 2006). The protein activities as well as the gene expression of these enzymes, along with other enzymes such as glutathione-s-transferase (GST), are regularly used as biomarkers of metal exposure in a wide variety of aquatic organisms including fish (Barata et al., 2005). Some recent evidence indicates that life-stage and species-specific differences in oxidative damage and antioxidant responses to metals like Cu and Cd occur in fish (Eyckmans et al., 2011; Tang et al., 2016). Antioxidant defense mechanisms in gills have also been suggested to play a role, albeit modest, in the tolerance observed in wild fish residing in metal contaminated waters (Uren Webster et al., 2013).

Another well-described biological response to the exposure with Cd is induction of heat shock proteins (e.g., HSP70) (Kwong et al., 2011; Matz and Krone, 2007). HSPs, especially HSP70s, have been proposed both as a biomarker of exposure as well as biomarker of effects in fish exposed to different classes of pollutants including metals (Elyse Ireland et al., 2004; Nadeau et al., 2001). Although the mechanism through which Cd activates HSP70 response is unknown, Cd induces the generation of denatured proteins, which could act as a trigger of HSP70 induction and heat shock response (Georgopoulos and Welch, 1993; Waisberg et al., 2003). Hence, HSP70 could act as an important protective mechanism against Cd toxicity.

Finally, metallothionein (MT) represents another biomarker that is induced by exposure to Cd (Chowdhury et al., 2005; De Smet et al., 2001; Hollis et al., 2001; Lange et al., 2002). MTs belong to a group of low molecular weight cytosolic proteins that act as a temporary storage of metals in cells. They are also considered as a useful biomarker for metal pollution because of generally good correlation between their expression and increasing metal body burdens (Kille et al., 1992; Van der Oost et al., 2003). Several studies have suggested that the increased ability of an organism to induce MT confers it tolerance to metals (Benson and Birge, 1985; Uren Webster et al., 2013).

Rainbow trout (*Oncorhynchus mykiss*), a commonly used model species for environmental testing, and white sturgeon (*Acipenser transmontanus*), an endangered species of aboriginal, commercial and recreational importance, have shown significant species and life-stage specific differences in the sensitivity to Cd in several studies (Calfee et al., 2014; Shekh et al., 2018; Tang et al., 2016; Vardy et al., 2014). We recently demonstrated that Cd uptake and the effect of Cd on Calcium (Ca) homeostasis could play an important role in species and life-stage specific

differences in the sensitivity to Cd in sturgeon and trout (Shekh et al., 2018). However, considering the diverse types of biological interactions that have been reported for Cd in fish (see previous paragraphs), this study aimed to expand these earlier observations by investigating the role of additional mechanisms in driving the species and/or life-stage specific differences in Cd sensitivity in rainbow trout and white sturgeon. Accordingly, the overall goal of our study was to develop a comprehensive understanding of the mechanistic underpinnings of species and life-stage specific differences in acute sensitivity to the exposure with Cd between two evolutionary distant fish species, rainbow trout and white sturgeon. For this purpose, we performed a comparative analysis of the accumulation of Cd, cellular lipid peroxidation, and mRNA expression of major antioxidant enzymes, Cd transporters, MT and HSP70s in different life-stages of these species.

5.3 Materials and Methods

5.3.1 Experimental Organisms and Test Chemicals

Embryos of white sturgeon and rainbow trout were obtained from the Nechako White Sturgeon Conservation Centre (BC, Canada) and Troutlodge (WA, USA), respectively. The embryos were reared in the Aquatic Toxicology Research Facility at the University of Saskatchewan in a flow-through system maintained at approximately 13 °C. Commercial frozen bloodworms (San Francisco Bay Brand, Newark, CA, USA) and trout food (Bio Vita Starter #0 Crumble, Bio-Oregon, British Columbia, Canada) were provided as food to white sturgeon and rainbow trout, respectively. Feeding was started at 10-12 days post hatch (dph). All the required permits were received before starting the experiments (Fisheries and Oceans Canada SARA Permit XRSF 20 2013/SARA 305). All procedures involving animals were approved by the University of Saskatchewan's University Council on Animal Care and Supply (Protocol 20140079). Cadmium chloride hemi-pentahydrate (CAS 7790-78-5; purity 99.999%) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and an initial stock solution was prepared in deionized water.

5.3.2 Exposure Protocol

All exposures were conducted in water that was a 3:1 mixture of reverse osmosis water and dechlorinated City of Saskatoon municipal tap water. The purpose of mixing the waters in this ratio was to maintain the hardness and alkalinity at a moderate level of ~65 and 40 mg/L as $CaCO_3$, respectively. Working Cd exposure solutions were prepared at least 24 hours before initiation of

the experiments and stored at 13 °C. Whole body Cd accumulation was estimated after 24 h exposure to 0, 1.25 and 5 µg/L (11.1 and 44.5 nM) of nominal Cd concentrations in four different early life-stages: larval (5 dph), swim-up (15 dph), early juvenile (45 dph), and juvenile (75 dph). Six individual 500 mL high-density polyethylene (HDPE) cups were used per exposure group for exposing each fish individually (n = 6). Half of the exposure media in each exposure chamber was replaced after 12 hours.

All other parameters (gene expressions and oxidative damage) were measured after an exposure period of 96 h in larval, swim-up, and early juvenile life-stages of each species. Fish were exposed to a Cd concentration of 50% of the 96h LC_{50} value for the respective life-stage and species (equivalent to 6.26, 1.06, 0.965 µg/L, and 8.8, 41.1, 8.1 µg/L of Cd in rainbow trout and white sturgeon, respectively, at larval, swim-up and early juvenile life-stages) under the same water chemistry conditions for 96 h. Six individual 500 mL HDPE cups per exposure group were used for exposure (n = 6). Within each cup, 5 fish were exposed in the larval life-stage experiment, whereas 2 fish were exposed for the other life-stages. Half of the exposure media was replaced with exposure water from the same stock every 12 hours. Following exposure to Cd, fish were euthanized with Aquacalm (metomidate hydrochloride) (Syndel Laboratories Ltd., BC, Canada). Four out of 5 fish from each exposure chamber of the larval stage and 1 out of 2 fish from all other life-stage exposures were kept for lipid peroxidation analysis, and one fish from each exposure chamber of all life-stages was kept for gene expression analysis. All samples were stored at -80 ºC until further analysis.

5.3.3 Water Chemistry

The major ion levels (Cl^-, DOC, Ca, Mg, K, Na, SO_4^{2-}) were analyzed in exposure water samples collected at the beginning and end of the experiments (Saskatchewan Research Council, Saskatoon, SK, Canada). Other water chemistry parameters such as hardness, alkalinity, dissolved oxygen, pH, and temperature were measured daily during each exposure. Hardness and alkalinity were determined by using Nutrafin Test kits (Hagen, Canada). Temperature, pH, and DO were measured by a YSI Quatro Multi-Parameter probe (Yellow Springs, OH). At the beginning and end of each exposure, water samples were collected randomly from 2 of the replicates in each exposure group. Water samples were filtered through a 0.45 µm polycarbonate filter, acidified (0.1 %) with trace metal grade nitric acid (Fisher Scientific, Canada), and analyzed for dissolved Cd

concentrations by graphite furnace atomic absorbance spectroscopy (AAnalyst 800, Perkin Elmer, USA) using appropriate method blanks and a certified Cd standard (Fisher Scientific, Canada).

5.3.4 Whole Body Cd Accumulation

At the end of exposure, each fish was euthanized with Aquacalm, washed twice for 30 s in deionized water, blotted dry, weighed and stored at -80 °C until further analysis. For whole body Cd analysis, each fish was digested for 48 h in 5 volumes of 2N trace metal grade Nitric acid (EMD Millipore, Billerica, MA, USA) at 60 ºC. Whole body Cd was measured using a graphite furnace atomic absorption spectrometer (AAnalyst 800, Perkin Elmer, USA) after appropriate dilution of samples in 0.1% nitric acid. The quality control/assurance of Cd analysis was ensured using appropriate method blanks, a certified Cd standard (Fisher Scientific, Canada) and a reference material (DOLT-4; National Research Council of Canada). The recovery percentage of Cd in the reference material was 96%.

5.3.5 Lipid Hydroperoxide (LPO) Assay

Because of the small size of the fish, organs were not distinguishable at the life-stages studied; hence, LPO levels were analyzed in the whole body. The measurements were performed using the Lipid Hydroperoxide Assay kit (ab133085, Abcam Inc, ON, Canada) following the manufacturer's protocol. Hydroperoxides were measured using 96-well plates at ambient temperature and based on their absorption spectrum at 500 nm recorded in a multimode plate reader (Verioskan Flash, Thermo Scientific, ON, Canada). The hydroperoxide concentrations were normalized on the basis of tissue protein concentration (mg/g). Bradford method was used to measure the protein content in each sample (Bradford, 1976).

5.3.6 Reverse Transcription Quantitative Real-time Polymerase Chain Reaction (qPCR) for Oxidative Stress, Metallothionein and HSP70 Genes

RNA extraction, cDNA synthesis and qPCR for all the oxidative stress, metallothionein and HSP70 genes were performed using commercial kits. Briefly, whole body total RNA was extracted by using the RNeasy Lipid Tissue Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. RNA concentration and purity (260/280 and 260/230 ratio) were quantified by a NanoDrop ND-1000 Micro-Volume UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All RNA samples were diluted and used for cDNA

synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada) as per the protocol recommended by the manufacturer. The qPCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 96-well plates. Each cDNA sample and primer combination were subjected to the following steps: A 50 µL reaction mixture was prepared with 2x concentrated QuantiFast SYBR green master mix, 2.5 µL of cDNA, 10 pmol gene-specific primers, and nuclease free water. Each cDNA sample was analyzed in duplicate with 20 µL reaction volumes per well. The reaction mixture for qPCR was denatured at 95 ºC for 10 min followed by a thermal cycle profile consisting of denaturing at 95 ºC for 10 s and extension for 1 min at 60 ºC for a total of 40 PCR cycles. Melting curve analysis was also conducted after 40 cycles to verify the specificity of amplicons. All primer sequences are available in Appendix (Table C5.S1). The term GPX as used in this manuscript refers to sturgeon GPX and trout GPX1. Similarly, the terms HSP70, MT and SOD represent sturgeon HSP70, MT, and SOD genes and trout HSP70a, MTa, and SOD1 genes, respectively. Multiple forms of GPX, HSP, MT, and SOD are known only in rainbow trout (GPX4a1, HSP70b, MTb, and SOD2) but not in sturgeon, and are included in Figure 5.4 accordingly.

5.3.7 Gene Expression Analysis of Apical Cd Transporters in Gills and Skin Epithelium of Developing Fish

Fish were not exposed to Cd for this experiment because the purpose here was to characterize the changes in the baseline expression of genes involved in Cd transport across different life-stages of both species. Fish were acclimated in normal exposure water (without Cd) for 5 days. At the end of 5 days, fish were euthanized, and skin was immediately removed by surgical blade and forceps under a dissection microscope and stored in RNA-later (Sigma Aldrich, St. Louis, MO, USA). Because of the small size of the fish, gill baskets could not be isolated. Hence, head was used as a proxy of the gill, and was separated from body and stored in RNA-later at 4 °C overnight and then transferred to -20 °C until RNA isolation. Total RNA extraction from the head and skin, cDNA synthesis and qPCR for all the genes of major apical transporters involved in Cd uptake (e.g., epithelial calcium channel (ECaC) and divalent metal transporter (DMT)) were performed with commercial kits using the same method as described previously.

5.3.8 Statistical Analysis

Whole body Cd accumulation, LPO and antioxidant gene expression data were analyzed by three-way ANOVA followed by further analysis of all ANOVA interaction terms and post-hoc testing with t-tests. The three independent variables in each ANOVA model were species, life-stages and exposure concentrations. After initially analyzing the data by three-way ANOVA, data from individual life-stages were isolated from both species and analyzed with two-way ANOVA, with species and exposure concentrations as independent variables. If the application of two-way ANOVA in a given life-stage revealed statistically significant two-way interactions between species and exposure concentration, it indicated the presence of species-specific differences in the effect of Cd exposure. In such cases, multiple comparison among groups were performed by using Tukey's test and these multiple comparisons allowed us to assess differences among all possible sets of groups, for example: control *versus* control across species or treatment *versus* treatment across species or control *versus* treatment within species for both species. The purpose of multiple comparison approach was to explain the origins of interactions determined by two-way ANOVA and to confirm the presence or absence of the exposure induced species-specific difference in a given life-stage. The data for transporters involved in Cd uptake were analysed by one-way ANOVA within a species, with life-stage as the independent variable. The trend in the gene expression changes for transporters with the growth of fish was discussed individually in each fish; hence, one-way ANOVA with life-stage as independent variable was the most suitable model.

Normality and homogeneity of variance of the dependant variables were determined by Shapiro−Wilk test and Bartlett's test, respectively, and square root transformed if required. A p value of less than 0.05 was considered as significant for all parameters.

5.4 Results

5.4.1 Exposure Verification and Water Quality

Measured Cd concentrations were comparable to the nominal concentration used in this study. The average temperature during the exposures was 13.4 ± 0.31 ºC. The average DO and pH were 7.3 ± 0.16 and 93.22 ± 4.64%, respectively. Measured concentrations of anions, cations, and DOC have been provided in the Appendix (Table C2.S1).

5.4.2 Whole Body Cd Accumulation

Three-way ANOVA revealed statistically significant three- way interaction ($F = 5.1$, $p < 0.001$). There were also two-way interactions between species-life stage ($F = 6.83$, $p < 0.001$), species-exposure ($F = 161.21$, $p < 0.001$), and life-stage-exposure ($F = 23.03$, $p < 0.001$). These three and two-way interactions suggest the presence of species and life-stage specific differences in whole-body accumulation of Cd. To fully characterize these interactions, we separated the whole-body accumulation data according to life-stages and analyzed the species-specific differences within each life-stage using two-way ANOVA.

The extent of Cd accumulation differed among life-stages for both species (Figure 5.1). In 5 dph rainbow trout, which have been demonstrated to be a comparatively less sensitive life-stage to Cd (Calfee et al., 2014; Shekh et al., 2018), no significant Cd accumulation occurred at any of the exposure concentrations tested relative to the control (adjusted $p = 0.99$ and 0.98 for 1.25 and 5 µg/L, respectively, DF = 30). In contrast, during later life-stages, which have been found to be more sensitive to Cd, significantly greater accumulation of Cd occurred at all exposure concentrations compared to the respective controls (Figure 5.1). White sturgeon showed significantly greater Cd accumulation at an exposure concentration of 5 ug/L Cd relative to the control during all life-stages (adjusted $p < 0.001$ and DF = 30 in all life-stages). Both species showed age-dependency in Cd accumulation properties with the older life-stage of each species demonstrating higher Cd accumulation than the preceding life-stage. For example, at the exposure concentration of 5 µg Cd/L, the Cd accumulation in white sturgeon was 0.29, 0.52, 0.88, and 1.28 nM/g wet weight in 5 dph, 15 dph, 45 dph and 75 dph fish, respectively (Figure 5.1).

From the perspective of species-specific differences, control groups did not show statistically significant difference in whole body Cd levels between rainbow trout and white sturgeon. However, when exposed to 5 µg/L of Cd, white sturgeon accumulated significantly greater concentrations of Cd compared to that in rainbow trout in all respective life-stages (Figure 5.1).

5.4.3 Gene Expression Analysis of Apical Transporters Involved in Cd Transport

In rainbow trout gills, a statistically significant upregulation of all the metal transporters was noted as the fish grew from 5 dph to 45 dph (adjusted $p < 0.05$ for all transporters, DF = 15) (Figure 5.2A). On the other hand, a significant downregulation of *ECaC* was observed in skin of

rainbow trout (adjusted $p < 0.001$, DF = 15) (Figure 5.2C). Similar patterns were also recorded in the skin of white sturgeon (Figure 5.2D). In the gills of white sturgeon there was a trend of greater upregulation of ECaC with increasing age; however, this was not statistically significant (adjusted $p = 0.15$, DF = 15), likely due to very high variance in the data (Figure 5.2B).

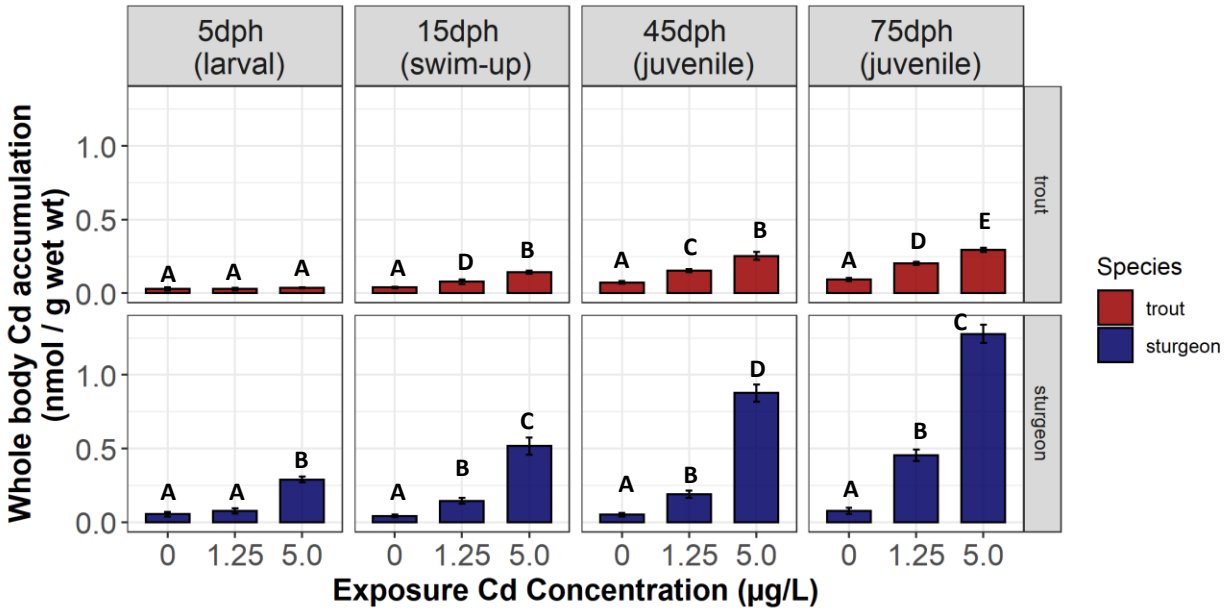

Figure 5. 1. Whole body Cd accumulation as a result of 24 h exposure to different waterborne Cd concentrations in rainbow trout and white sturgeon across different life-stages. Within each life-stage, data are presented as mean ± SEM of absolute values of Cd accumulation (n = 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Statistically significant difference among groups is indicated by different letters ($p < 0.05$).

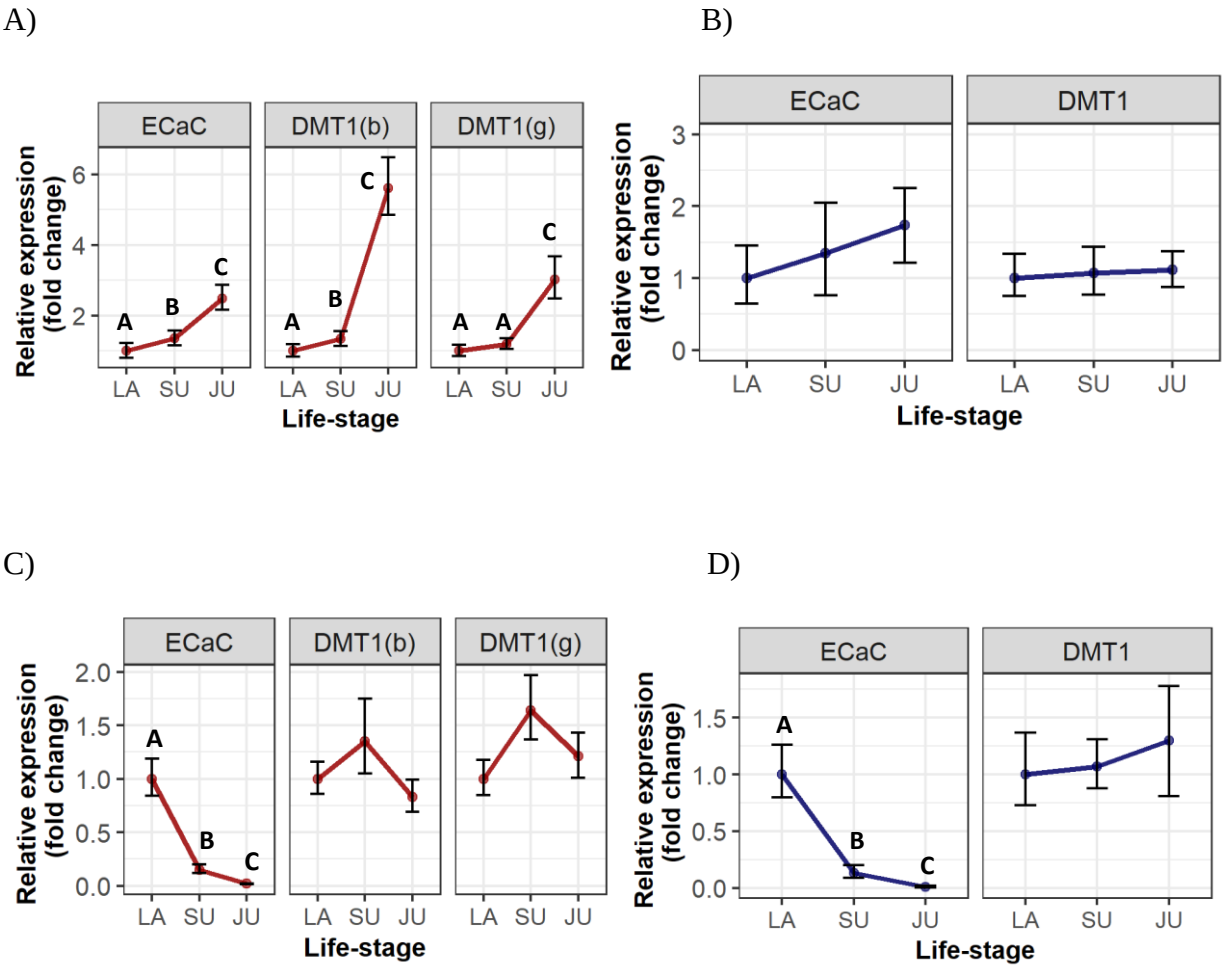

Figure 5. 2. Changes in the transcript abundance of genes for Cd uptake transporters/channels (*ECaC* and *DMT*) in rainbow trout **(A)** gills **(C)** skin and white sturgeon (**B)** gills and **(D)** skin during the course of development through early life-stages. Fold changes in two later life-stages were calculated with reference to first life stage (5dph). Results are presented as relative expression (mean ± SD, n = 6). Relative expressions with different upper-case letters are significantly different from each other ($p < 0.05$). The terms DMT1(b) and DMT1(g) represent NRAMPb and NRAMPg genes in rainbow trout. LA, SU and JU on the axis represent larval, swim-up and juvenile, respectively.

5.4.4 Lipid Peroxidation (LPO)

Analysis of the LPO data by three-way ANOVA demonstrated significant two-way interactions between species and exposure ($F = 14.41$, $p < 0.001$) as well as between life-stage and exposure ($F = 5.63$, $p < 0.001$). Species-specific differences in the LPO response to Cd exposure were further analyzed by applying two-way ANOVA within each life-stage followed by the post-hoc tests among different groups.

At 50% of the respective 96 h LC_{50} concentration (Shekh et al., 2018), larval and early juvenile life-stages of rainbow trout demonstrated significantly higher LPO relative to the control (adjusted $p < 0.001$ in both, DF = 30). In contrast, sturgeon showed no significant increase in LPO at the larval life-stage (adjusted $p = 0.9$, DF = 30) but exhibited a significant but modest induction of LPO at the juvenile stage relative to that in rainbow trout (1.93-times in white sturgeon vs. 3.3-times in rainbow trout) (Figure 5.3). In both larval and juvenile life-stages, there was a significant two-way interaction between species and exposure ($p = 0.001$ and <0.001 in larval and juvenile, respectively), indicating that exposure to Cd caused LPO induction in a species-specific manner. Post-hoc tests showed that the LPO induced by Cd exposure was significantly higher in white sturgeon as compared to rainbow trout in both larval and juvenile life-stages (adjusted $p = 0.002$ and <0.001, respectively, DF = 30).

At the swim-up life-stage, the species and exposure main effect was not statistically significant ($F = 1.94$ and 0.93, $p = 0.17$ and 0.40, respectively). Moreover, there was no significant two-way interaction between species and exposure concentration ($F = 1.39$, $p = 0.27$). These observations suggested that there was no species-specific difference in Cd induced LPO at the swim-up life-stage (Figure 5.3).

5.4.5 Metallothionein Gene Expression Response

Larval life-stage fish showed no effect of Cd on MT gene expression in both species (adjusted $p = 0.76$ and 1.00, DF = 16). In other words, there was no species-specific difference in the response of this life-stage, as demonstrated by non- significant two-way interactions between species and exposure concentration ($F = 0.49$, $p = 0.50$) as well as a non-significant main effect of exposure concentration ($F = 0.48$, $p = 0.49$) (Table 5.1 and Figure 5.4). In both species, the upregulation of the MT gene following Cd exposure was significantly higher compared to the

control at the swim-up and juvenile life-stages (2.0- to 4.9-fold, adjusted $p<0.05$, DF = 16 in each life-stage). However, in both life-stages, rainbow trout demonstrated a significantly higher Cd-induced upregulation of MT compared to white sturgeon (about 4- to 5-fold vs. 2-fold, respectively) (Table 5.1 and Figure 5.4). Expression of MTb in rainbow trout demonstrated no significant change following Cd exposure (Figure 5.4).

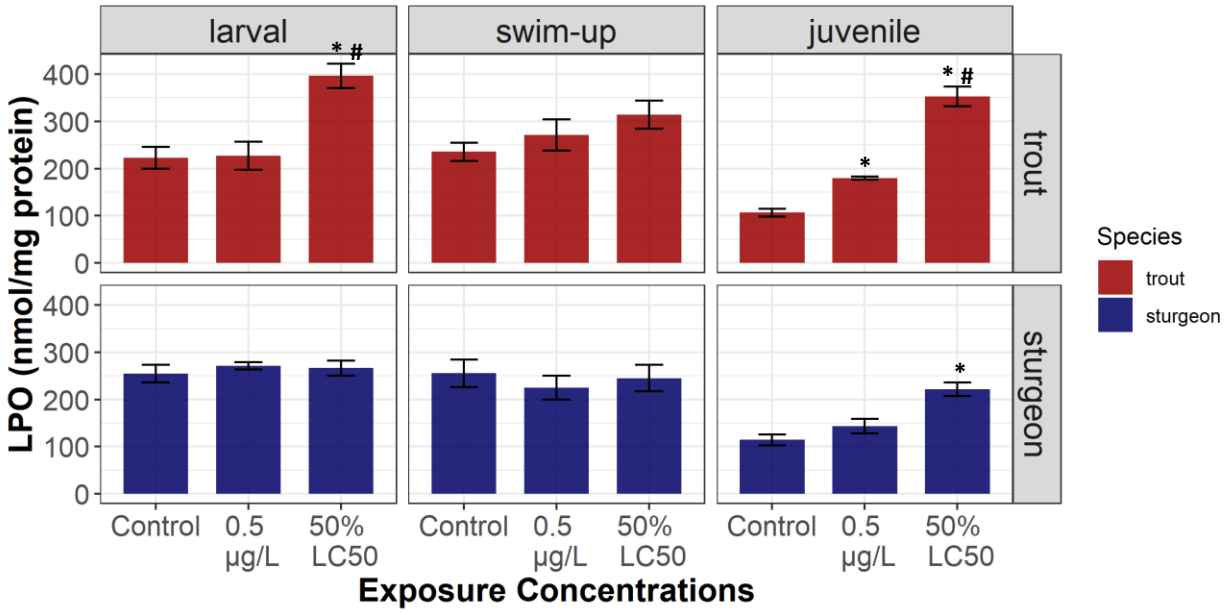

Figure 5. 3. Levels of lipid peroxidation (LPO) across different life-stages of white sturgeon and rainbow trout following 96 h exposure to Cd. Data are presented as mean ± SEM of LPO/mg protein (n = 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). A number symbol (#) on rainbow trout bars represents a significant difference between rainbow trout and white sturgeon for the corresponding exposure group ($p < 0.05$).

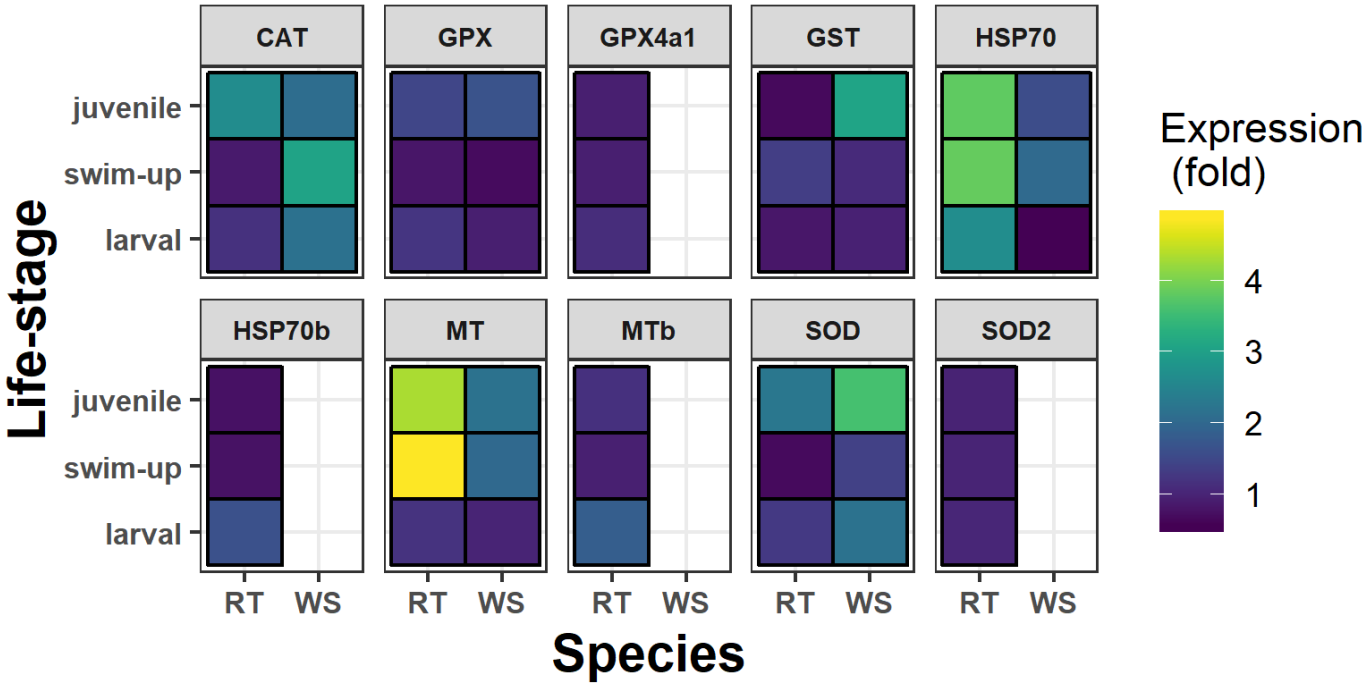

Figure 5. 4. Heat map of antioxidative, MT and HSP genes with each box representing the changes in expression in each life-stage as a result of Cd exposure (50% of LC50). From blue to yellow, data represents fold upregulation compared to respective control, according to the scale shown in the legend. The term GPX, HSP70, MT and SOD represent sturgeon genes GPX, HSP70, MT, and trout genes GPX1a, HSP70a, MTa, and SOD1. Multiple forms of GPX, HSP, MT, and SOD are not known in sturgeon but are known in trout (GPX4a1, HSP70b, MTb, and SOD2) and are included in this figure accordingly.

5.4.6 Heat Shock Protein (HSP70) Gene Expression Response

The application of three-way ANOVA to overall HSP70 data followed by two-way ANOVA in individual life-stages demonstrated a significant two-way interaction between species and exposure in all life-stages, which indicated species-specific differences in HSP70 response to Cd exposure in each life-stage ($F = 32.93, 5.63, 10.15$ and $p < 0.001, 0.03, 0.01$, for larval, swim-up and juvenile, respectively). In rainbow trout, the maximum change in HSP70 gene expression (compared to control) was observed during the swim-up life-stage (3.9-fold upregulation, adjusted $p<0.001$). In white sturgeon, the only significant change in HSP70 expression as compared to control, was found at the swim-up life stage (2.1-fold upregulation, adjusted $p = 0.008$, $DF = 16$). With respect to species-specific differences, rainbow trout demonstrated a higher upregulation of the HSP70 gene in all life-stages compared to white sturgeon (2.7- to 3.9-fold versus 0.6- to 2.1-fold). This observation clearly explained the significant two-way interaction observed in all life-stages (Table 5.1 and Figure 5.4). The HSP70b gene, which is specific to rainbow trout, demonstrated no significant change in its expression following exposure to Cd (Figure 5.4).

5.4.7 Antioxidant Gene Expression Responses

The application of three-way ANOVA followed by the analysis of individual life-stages demonstrated that in rainbow trout, exposure to Cd resulted in a significant upregulation in the expression of CAT and SOD mRNA at the juvenile life-stage (2.6- and 2.3-fold). In white sturgeon, upregulation in CAT was significant at all life-stages (2.1- to 3.1-fold). Other antioxidative enzymes in white sturgeon showed responses in a life-stage specific manner, such as 3.1- and 3.6-fold upregulation of GST and SOD, respectively, during the early juvenile stage but comparatively less or no upregulation in other life-stages (Table 5.1 and Figure 5.4).

In terms of species-specific differences in the antioxidant enzyme responses, overall, white sturgeon were able to mount a greater antioxidative response at the gene expression level. The larval life-stage of white sturgeon showed an approximately 2.2-fold upregulation in the expression of both CAT and SOD genes as compared to 1.2- and 1.3-fold in rainbow trout. During the swim-up life-stage of white sturgeon, a 3.1-fold upregulation of CAT was observed compared to 0.9-fold in rainbow trout, which explained the two-way interaction between species and exposure for CAT upregulation ($F = 20.44$, $p <0.001$). At the early juvenile life-stage of white sturgeon, GST demonstrated 3.1-fold upregulation, which was significantly higher as compared to

0.7-fold in rainbow trout (two-way interaction, $F = 20.73$, $p < 0.001$). At the juvenile stage, CAT demonstrated similar response in both species (2.1- and 2.6-fold induction, respectively; both adjusted $p < 0.05$); however, there was no significant species-specific differences ($F = 0.66$, $p = 0.43$). No significant effect of Cd exposure was found on the expression of GPX in either white sturgeon or

Table 5. 1. Fold change ± SD in antioxidant genes, HSP70, and MT genes across different life-stages in both species as a result of Cd exposure (50% of LC50). Statistically significant difference between exposure group and control group within each species and life-stage is indicated by an asterisk (*) (n = 6, p <0.05). The term GPX, HSP70, MT and SOD represent GPX, HSP70, MT, and SOD gene of sturgeon and GPX1a, HSP70a, MTa, and SOD1 gene of trout

Life-stage	**Exposure metric**	**Fold change in gene expression as compared to control ± SD**											
		CAT		**GPX**		**GST**		**SOD**		**MT**		**HSP70**	
		Trout	**sturgeon**	**trout**	**sturgeon**	**Trout**	**sturgeon**	**Trout**	**sturgeon**	**trout**	**sturgeon**	**Trout**	**sturgeon**
larval	**50% of LC50**	1.2 ±0.6	2.2 ±0.4*	1.2 ±0.4	0.9 ±0.4	0.8 ±0.3	0.9 ±0.4	1.3 ±0.5	2.2 ±0.5*	1.2 ±0.5	0.9 ±0.4	2.7 ±0.3*	0.6 ±0.4
swim-up	**50% of LC50**	0.9 ±0.6	3.1 ±0.2*	0.8 ±0.5	0.7 ±0.3	1.3 ±0.6	1.1 ±0.6	0.7 ±0.6	1.4 ±0.5	4.9 ±0.5*	2.0 ±0.3*	3.9 ±0.4*	2.1 ±0.6*
juvenile	**50% of LC50**	2.6 ±0.5*	2.1 ±0.4*	1.5 ±0.4	1.7 ±0.4	0.7 ±0.4	3.1 ±0.4*	2.3 ±0.6*	3.6 ±0.4*	4.3 ±0.6*	2.2 ±0.2*	3.8 ±0.5*	1.6 ±0.4

rainbow trout at any life-stage (changes were between 0.7- and 1.7-fold) (Table 5.1 and Figure 5.4).

5.5 Discussion

Several recent studies with white sturgeon and rainbow trout have demonstrated significant life-stage and species-specific differences in their sensitivity to Cd. In a parallel study performed under the same water chemistry conditions as the present study, rainbow trout demonstrated a greater sensitivity to the exposure with Cd relative to white sturgeon during four early life-stages, and the difference in tolerance was highest at 15 dph and 75 dph (35.5- and >27.3-times, respectively) (Shekh et al., 2018) (Chapter 2). The higher susceptibility of trout as compared to sturgeon may stem from an interplay of many factors including toxicokinetic and toxicodynamic properties, storage or detoxification of metals in fish (Buchwalter et al., 2008; Rainbow, 2002).

Based on its greater sensitivity to Cd, we hypothesized that the accumulation of Cd will be higher in rainbow trout as compared to white sturgeon. However, the pattern of whole-body Cd accumulation observed in this study across life-stages and species did not demonstrate a consistent relationship with observed differences in acute sensitivity to Cd. Although relatively more sensitive life-stages of rainbow trout (Shekh et al., 2018) accumulated more Cd compared to that in the less sensitive life-stages, this pattern was not observed in white sturgeon. In white sturgeon, each older life-stage accumulated greater concentrations of Cd than the preceding life-stage. If higher Cd accumulation is assumed to produce greater toxic effects, the life-stage specific sensitivity pattern in white sturgeon cannot be explained by the Cd accumulation pattern observed in the present study. Despite highest Cd accumulation at 75 dph (Figure 5.1), sturgeon showed lesser sensitivity at this life-stage compared to other life-stages (Shekh et al., 2018) (Chapter 2). Between the two species, rainbow trout, which has been shown to be more sensitive to Cd, accumulated significantly less Cd relative to white sturgeon when exposed to the same Cd concentration for 24 h. Inconsistent relationships between metal sensitivity and metal accumulation have also been reported in other fishes and aquatic invertebrates (Shaukat and Javed, 2013; US EPA, 2016). Such inconsistent relationship between whole body Cd accumulation and toxic effect might be mediated by the differences in sequestration of the metal load in the metabolically detoxified fraction such as metallothionein and metallothionein like proteins, and metal rich granules (Liu et al., 2007; McGeer et al., 2012).

Changes in gene expression of branchial transporters support our observation on life-stage dependent increases in Cd accumulation (Figure 5.1 and 5.2). The gene expression results are also in agreement with the short-term Cd uptake (maximum capacity of Cd uptake (J_{max})) reported in our recent study (Shekh et al., 2018). The expression of branchial transporters involved in Cd uptake increased gradually as the rainbow trout grew in age and size (Figure 5.2A), which could have contributed to a gradual increase in Cd accumulation (Figure 5.1) and J_{max} values (Shekh et al., 2018) across different life-stages. Previous studies have also demonstrated a positive correlation between the expression of metal transporter genes and the uptake of metals, providing further evidence of potential linkage between the expression of metal transporters and Cd accumulation in fish (Bai et al., 2012; Tallkvist et al., 2003). The same argument could be applied to white sturgeon as well because an increasing trend in the branchial expression of *ECaC* was also observed in this species; however, as mentioned previously, the increased expression was not statistically significant, most likely due to the high variance in the data (Figure 5.2B). Interestingly, we observed a significant decrease in the expression of *ECaC* in the skin epithelium in both species as the fish grew from the larval to the juvenile stage. This indicates that while ECaC could play important role in Cd uptake through skin in larval stage, its role becomes less prominent during the juvenile stage and is superseded by the uptake of Cd through the transporters present in gill epithelium.

In a recent study with white sturgeon, oxidative damage to cellular lipids, measured as LPO, was shown to contribute to the life-stage specific sensitivity differences to Cd (Tang et al., 2016). The present study partially confirmed these previous findings. Early juvenile white sturgeon (45 dph), which have been suggested to be the most sensitive life-stage of this species, demonstrated a significantly higher Cd-induced LPO level compared to that at the swim-up stage (Figure 5.3). This observation was in agreement with the previous observation (Tang et al., 2016). However, in the present study, the larval stage (5 dph) of white sturgeon demonstrated no significant increase in LPO levels, despite this life-stage having comparable sensitivity to Cd as the 45 dph life-stage. Similarly, in rainbow trout, no life-stage specific relationship between LPO levels and acute Cd sensitivity was found. For example, the swim-up life-stage (15 dph) in trout, which was several times more sensitive to Cd than the larval life-stage (5 dph) (Shekh et al., 2018), exhibited no significant increase in LPO levels compared to that in the larval life-stage during acute Cd exposure (Figure 5.3). These observations suggest that LPO might not always be a good

indicator of life-stage specific differences in metal sensitivity in fish as previously suggested. On the other hand, a direct comparison of LPO levels between the two species clearly demonstrated that the more sensitive species, rainbow trout, generally produced greater LPO levels compared to white sturgeon during acute Cd exposure (Figure 5.3). No other previous studies compared the species-specific differences in Cd induced LPO production in fish. However, reduced ascorbate, a cellular antioxidant molecule, has been shown to be present at different baseline levels in three different fish species: rainbow trout, common carp, and gibel carp. It has also been suggested that differences in baseline ascorbate levels are indicative of the differences in the sensitivity of these species to Cu (Eyckmans et al., 2011). Direct oxidative damage observed in our study and the response of antioxidant molecules in previous studies suggest possible links between oxidative stress pathways and species-specific differences in the sensitivity of fish to metals such as Cd and Cu.

In the present study, the major antioxidant genes, SOD, CAT and GST, generally demonstrated significantly greater expressions in white sturgeon relative to rainbow trout after exposure to Cd. This suggests that these enzymes could have provided a greater level of protection to white sturgeon against Cd induced oxidative stress. This might also explain the lower LPO levels recorded in white sturgeon in our study. For example, at the juvenile life-stage, GST demonstrated a significantly higher upregulation in white sturgeon relative to that in rainbow trout (Table 5.1). Similarly, SOD in larval and CAT in larval and swim-up life-stages showed greater upregulation in sturgeon than in trout.

The response in the induction of MT mRNA following Cd exposure was different among life-stages and also between the two species. Between species, MT induction in the Cd exposure group relative to the control group was significantly greater in rainbow trout than in white sturgeon for all comparable life-stages except larvae (4.3- to 4.9-fold, and 2.0- to 2.2-fold in trout and sturgeon, respectively) (Figure 5.4). These results are consistent with previous observations that demonstrated that sturgeon elicit lesser maximal MT gene response compared to salmonids (Doering et al., 2015). In our study, all qPCR reactions were run on the cDNA product of equal initial RNA concentrations, but it still resulted in different C_t values (cycle threshold) in the control groups of both species (average C_t values of 21.7±0.3 and 24.3±0.7 in trout and sturgeon, respectively (results not shown)). The difference of approximately three cycles between trout and sturgeon suggests that the basal (control) expression of MT in trout could be several times higher,

which further implies that a 4.3- to 4.9-fold increase in MT gene expression in trout would result in significantly higher number of MT gene copies as compared to sturgeon (2.0- to 2.2-fold increase). Therefore, it is expected that MT in rainbow trout will chelate more atoms of free Cd than in white sturgeon. In the Cd accumulation experiment, we observed that white sturgeon accumulated significantly higher amount of Cd in the whole body (Figure 5.1). This observation, combined with the previous finding that sturgeon is less sensitive to Cd than trout, led us to hypothesize that the capacity of MT induction could be greater in white sturgeon than rainbow trout, which in turn would provide sturgeon greater protection against the Cd body burden. Thus, Cd-induced MT gene induction results between the two species were surprising because the MT gene expression was higher in rainbow trout rather than in white sturgeon. This implies that the greater tolerance of white sturgeon to Cd toxicity cannot be explained on the basis of MT gene induction. Although whole-body Cd accumulation and MT gene upregulation did not explain the species-specific differences in Cd sensitivity, the combined results of Cd accumulation and MT gene induction observed in this study have wider implications. Higher Cd accumulation in conjunction with a lack of MT induction in less sensitive (more tolerant) species warrants further investigation into the subcellular distribution of Cd. It is possible that the species-specific difference in Cd sensitivity is mediated by the differences in Cd binding to the sensitive fractions of cellular organelles among different fish species.

For rainbow trout, more sensitive life-stages demonstrated a greater upregulation of the MT gene relative to the less sensitive life-stage (larval), which again contradicts the widely reported protective effect of MT against metal toxicity. Based on our observations, it appears that MT gene induction alone can not explain the life-stage specific differences in the sensitivity to Cd in rainbow trout. Similarly, MT gene induction can not explain the life-stage specific differences in the accumulation (Figure 5.1) and sensitivity of white sturgeon to Cd (Shekh et al., 2018). For example, despite the large difference in the accumulation and sensitivity to Cd during swim-up and early juvenile life-stages, the degree of MT upregulation in both of these life stages was comparable (Figure 5.4). A previous study with white sturgeon reported that the early life-stage of white sturgeon (up to 48 dph) had lesser capacity to induce MT gene relative to the later life stage sturgeon (139 dph) (Tang et al., 2016). This suggested the potential role of MT gene induction in providing higher tolerance to Cd in older white sturgeon compared to very early life-stage sturgeon

(Tang et al., 2016). However, 139 dph life-stage of white sturgeon was not examined in the present study.

Although our results did not reveal any apparent relationship between HSP70 mRNA expression and life-stage specific differences in the sensitivity to Cd, species-specific differences in the sensitivity were explained well by HSP70 gene expression. For example, species-specific comparison revealed that the more sensitive species, rainbow trout, upregulated HSP70 gene to a greater degree than the less sensitive species, white sturgeon, in all comparable life-stages (Figure 5.4 and Table 5.1). Exposure to Cd is known to induce denaturation of proteins in cells (Jungmann et al., 1993; Waisberg et al., 2003). These denatured proteins act as a signal for the activation of HSP genes such as HSP70 (Ananthan et al., 1986). The major function of HSP proteins is to bind to the denatured proteins in the cell and to aid in their refolding to the native state (Palleros et al., 1991). Higher HSP70 upregulation in rainbow trout could be an indicator of unregulated and persistent Cd induced protein misfolding compared to white sturgeon, where either the effect of Cd was less potent, or Cd was not available to the sensitive protein fractions within the cells. Hence, based on our observations in the current study, HSP70 gene expression could be an indicator of species-specific differences in the sensitivity to Cd in fish. However, further research with other metals and fish species differing in metal sensitivity is required to confirm or refute the usefulness of HSP70 or other members of HSP family as markers of species-specific differences in sensitivity to metals.

5.6 Conclusions

In a recent analogous study, we demonstrated that the differences in Cd uptake parameters and Cd induced disruption of Ca uptake and whole-body Ca balance could be important factors in determining species and/or life-stage specific differences in the acute sensitivity to Cd (Shekh et al., 2018). In the present study, the concentrations that were chosen for exposure were 50% of the respective LC_{50} values, which were 6.2, 1.1, and 0.97 µg/L for larval, swim-up and juvenile rainbow trout, respectively and 8.33, 37.64, and 6.68 µg/L for larval, swim-up and juvenile white sturgeon. In other words, the exposure concentrations were lower in rainbow trout, yet the oxidative damage (LPO) and HSP70 gene expression responses were in general higher in rainbow trout as compared to white sturgeon. Hence, based on the observations from this study, it appears that species-specific differences in acute Cd sensitivity could be explained, at least in part, by

species-specific differences in HSP70 gene response and oxidative damage induced by Cd exposure. Based on observations in current chapter and Chapter 2, it is apparent that species- and life-stage specific differences in the sensitivity to Cd and possibly other metals in fish are governed by multiple toxicokinetic and toxicodynamic factors.

6 CHAPTER 6: ROLE OF COPPER INDUCED OXIDATIVE STRESS AND METALLOTHIONEIN RESPONSE IN SPECIES AND LIFE-STAGE SPECIFIC DIFFERENCES IN THE SENSITIVITY OF WHITE STURGEON AND RAINBOW TROUT TO COPPER

PREFACE

Chapter 3 showed that ion physiology parameters such as Na uptake and whole-body Na levels explain higher sensitivity of white sturgeon to Cu as compared to rainbow trout. However, oxidative stress and metallothionein induction are well documented responses of Cu exposure and their involvement in the species-specific differences in the sensitivity to Cu cannot be ruled out. Therefore, the aim of chapter 6 was to compare the differences in Cu induced oxidative stress, antioxidant mechanisms, and metallothionein response between rainbow trout and white sturgeon across multiple early life-stages. Species-specific differences in these parameters were evaluated because these parameters are known to be important pathways through which Cu causes toxicity. Hence, quantitative differences in Cu induced oxidative stress, antioxidant mechanisms, and metallothionein response could be important contributing factors in life-stage and species-specific differences in the sensitivity to Cu.

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Author contributions:

Kamran Shekh (University of Saskatchewan) designed and conducted the experiment, analysed and visualised the data, and drafted the manuscript.

Alper James Alcaraz (University of Saskatchewan) helped with the experiment and commented on the manuscript.

Drs. Markus Hecker and Som Niyogi (both University of Saskatchewan) provided inspiration, guidance, and scientific input, commented on the manuscript, and provided funding for the research.

6.1 Abstract

Recent studies have shown that early life-stages of the endangered white sturgeon (*Acipenser transmontanus*) are possibly one of the most sensitive fishes to aqueous copper (Cu) exposure. Cu is a physiologically essential element; however, it is highly toxic to aquatic organisms at elevated concentrations. Oxidative stress is considered to be an important mechanism of Cu toxicity. However, little is known about the specific mechanism by which oxidative stress drives the sensitivity of species to metals including Cu. In the present study, rainbow trout (*Oncorhynchus mykiss*) and white sturgeon from three distinct early-life-stages were exposed to waterborne Cu (at 50% of respective 96h LC_{50}s) for 96 hours. Following exposure to Cu, major enzymatic and non-enzymatic antioxidant parameters, lipid peroxidation and metallothionein (MT) gene expression were evaluated. Our results indicated that in larval and swim-up life-stages, exposure to Cu caused much greater oxidative damage in white sturgeon than in rainbow trout. Moreover, baseline glutathione (GSH) was significantly greater in rainbow trout than white sturgeon. Our observations also suggested that rainbow trout exceedingly relied on GSH to combat Cu-induced oxidative stress as they grew older. In contrast, white sturgeon appeared to recruit an increasing level of MT expression as the primary mechanism to neutralize Cu-induced oxidative stress and/or Cu loading. In a recent complimentary study, we showed that in white sturgeon, Na uptake and whole-body Na levels are significantly more vulnerable than that in rainbow trout, which explained the higher sensitivity of sturgeon to Cu. Overall, our findings indicate that oxidative damage and Na homeostasis, together explain the higher sensitivity of white sturgeon to Cu. The higher sensitivities of larval and swim-up life-stages in white sturgeon were explained by susceptibility of Na processes along with Cu-induced oxidative damage, whereas, higher sensitivity of juvenile life-stage was only explained by susceptibility of Na processes.

6.2 Introduction

Copper (Cu) is extracted in very high quantities across the world due to its significant economic importance (Patterson et al., 1998). Although physiologically essential, Cu is also considered to be a potent toxicant at elevated concentrations (Wang et al., 2011). Due to its continuous release from anthropogenic sources such as mining and smelting activities and its high toxicity, Cu is a frequent cause of impaired water quality in many regions (Reiley, 2007). Cu is an essential element mainly because its redox potential is utilized by a number of enzymes (Solomon and Lowery, 1993). However, the redox properties of Cu, along with its capacity to inhibit antioxidant enzymes, can also lead to the accumulation of reactive oxygen species (ROS) and ultimately result in oxidative stress (Grosell, 2012). Hence, oxidative stress is considered an important mode of toxic action for Cu in fish, along with other potentially important toxic mechanisms such the disruption of sodium (Na) homeostasis, ammonia excretion and acid-base balance, and olfactory impairment (Grosell, 2012).

It has been demonstrated in several studies that exposure to Cu induces oxidative stress response in fish during short as well as long term exposures (Paris-Palacios et al., 2000; Pedrajas et al., 1995; Sanchez et al., 2005). Cu is known to enhance the formation of ROS through Fenton reaction (Grosell, 2012). ROS is also formed naturally in cells as a by-product of natural metabolic processes. However, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and non-enzymatic antioxidant compounds such as glutathione (GSH) and ascorbate (ASC) act as defence mechanisms against oxidative injuries (Birben et al., 2012). SOD, GPX and CAT protect against the oxidative stress by removing superoxide anions and hydroperoxides, respectively, from cells. In addition, GPX prevents oxidative damage by utilizing GSH and in the process, converts GSH to oxidized glutathione (GSSG) (Birben et al., 2012; Hansen et al., 2006).

Similar to other contaminants, water quality guidelines that regulate the release or presence of metals in surface waters are mainly governed by toxicity data from model data-rich species (CCME, 2014; USEPA, 2007). However, there is significant uncertainty with regard to the status of protection of these criteria for many non-model species due to the lack of information on their sensitivity to metals including Cu. White sturgeon (*Acipenser transmontanus*) is one such North American native non-model fish species. Many populations of white sturgeon are declining in the

north-western parts of the USA and this species have also been listed as endangered in Canada (COSEWIC, 2012; Vardy et al., 2013). Recent studies have shown that early life-stages of white sturgeon are one of the most sensitive organisms to aqueous copper Cu exposure (Calfee et al., 2014; Vardy et al., 2013).

Significant differences in the sensitivity to metals including Cu have been shown for both apical and physiological endpoints across other fish species (De Boeck et al., 2004; Eyckmans et al., 2010; Grosell et al., 2002; Nemcsók and Boross, 1982; Shuhaimi-Othman et al., 2015; Song et al., 2015). Physiological parameters such as Na turnover and baseline Na uptake rate are correlated with the species-specific differences in the sensitivity to metals like Cu and Ag (Bianchini et al., 2002; Grosell et al., 2002). Biochemical mechanisms such as the capacity to mobilize antioxidative response as well as to induce metallothionein (MT) production can strongly influence the detoxification and toxicity of Cu in organisms. However, the relative contribution of these biomarker responses in species-specific differences in the sensitivity to Cu has only been studied sporadically. Some recent studies indicated that differences in metal-induced oxidative stress and antioxidant response could at least partially explain life-stage and species-specific differences in the sensitivity to metals such as Cu and Cd (Eyckmans et al., 2011; Tang et al., 2016). Cu-induced oxidative stress response has been studied extensively in many species; however, direct comparison among these species from different studies is challenging because toxic effects of metals are not only dependant on exposure concentrations but also on water chemistry (Niyogi and Wood, 2004b). Moreover, a comparative evaluation of oxidative stress between rainbow trout and white sturgeon has not been performed before. We have recently showed that differences in the susceptibility of Na homeostasis between white sturgeon and rainbow trout can explain the higher sensitivity of white sturgeon to Cu (Shekh et al., 2019); however, involvement of additional mechanisms such as oxidative stress and detoxification cannot be ruled out because of their significant contribution in the toxicity outcome of metals. Hence, a comparative examination of metal-induced oxidative stress response and MT induction in different species under an identical water chemistry condition is required to fully understand the relative contribution of these biochemical parameters to species-specific differences in Cu sensitivity.

The major objectives of the current study were two-fold: (i) to investigate the species and life-stage specific differences observed in oxidative stress and detoxification response during acute

aqueous exposure to Cu in two evolutionary distinct fish species, rainbow trout and white sturgeon, and (ii) to determine if differences in oxidative stress and detoxification response can explain the differences in Cu sensitivity across different life stages between these two species. To address these objectives, fish were exposed to waterborne Cu for 96 hours and subsequently the activities of antioxidant enzymes (SOD, CAT and GPX), the concentrations of GSH and ASC, and the mRNA expression of MT were evaluated. In addition, the GSH/GSSG ratio and Lipid Hydroperoxide (LPO) content were also determined to assess the relative differences in Cu-induced oxidative damage between the two species.

6.3 Materials and Methods

6.3.1 Experimental Organisms and Test Chemicals

Embryos of white sturgeon were obtained from the Nechako White Sturgeon Conservation Centre (BC, Canada) and embryos of rainbow trout were obtained from Troutlodge (WA, USA). All embryos were reared in the Aquatic Toxicology Research Facility at the University of Saskatchewan in a flow-through system maintained at approximately 13 °C.

Commercial frozen bloodworms (San Francisco Bay Brand, Newark, CA, USA) were fed to sturgeon starting at 8 days post hatch (dph). Similarly, rainbow trout were fed commercial trout food from 8 dph onwards (Bio Vita Starter #0 Crumble, Bio-Oregon, British Columbia, Canada). Proper permits were obtained before research commenced (Fisheries and Oceans Canada SARA Permit XRSF 20 2013/SARA 305), and all procedures involving animals were approved by the University of Saskatchewan's University Council on Animal Care and Supply (Protocol 20140079). Copper (II) sulfate pentahydrate (CAS# 7758-99-8; purity, 99.995%) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

6.3.2 Exposure Protocol

All exposures were performed in water prepared by mixing dechlorinated city of Saskatoon municipal tap water with reverse osmosis tap water in the ratio of 1:3, which resulted in a hardness and alkalinity of ~65 and 40 mg/L as $CaCO_3$, respectively. Stock solutions of Cu were also prepared in this water and stored at 13 °C for at least 24 hours before exposure.

Three life-stages of each species: larval (7 dph), swim-up (30 dph), and juvenile (70 dph) were utilized for this study. Both species of fish were exposed to Cu concentrations that

represented 50% of the 96h LC_{50} values for the respective life-stages, as determined previously (Shekh et al., 2019). Specifically, larval, swim-up and juvenile life-stages were exposed to 30.3, 37.2, and 41.5 µg/L, respectively, for rainbow trout, and 18.2, 12.1, and 17.8 µg/L of Cu, respectively, for white sturgeon. Exposures were conducted in 5 L HDPE buckets and 6 replicate buckets per exposure group were used (n = 6), each containing 8 fish. Half of the exposure media was replaced with exposure water from the same stock every 24 hours. At the end of the exposure period, fish were euthanized, and samples were collected separately for each of the parameters described below. Samples were flash frozen in liquid nitrogen followed by storage at −80 ◦C until further analysis. Because of the very small size of the larval and swim-up life-stage fish, it was not possible to isolate their gills, so the whole head was used as a proxy for the gills. In order to maintain consistency across different life stages, the whole head was chosen as a substitute of isolated gills for the juvenile life-stage as well.

6.3.3 Water Chemistry

Water chemistry parameters (Ca, Mg, K, Na, Cl, SO_4 and dissolved organic carbon (DOC)) were analyzed in the water samples collected at the beginning and end of the exposure period by the analytical laboratory at the Saskatchewan Research Council, Saskatoon, SK, Canada (see Table C2.S1). Parameters such as hardness, alkalinity, dissolved oxygen, pH, and temperature were measured daily as described elsewhere (Shekh et al., 2018). Dissolved Cu concentrations were measured in water samples collected at the beginning and the end of exposure. These water samples were randomly taken from two of the replicates in each exposure group, filtered through a 0.45 µm polycarbonate filter, acidified (0.2 %) with trace metal grade nitric acid, and dissolved Cu concentrations were measured by graphite furnace atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer, USA). The quality control and quality assurance of the Cu measurement was maintained by using a certified Cu standard (Perkin Elmer, USA) and a certified reference material (SLRS-6; National Research Council, Canada). The recovery rate of Cu in the reference material was 96%.

6.3.4 Measurement of Antioxidant Enzyme Activities

The activities of SOD, CAT and GPX were measured using commercially available kits (Cayman Chemical Company Inc, MI, USA), following the instructions provided by the manufacturer. The frozen samples were thawed and immediately homogenized on ice using a

hand-held homogenizer. The homogenates were centrifuged at 25,000 ×g for 20 min at 4 °C and the supernatants were used for measuring the activities of all enzymes. The enzyme activities in different tissues were normalized against the respective protein concentration. The protein concentrations in tissues were measured using Bradford reagent, as described previously (Bradford, 1976). The units of measurement were nmol/min/mg protein for CAT and GPX. For SOD, units of measurement were U/mg protein, where 1 U is defined as amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

6.3.5 Measurement of ASC

Tissue content of ASC was measured following a protocol described previously (Zannoni, 1974). All tissues were homogenized in 5% Trichloroacetic acid (CAS# 76-03-9, Sigma-Aldrich, MO, USA) followed by centrifugation at 15,000 g at 4°C for 15 minutes. The supernatants were collected and stored at -80 until analysis. The final reaction mixture was prepared in a 96 well plate, where each well contained 150 µL supernatant, 10 µL ortho-phosphoric acid (CAS# 7664-38-2, Thermo Scientific, ON, Canada), 80 µL 2,2'-Bipyridyl (CAS# 366-18-7, VWR, ON, Canada), and 10 µL ferric chloride (CAS# 7705-08-0, VWR, ON, Canada). A standard curve was prepared using high purity commercially available ascorbic acid (Alfa Aesar, MA, USA) and a reagent blank was also included in the plate. The plate was incubated for 15 minutes at room temperature and absorbance was measured in a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Finland) at 525 nm wavelength. ASC content (µg) in different tissues were normalized against the respective tissue weights.

6.3.6 Measurement of GSH and GSH/GSSG Ratio

The concentration of GSH and GSSG in tissue samples was measured using the method previously described (Jamwal et al., 2016). The method is based on fluorometric detection. Fresh tissue samples were homogenized in phosphate−EDTA buffer (0.1 M sodium phosphate−0.005 M EDTA, pH 8.0). The homogenate was centrifuged at 10,000g for 10 minutes at 4°C and the supernatant was divided into two fractions (200 µL for GSSG and the rest for GSH). In the GSSG fraction, 80 µL of 0.04 M of N-ethylmaleimide was added. Both fractions were then stored at -80°C until further analysis. For measuring GSH content, the final reaction mixture contained 180 µL of phosphate−EDTA buffer (0.1 M sodium phosphate−0.005 M EDTA, pH 8.0), 10 µL of O-phthalaldehyde (OPT, 100 µg per 100 µL of methanol), and 10 µL of sample. Similarly, the final

reaction mixture for GSSG measurement contained 140 µL of 0.1 N NaOH, 20 µL of OPT, and 40 µL of sample. The plates were incubated for 15 minutes at room temperature and the fluorescence intensity was measured in a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Finland) at excitation and emission wavelengths of 350 and 450 nm, respectively. The GSH and GSSG contents were normalized against the respective protein concentrations in each tissue sample.

6.3.7 Measurement of Lipid Hydroperoxide (LPO)

The measurement of LPO in tissue samples was performed using the Lipid Hydroperoxide Assay kit (ab133085, Abcam Inc, ON, Canada), following the manufacturer's protocol. The method was based on the detection of LPO at 500 nm absorption wavelength in a 96-well plate microplate reader (Verioskan Flash, Thermo Scientific, ON, Canada) at the ambient temperature. LPO contents (nanomole) were normalized on the basis of per mg protein concentration in respective tissue.

6.3.8 Reverse Transcription Quantitative Real-time Polymerase Chain Reaction (qPCR) for MT Gene

Total RNA was extracted by using the RNeasy Lipid Tissue Kit (Qiagen, Mississauga, ON, Canada). RNA concentration and purity were confirmed by a NanoDrop ND-1000 Micro-Volume UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA samples were used for synthesising cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada). A 50 µL reaction mixture was prepared with 2× concentrated QuantiFast SYBR green master mix, 2.5 µL of cDNA, 10 pmol gene-specific primers, and nuclease free water. Reaction mixtures were prepared in 96-well plates and qPCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each cDNA sample was analyzed in duplicate with 20 µL reaction volumes per well. The following method was used for qPCR: denaturation at 95 °C for 10 min, followed by a thermal cycle profile consisting of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. After the 40 cycles of qPCR was completed, a melting curve analysis was also conducted to verify the specificity of amplicons. The primer sequences for MT in rainbow trout and white sturgeon were obtained from literature (Kwong et al., 2011; Tang et al., 2016). Multiple forms of MT are known

only in rainbow trout (MTa and MTb) but not in sturgeon and are included in Figure 6.3B accordingly.

6.3.9 Statistical Analyses

The data for LPO and ASC content, the activity of antioxidant enzymes, MT gene expression, and GSH/GSSG ratio were analyzed by three-way ANOVA followed by further analysis of all ANOVA interaction terms and post-hoc testing with t-tests. The three independent variables in each ANOVA model were species, life-stages and exposure concentrations. After initially analyzing the data by three-way ANOVA, data from individual life-stages were isolated from both species and analyzed with two-way ANOVA, with species and exposure concentrations as independent variables. If the application of two-way ANOVA in a given life-stage revealed statistically significant two-way interactions between species and exposure concentration, it indicated the presence of species-specific difference in the effect of Cu exposure. In such cases, multiple comparison among groups were performed by using Tukey's test and these multiple comparisons allowed us to assess differences among all possible sets of groups, for example: control *versus* control across species or treatment *versus* treatment across species or control *versus* treatment within species for both species. The purpose of multiple comparison approach was to explain the origins of interactions determined by two-way ANOVA and to confirm the presence or absence of the exposure induced species-specific difference in a given life-stage. Baseline GSH level was analyzed using two-way ANOVA, with species and life-stage as independent variables. Normality and homogeneity of variance of the dependant variables were determined by Shapiro−Wilk test and Bartlett's test, respectively, and square root transformed if required. A p value of <0.05 was considered as statistically significant.

6.4 Results

6.4.1 Exposure Verification and Water Quality

Measured Cu concentrations were comparable to the nominal concentration used in this study. The average temperature during the exposures was 13.1 ± 0.29 ºC. The average pH and DO were 7.2 ± 0.14 and 94.17 ± 3.57%, respectively. Measured concentrations of anions, cations, and DOC have been provided in the Appendix (Table C2.S1).

6.4.2 Lipid Hydroperoxide (LPO)

Three-way ANOVA revealed significant three-way interactions among species, life-stages, and exposure ($F = 2.74$, $p = 0.034$) as well as significant two-way interactions between species and exposure ($F = 17.76$, $p < 0.001$), and life-stage and exposure ($F = 6.35$, $p < 0.001$). Two-way ANOVA was applied in each life-stage with species and exposure as independent variables. In the larval life-stage, there was a species-specific difference in Cu-induced LPO between rainbow trout and white sturgeon, as indicated by a significant two-way interaction between species and exposure ($F = 7.36$, $p = 0.003$). The Cu-induced LPO level was significantly higher in sturgeon relative to that in trout (4.7- versus 2.1-times, compared to the control), which explains the significant two-way interaction observed in larval life-stage (Figure 6.1). Similarly, during the swim-up life stage, the effect of Cu on LPO was significantly greater in white sturgeon, as indicated by a significant two-way interaction ($F = 11.71$, $p < 0.001$). A 4.8-times increase in LPO was recorded in sturgeon while only a 1.7-times increase was observed in trout. At juvenile life-stage, exposure to Cu caused a significant increase in LPO in both species. For example, exposure to high concentrations (17.8 and 41.6 µg/L) of Cu in sturgeon and trout (50% of respective LC_{50}) caused a significant increase in LPO in both species ($p = 0.008$ and 0.007, respectively and $DF = 30$); however, there was no difference in LPO induction between the two species at any tested exposure concentration (adjusted $p = 0.13$ and 0.75 for 10 and 50% of LC_{50} exposure concentrations, respectively and $DF = 30$).

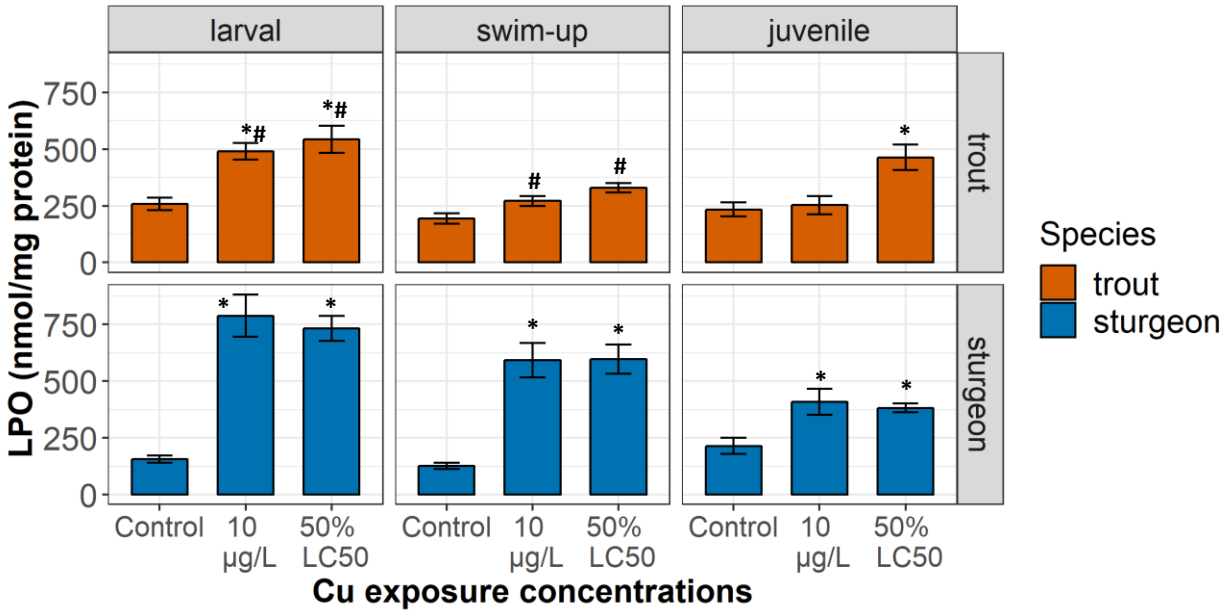

Figure 6. 1. Levels of lipid peroxidation (LPO) across different life-stages of white sturgeon and rainbow trout following 96 h exposure to Cu. Data is presented as mean ± SEM of LPO (nmol/mg protein) (n= 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). A number symbol (#) on rainbow trout bars represents a significant difference between rainbow trout and white sturgeon for the corresponding exposure group ($p < 0.05$).

6.4.3 GSH/GSSG Ratio and GSH

Three-way ANOVA did not reveal significant three-way interactions among species, life-stage and Cu exposure ($F = 0.22$, $p = 0.81$). Also, there was no significant two-way interaction between species and exposure ($F = 3.98$, $p = 0.051$) and species and life-stage ($F = 2.82$, $p = 0.07$), which suggests that there might not be a species-specific difference in the GSH/GSSG ratio as a result of Cu exposure. To confirm this further, we applied two-way ANOVA between the species in each life-stage separately. There was no significant two-way interaction between species and exposure regardless of the life stage ($F = 1.46, 2.27, 0.50$ and $p = 0.24, 0.15, 0.49$ for larval, swim-up and juvenile, respectively); however, the main exposure effect was statistically significant in all life-stages ($p < 0.001$). This observation indicates that although exposure to Cu caused statistically significant reduction in GSH/GSSG ratio in all life-stages, there was no species-specific difference (Figure 6.2).

A two-way ANOVA test for the baseline GSH levels between rainbow trout using species and life-stages as independent variables showed a significant two-way interaction ($F = 5.5$, $p = 0.01$). In all life-stages, the post-hoc tests showed that the baseline GSH levels were significantly greater in rainbow trout relative to the respective life-stages of white sturgeon (adjusted $p < 0.05$, DF = 30) (Figure 6.3A). Moreover, there was a significant increase in the GSH content in rainbow trout with increasing age, as indicated by one-way ANOVA applied among different life-stages within rainbow trout (adjusted $p < 0.001$, DF = 30) (Figure 6.3A). On the other hand, the GSH levels remained unchanged in white sturgeon across different life stages (adjusted $p = 0.99$, DF = 30) (Figure 6.3A).

6.4.4 Ascorbate (ASC)

Three-way ANOVA did not reveal a significant three-way interaction among the parameters: species, life-stage and exposure ($F = 0.06$, $p = 0.95$). Moreover, there were no statistically significant two-way interactions between species and exposure ($F = 0.55$, $p = 0.46$) or life-stage and exposure ($F = 1.28$, $p = 0.29$). These observations suggest that there were no species or life-stage specific differences in the effects of Cu exposure on ASC level. These observations were further confirmed by applying two-way ANOVA separately between species in all life-stages. There were no significant two-way interactions between species and exposure in any of the life-stages tested ($F = 0.67, 0.09$, and 0.31, $p = 0.67, 0.77$, and 0.59 in larval, swim-up and juvenile

stage, respectively), which suggested that exposure to Cu did not cause differential effect on the ASC content between rainbow trout and white sturgeon. Moreover, the main effect of Cu exposure on ASC content was not significant in any of the life-stages in both species. The main effect of the species was significantly different in swim-up and juvenile life- stage, which suggested that the baseline ASC levels between the two species were different in these two life-stages (Figure 6.4); however, the levels were not affected by Cu exposure.

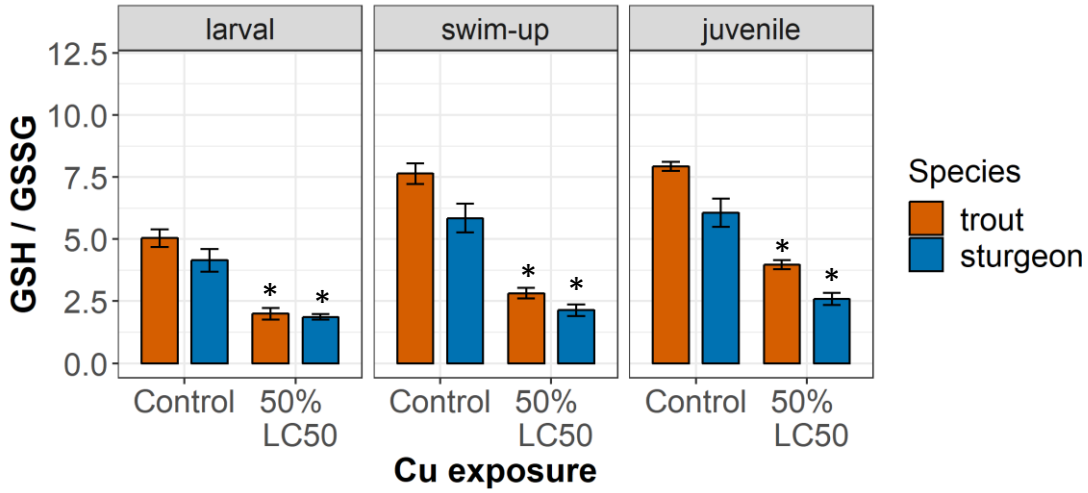

Figure 6. 2. Changes in thiol redox status, expressed as a ratio of glutathione (GSH) to oxidized (GSSG) glutathione, in rainbow trout and white sturgeon across difference life-stages following 96 h exposure to Cu. Data are presented as mean ± SEM of GSH/GSSG ratio (n= 6). Within each life-stage, significant differences compared to controls are indicated by * ($p < 0.05$). No species-specific difference was observed in the response.

A) B)

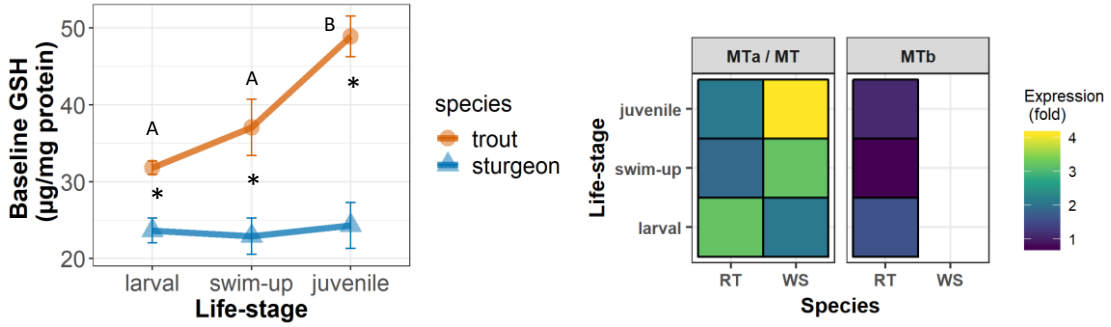

Figure 6. 3. Changes in (A) baseline GSH levels across life-stages, expressed as µg/mg protein and (B) MT upregulation as a result of Cu exposure, in rainbow trout (RT) and white sturgeon (WS) across different life-stages following 96 h exposure to Cu. GSH Data is presented as mean ± SEM of GSH level (µg/mg protein) (n= 6). In figure (A), within each life-stage, significant differences between species are indicated by * ($p < 0.05$). The life-stage specific differences with in rainbow trout are shown by different alphabets. There was no life-stage specific difference in white sturgeon. Statistical significance of MT response is discussed in the results section.

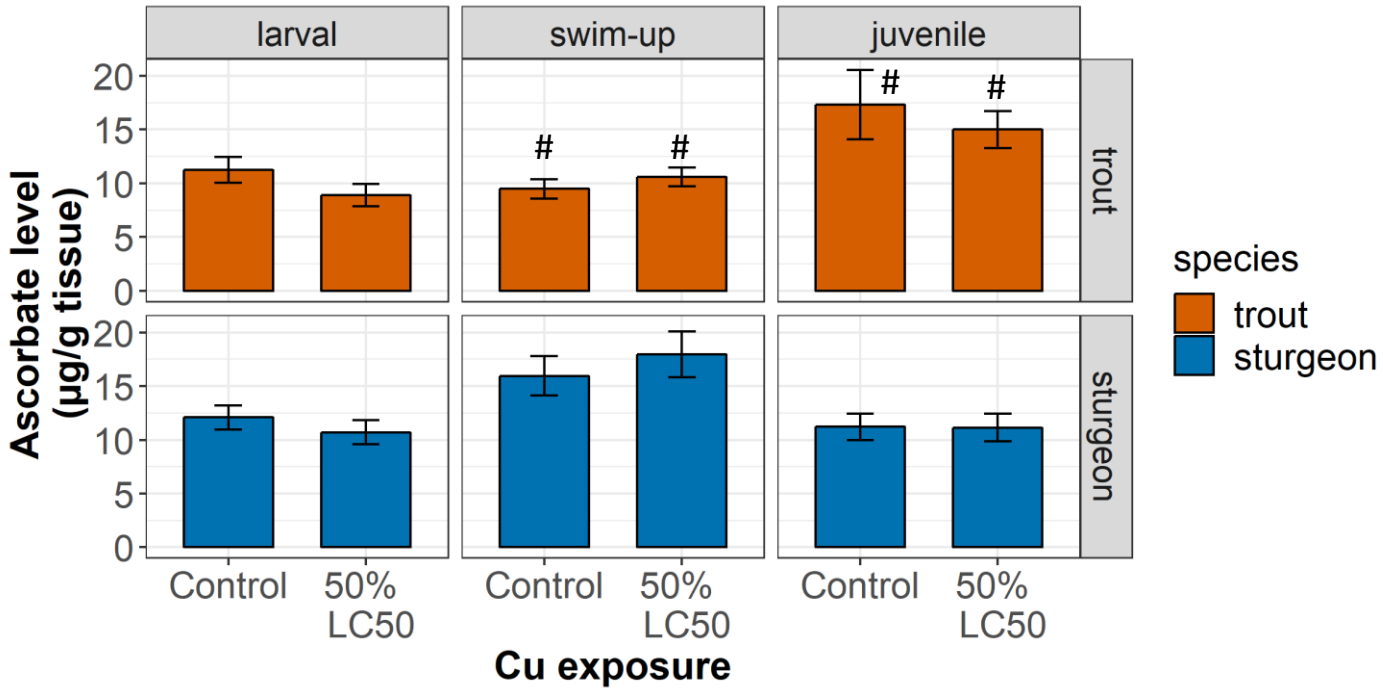

Figure 6. 4. Levels of ascorbate (ASC) across different life-stages of white sturgeon and rainbow trout following 96 h exposure to Cu. Data is presented as mean ± SEM of ASC µg/g tissue (n= 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. A number symbol (#) on rainbow trout bars represents a significant difference between rainbow trout and white sturgeon for the corresponding exposure group ($p < 0.05$). In both species, exposure to Cu did not produce any significant effect on the level of ASC as compared to the respective control.

6.4.5 Metallothionein (MT)

Similar to other parameters discussed above, three-way interaction among species, life-stage and exposure was not statistically significant ($F = 0.79$, $p = 0.46$). However, there were significant two-way interactions between species and exposure ($F = 7.6$, $p = 0.008$) as well as life-stage and exposure ($F = 4.02$, $p = 0.02$). Two way ANOVA was applied between the species within each life-stage, which showed that during the larval life-stage, Cu exposure caused a significant upregulation in MT gene in both rainbow trout (3.2-fold, adjusted $p < 0.001$, DF = 16) and white sturgeon (2.1-fold, adjusted $p = 0.002$, DF = 16) (Figure 6.3B); however, the upregulation of the MT gene was not significantly different between the two species (two-way interaction $p = 0.43$, $F = 4.86$). In swim-up life-stage, Cu exposure caused a significant upregulation in both species (Figure 6.3B); moreover, there was also a species-specific difference in the response between the two species (significant two-way interaction, $p = 0.02$, $F = 6.39$), with a 3.24-fold upregulation in white sturgeon (adjusted $p < 0.001$, DF = 16), but only a 1.85-fold increase in rainbow trout (adjusted $p = 0.01$, DF = 16). Similar to swim-up life-stage, juveniles also showed a significant species-specific difference in the effect of Cu on MT gene expression (two-way interaction, $p = 0.04$, $F = 5.11$), with a 4.14-fold upregulation in sturgeon (adjusted $p < 0.001$, DF = 16) but only a 2.05-fold upregulation in trout (adjusted $p = 0.02$) (Figure 6.3B). Overall, MT response in white sturgeon was significantly higher than rainbow trout at the swim-up and juvenile life-stages, but not at the larval life-stage (Figure 6.3B). It is notable that in rainbow trout, responses in MT gene expression as a result of Cu exposure decreased progressively (from 3.22- to 1.85-fold) with the increase in age, whereas the MT response in white sturgeon increased from 2.08-fold to 4.14-fold from larval to juvenile life stages. Exposure to Cu did not cause any statistically significant upregulation in the MTb gene in rainbow trout (Figure 6.3B).

6.4.6 Antioxidant Enzymes

In both white sturgeon and rainbow trout, exposure to Cu caused a significant increase in the activity of the SOD enzyme at the swim-up life-stage (adjusted $p = 0.01$ and <0.001, respectively, DF = 20), but not at the larval and juvenile life-stages (Figure 6.5). Moreover, exposure to Cu did not induce species-specific differences in the response at the larval and juvenile life-stage, because there were no significant interactions between species and exposure at these life-stages ($F = 0.09$ and 0.16 and $p = 0.76$ and 0.69, respectively). Although, in the swim-up life-

stage, the levels of SOD induction were comparable in both species (2.3- and 2.6-times increase), the two-way interaction was statistically significant ($F = 10.18$, $p = 0.005$), indicating that the effect of Cu was species-specific (Figure 6.5).

Larval and juvenile life-stages demonstrated a significant species-specific difference in the effect of Cu on CAT activity, as indicated by the significant two-way interactions between species and exposure ($F = 9.13$ and 17.99, $p = 0.007$ and <0.001 in larvae and juvenile, respectively) (Figure 6.6). There was no species-specific difference in CAT response at swim-up life-stage as indicated by a non-significant two- way interaction ($F = 0.42$, $p = 0.42$). Exposure to Cu increased the activity of CAT in all life-stages of white sturgeon (adjusted $p = 0.005$, 0.001, 0.01 in larval, swim-up and juvenile fish, respectively), whereas in rainbow trout, the increase in activity was only observed at swim-up and juvenile life-stages (adjusted $p = 0.02$ and < 0.001, respectively).

Baseline GPX activity in both species was very low in larval, swim-up and juvenile life-stages (42 – 57 nmol/min/mL) and exposure to Cu did not cause any significant change in the activity in any of the life-stages (data not shown). According to the manufacturer's protocol of the kit used for the analysis, such low activities of GPX cannot be reliably measured.

6.5 Discussion

The present study showed that the greater sensitivity to Cu of larval and swim-up life-stages of white sturgeon can be explained, at least partly, by the greater oxidative damage caused by Cu in this species when compared to rainbow trout. However, during the juvenile life-stage, Cu-induced oxidative damage did not show any difference between these two species (Figure 6.1) despite the fact that white sturgeon showed significantly greater mortality than rainbow trout to Cu during this life-stage (Shekh et al., 2019). The data also showed that in both species, although oxidative damage was significant at all life-stages, the effect level was markedly reduced during the juvenile life-stage (Figure 6.1). Another recent study similarly demonstrated that the larval life-stage of white sturgeon was more sensitive to LPO induction when exposed to Cu while no significant increase occurred in juvenile fish (Tang et al., 2016). These patterns in LPO response suggest that both species are not able to efficiently mount a robust antioxidant response until they reach the juvenile life-stage. Although evidence for life-stage specific changes in antioxidant mechanisms is mostly lacking, a recent review suggested that newly hatched fish larvae only demonstrate significant increase in the activity of endogenous antioxidant defense mechanisms

after several weeks post hatching (Birnie-Gauvin et al., 2017). For example, when Siberian sturgeon were fed oxidized lipid, there was no increase in the SOD activity in 7 dph and 20 dph fish; however, there was a significant increase in 27 dph fish (Fontagné et al., 2006). Another recent study showed that the antioxidant capacity (GSH level) in sockeye salmon increased significantly from pre-fertilized eggs to emergent fry (Taylor et al., 2015).

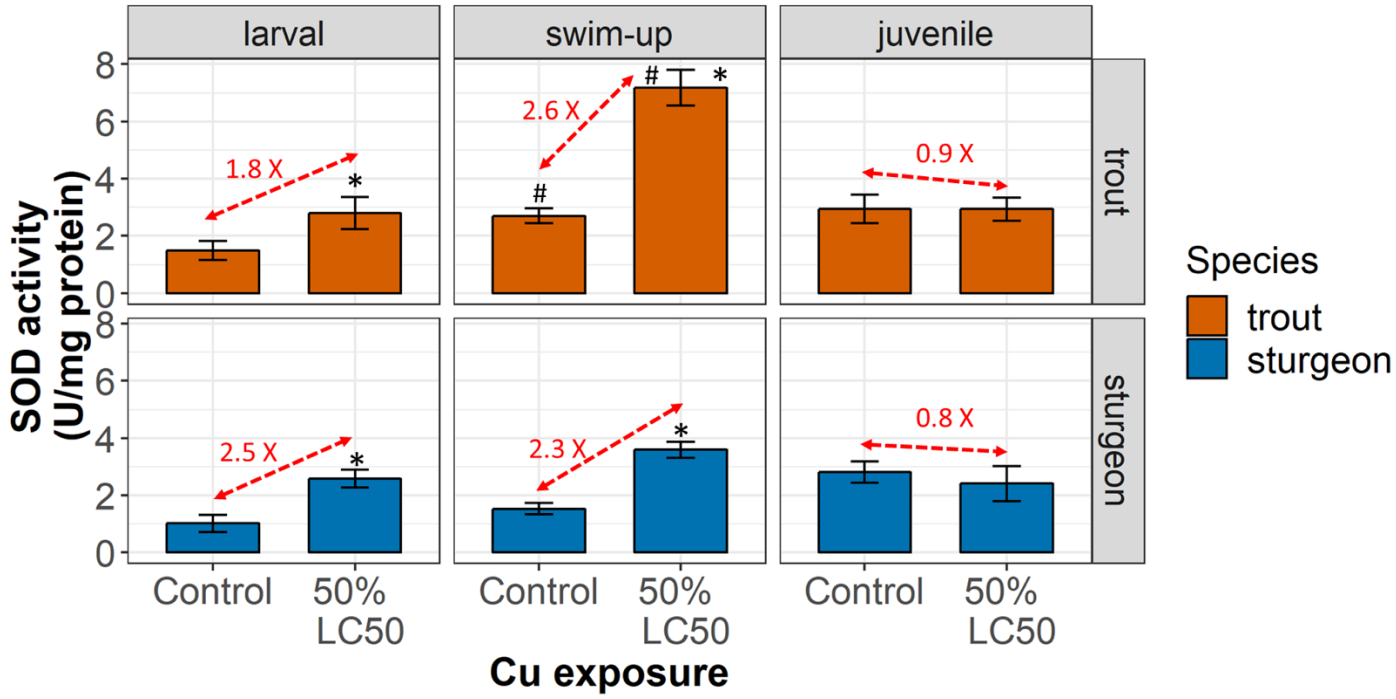

Figure 6. 5. Levels of superoxide dismutase (SOD) activity across different life-stages of white sturgeon and rainbow trout following 96 h exposure to Cu. Data are presented as mean ± SEM of SOD activity (U/mg protein) (n= 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). A number symbol (#) on rainbow trout bars represents a significant difference between species for the corresponding exposure group ($p < 0.05$).

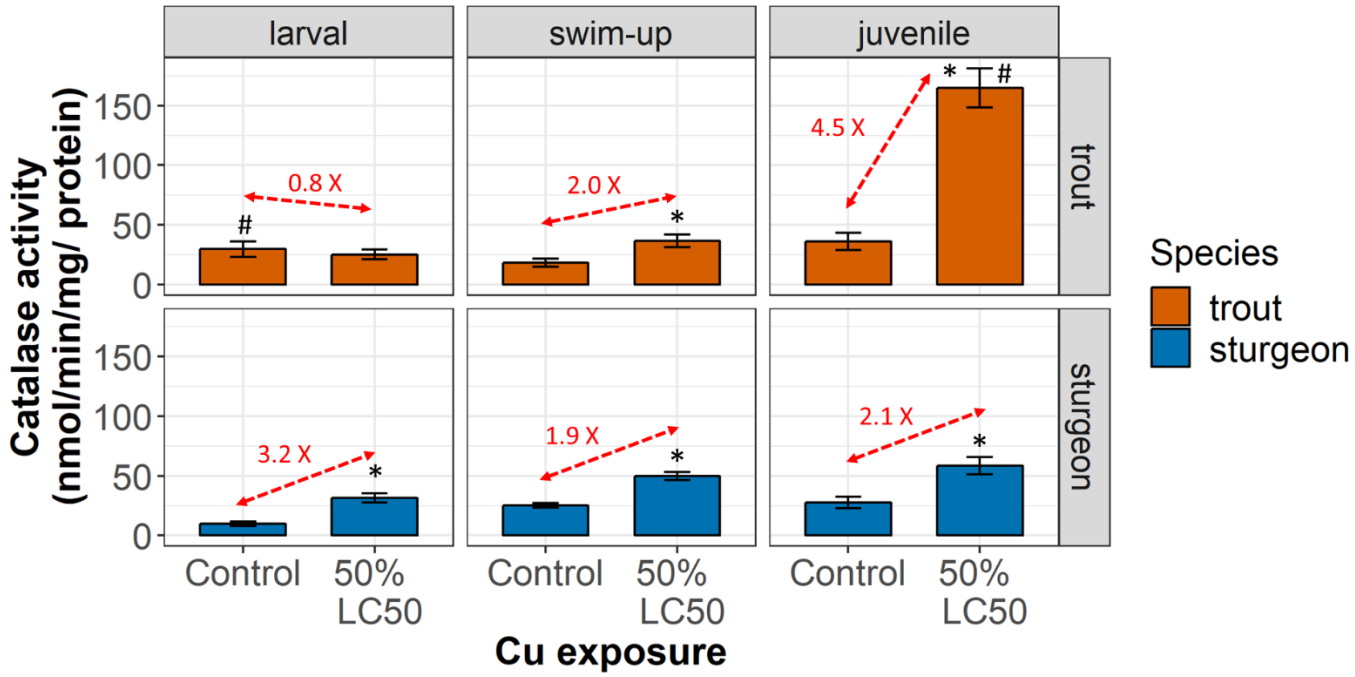

Figure 6. 6. Levels of catalase (CAT) activity across different life-stages of white sturgeon and rainbow trout following 96 h exposure to Cu. Data are presented as mean ± SEM of CAT activity (nmol/min/mg protein) (n= 6). Data were analysed using three-way ANOVA followed by analysis of two-way interactions between species and exposure in all life-stages. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$). A number symbol (#) on rainbow trout bars represents a significant difference between species for the corresponding exposure group ($p < 0.05$).

GSH is one of the most prevalent antioxidant cellular thiols. In oxidative environments, GSH is converted to its oxidized form (GSSG), thus protecting the cells from oxidative injury (Carlberg and Mannervik, 1985; Mannervik, 1985). The reduced ratio of GSH/GSSG is considered to be a reliable indicator of the redox status of the cellular environment and used as a biomarker of oxidative stress in animals including fish (Massarsky et al., 2017). In all life-stages, both species demonstrated significant reduction in GSH/GSSG ratio, indicating that they were suffering from oxidative stress following exposure to Cu. However, there was no species-specific difference in the GSH/GSSG ratio (no significant two-way interaction) at any life stages. Hence, species-specific differences in oxidative damage (LPO) cannot be explained by the GSH/GSSG ratio. However, baseline (control) levels of GSH were significantly higher in rainbow trout compared to white sturgeon in all life-stages (Figure 6.3A). Higher baseline GSH values in rainbow trout suggest that this species may be better protected against oxidative damage relative to white sturgeon. This is also reflected in the LPO data, where we observed significantly less oxidative damage in rainbow trout, especially in early life-stages (Figure 6.1). Our findings are consistent with a previous comparative study which showed that rainbow trout have a significantly higher baseline GSH content when compared to other species such as common carp and gibel carp (Eyckmans et al., 2011).

Life-stage specific differences in ASC requirements have been suggested previously in fish (Knox et al., 1988). Moreover, baseline ASC levels have also been shown to differ significantly among various fish species (Eyckmans et al., 2011). Our study showed that there were no species-specific differences in the baseline ASC levels in larval fish. However, at the swim-up life-stage, ASC level was significantly higher in white sturgeon compared to rainbow trout, whereas at the juvenile life-stage, ASC level was significantly higher in rainbow trout (Figure 6.4). Nonetheless, exposure to Cu did not cause any significant change in ASC levels in both species, which suggests that ASC levels did not play a significant role in countering the oxidative stress in both species. Previous studies indicated that the relative contribution of various antioxidant molecules may differ among species; with GSH as the molecule of preference for defense in some species whereas ASC is more prevalent in others (Eyckmans et al., 2011; Sinha et al., 2014). Our data indicates that in both rainbow trout and white sturgeon, GSH was used preferentially over ASC as a defense against oxidative stress. Selective preference between GSH and MT for metal detoxification may also differ among species, as shown in previous studies (De Boeck et al., 2003; Eyckmans et al.,

2011). The authors showed that there was a significant correlation between gill Cu accumulation and MT expression in gibel carb but not in rainbow trout and common carp, suggesting that some species rely on MT, whereas others rely more on other metal-chelating compounds such as GSH for metal detoxification. The levels of baseline GSH and induction of MT by Cu provide useful information regarding the potential roles of these molecules in dealing with Cu stress across different life-stages (Figure 6.3A and B). In this study, the response of MT to Cu was negatively related to the baseline GSH concentration as a function of life-stage. As fish developed from the larval to the juvenile life-stages, GSH concentration increased significantly in rainbow trout but remained stable in white sturgeon (Figure 6.3A). Concurrently, the response of MT to Cu decreased from larval trout to swim-up and juvenile trout; whereas, the MT response to Cu increased across the life-stages in white sturgeon (Figure 6.3B). This observation confirms the previous hypothesis that there might be species- and life-stage-specific differences in the relative dependence on GSH and MT against metal exposure.

Oxidative stress has been shown to influence the activities of antioxidant enzymes such as SOD and/or CAT in several fresh- and saltwater fish species (C. Fernandes et al., 2008; Livingstone, 2001; Pandey et al., 2003). The induction of these antioxidant enzymes is considered as an adaptive mechanism against oxidative stress. Hence, we hypothesised that the differences in the activity of these enzymes could explain the species-specific differences in oxidative stress response between white sturgeon and rainbow trout. Both species demonstrated an increase in SOD activity at the larval and swim-up life-stages but not at the juvenile life-stage. This suggested that oxidative stress was prevalent in both species during early life-stages but subsided at later life-stages. This observation is also supported by the LPO data, as both species showed high induction in the LPO at the larval life-stage, but the levels were reduced in later life-stages. Interestingly, there was no notable difference in SOD activity between the two species at any life-stage when exposed to Cu. Thus, SOD could not explain species-specific differences in Cu-induced oxidative damage observed in the present study (Figure 6.5). Similarly, patterns in CAT activities also did not correspond with the species-specific differences in Cu-induced oxidative damage in a consistent manner (Figure 6.6). For example, larval and juvenile life-stages showed a significant species-specific difference in the CAT activity following exposure to Cu (two-way interaction, $p = 0.005$, and <0.001, respectively), however there was no species-specific difference at the swim-up life-stage. Overall, the differences in antioxidant enzyme activities between the two species did

not show a consistent response across life-stages, therefore likely did not contribute to the species-specific difference in oxidative damage caused by Cu exposure. The activity of antioxidant enzymes has been shown to be very dynamic, and therefore can vary considerably at different time points during the exposure to metals (Eyckmans et al., 2011). This is probably the reason why previous studies on antioxidant enzymes have been inconsistent, as both increase and decrease in the activities of these enzymes have been reported in fish following exposure to metals (Sevcikova et al., 2011). It should be noted though that we analyzed antioxidant enzyme activities at a single time point (96 h) in the present study, and the responses of these enzymes at multiple time points over the entire exposure period should be examined in future studies to fully understand the influence of antioxidant enzymes on the species-specific differences in Cu-induced oxidative damage.

6.6 Conclusion

In conclusion, we observed that in early larval and swim-up life-stages, exposure to Cu caused much greater oxidative damage in white sturgeon than in rainbow trout, which is consistent with the greater sensitivity of early life-stages of sturgeon to Cu. Although Cu-induced changes in GSH/GSSG ratios were similar in both species across all life-stages, the baseline GSH was significantly greater in rainbow trout than white sturgeon, which is likely the reason why the degree of oxidative damage in rainbow trout was lower than white sturgeon. Comparison of GSH and MT levels across life-stages indicated that as the growth occurs, trout rely predominantly on GSH, whereas sturgeon appear to recruit an increasing level of MT as the primary mechanism to neutralize oxidative stress and/or Cu loading. These diversions in the physiological response to Cu exposure could govern the species-specific differences in the sensitivity to Cu between rainbow trout and white sturgeon. In a recent analogous study with Cu, we showed that in white sturgeon, Na uptake and whole-body Na levels are significantly more vulnerable than that in rainbow trout, which explained the higher sensitivity of sturgeon to Cu (Shekh et al., 2019). Overall, our findings indicate that oxidative damage and Na homeostasis, together explain the higher sensitivity of white sturgeon to Cu. The differences in sensitivity of larval and swim-up life-stages were explained by the susceptibility of Na processes along with Cu-induced oxidative damage, whereas the sensitivity of juvenile life-stage was only explained by the susceptibility of Na processes.

This study has important implications in understanding the biochemical basis of life-stage and species-specific differences in the sensitivity to Cu and possibly other metals. An enhanced understanding of the physiological, biochemical and molecular underpinnings of the differences in sensitivity to contaminants could be useful in developing screening methods that could readily identify whether a resident species is expected to be more or less sensitive to a contaminant relative to a well characterized model species. Such an approach could be highly effective in prioritizing toxicity testing of metals in native, non-model species of regulatory concern, including endangered species such as white sturgeon.

7 CHAPTER 7: GENERAL DISCUSSION

7.1 Introduction

In the U.S., acute ambient water quality criteria (AWQC) for metals are generally derived from species sensitivity distributions (SSDs), which involves plotting toxicity test data (such as LC_{50}) from different species and genera against percentage rank. AWQC values are then derived as either Final Acute Value (the 5^{th} percentile of all LC_{50} values across the species considered in the analysis) or Criterion Maximum Concentration (the final acute value divided by factor of 2) (USEPA, 1985). Canadian water guidelines (WQG) are also derived using the SSD approach by estimating the 5^{th} percentile of the SSD (CCME, 2007). Very often, AWQCs and WQG are governed by data from invertebrates because of their higher sensitivity to certain toxicants compared to fish species; however, this is not always the case. For example, the SSD used for deriving the Canadian short term (acute) WQG for cadmium (Cd) demonstrated that among the fish species for which reliable toxicity data on Cd are available, rainbow trout is the most sensitive species (CCME, 2014). To derive appropriate and universally protective AWQCs, the knowledge of life-stage and species-specific differences in the sensitivity to contaminants including metals is essential. However, many non-model native fish species are often under-represented in SSD curves because of the unavailability of toxicity data from these species. Therefore, the status of protection of data-poor species remains uncertain especially if a species is more sensitive than the model species. For example, some recent studies have indicated that white sturgeon is more sensitive to acute waterborne copper (Cu) exposure than rainbow trout (Calfee et al., 2014; Vardy et al., 2013). In fact, several life-stages of white sturgeon have been shown to be the most sensitive relative to other fish species on the SSD curve derived from data on 59 freshwater fish species, thus questioning the status of protection of white sturgeon by current AWQCs (Vardy et al., 2013). In a study conducted under similar water chemistry conditions as this study (Vardy et al., 2013), the acute criterion for Cu, recommended by the US EPA for protection of aquatic life (USEPA, 2007), was between 6.4 and 9.5 µg/L, indicating that the swim-up life-stage of white sturgeon will only be borderline protected based on the acute Cu LC_{50} data presented in chapter 3 ($1/2 \times LC_{50} = 12.08$ µg/L).

Large differences in sensitivity among life-stages and species have been observed for many metals (Besser et al., 2007; Buhl and Hamilton, 1991; Calfee et al., 2014; CCME, 2014; Chapman, 1978; Domingues et al., 2010; Dwyer et al., 2005; Hansen et al., 2002; Marr et al., 1995; Mebane,

2012; Mohammed, 2013; Niyogi and Wood, 2004a; Tang et al., 2016; USEPA, 2007; Vardy et al., 2013). White sturgeon have demonstrated a peculiar pattern of sensitivity to metals such as Cd and Cu. Compared to rainbow trout, white sturgeon showed greater sensitivity to acute waterborne Cu exposure, whereas the trend in sensitivity was reversed with acute waterborne Cd exposure (Calfee et al., 2014; Vardy et al., 2014, 2013). However, the mechanisms of life-stage and species-specific differences in the sensitivity among fish species have not been well understood to date, especially for data-poor non-model species such as white sturgeon, and which motivated the studies conducted under this thesis.

Emerging evidence from different studies has indicated that baseline ion (e.g., Ca^{2+}, Na^{+}) uptake rate, ionic turnover, metal detoxification mechanisms, and oxidative stress could play important roles in life-stage and species-specific differences in the sensitivity among fishes to the exposure to metals (Doering et al., 2015; Eyckmans et al., 2011; George et al., 1996; Grosell et al., 2002; Niyogi and Wood, 2004a). However, the majority of comparative toxicology studies have usually been conducted with only one life-stage and addressed only one of the multiple mechanisms of metal toxicity. Due to these shortcomings, a complete understanding of the specific mechanistic drivers of the species-specific differences in the sensitivity of fishes to metals is still lacking. Therefore, the overall objective of my study was to generate novel knowledge about the physiological, biochemical and molecular underpinnings of life-stage and species-specific differences in the sensitivity to Cd and Cu, two contaminants of particular concern in Canadian natural waters. White sturgeon and rainbow trout were selected as the test species for this study because some recent studies have shown that marked life-stage and species-specific differences exist in these species for Cd and Cu (Calfee et al., 2014; Vardy et al., 2014, 2013). White sturgeon and rainbow trout are evolutionarily distinct species and hence, they are good models to study biochemical and molecular differences. Moreover, white sturgeon and rainbow trout are freshwater fishes of commercial, cultural, and recreational importance to Canada, and serve as indicators for the health of freshwater ecosystems. In my study, white sturgeon was found to be more sensitive to Cu than rainbow trout. On the other hand, rainbow trout in general was more sensitive to Cd than white sturgeon except in larval life-stage, where the sensitivity to Cd in both species were very similar to each other. With Cd, extremely high life-stage specific differences in the sensitivity were recorded with white sturgeon, with swim-up and late juvenile life-stages being several times less sensitive than larval and early juvenile (chapter 2). The pattern in sensitivity observed in my

study were very similar to the pattern recorded in a previous study (Calfee et al., 2014). Hence, based on the observations in the current study and similar observations in several previous studies, it appears that there is indeed very high life-stages specific differences in the sensitivity to Cd in white sturgeon.

The major focus of Chapters 2 and 3 was to characterize the life-stage and species-specific effects of Cd and Cu on ion homeostasis by measuring ion uptake and whole-body ions (Ca and Na, respectively). Whole body Cu (Chapter 3) and Cd (Chapter 5) accumulation were also measured. The role of oxidative stress in life-stage and species-specific differences in the sensitivity to Cu and Cd was evaluated by measuring lipid hydroperoxides (LPO) induced by Cd (Chapter 5) and Cu (Chapter 6). The further aims of Chapter 5 and 6 were to understand the mechanisms of anti-oxidant defense, detoxification capacity of Cu and Cd, and life-stage dependent changes in metal uptake transporters in white sturgeon and rainbow trout.

7.2 Ionoregulatory Basis of Sensitivity to Cd and Cu in Rainbow Trout and White Sturgeon

In this study, I showed that the species-specific differences in the acute sensitivity of rainbow trout and white sturgeon to metals can be explained by the metal-induced inhibition of ion (Na^+ or Ca^{2+}) uptake processes and the resulting reduction in the whole-body ion levels.

Greater sensitivity of white sturgeon to Cu was explained by greater reduction of its waterborne Na uptake compared to rainbow trout, when exposed to the same waterborne Cu concentration (50 µg/L for 4.5h) (Chapter 3). Similarly, higher sensitivity of rainbow trout to Cd was explained by significantly higher reduction in Ca uptake when exposed to waterborne Cd (40 µg/L for 3h) (Chapter 2). With both Cu and Cd, the reduction in whole-body ion uptake resulted in a reduction in whole-body Na and Ca levels, respectively, and the magnitude of effect was significantly greater in the more sensitive species. Most importantly, whole body ion uptake and ion levels explained the species-specific differences in sensitivity irrespective of life-stages, which indicated that ionoregulatory endpoints could be used reliably to explain species-specific differences in the acute sensitivity to metals. The findings from this study provided answers to a long-standing conundrum concerning the reasons behind greater sensitivity of white sturgeon to Cu (Calfee et al., 2014; Vardy et al., 2013). Additionally, these findings have general implications for our understanding of the variability of responses in the apical outcomes to metal exposure in fish species.

Despite solid evidence of the role of ionoregulatory mechanisms in species-specific differences in sensitivity, the involvement of additional mechanisms such as oxidative stress and detoxification could not be ruled out because of their known significant contribution in the toxicity outcome of metals. Hence, a comparative examination of acute metal-induced oxidative stress response, MT induction, and heat shock response was required to fully understand the relative contribution of these biochemical parameters to species-specific differences in metal sensitivity.

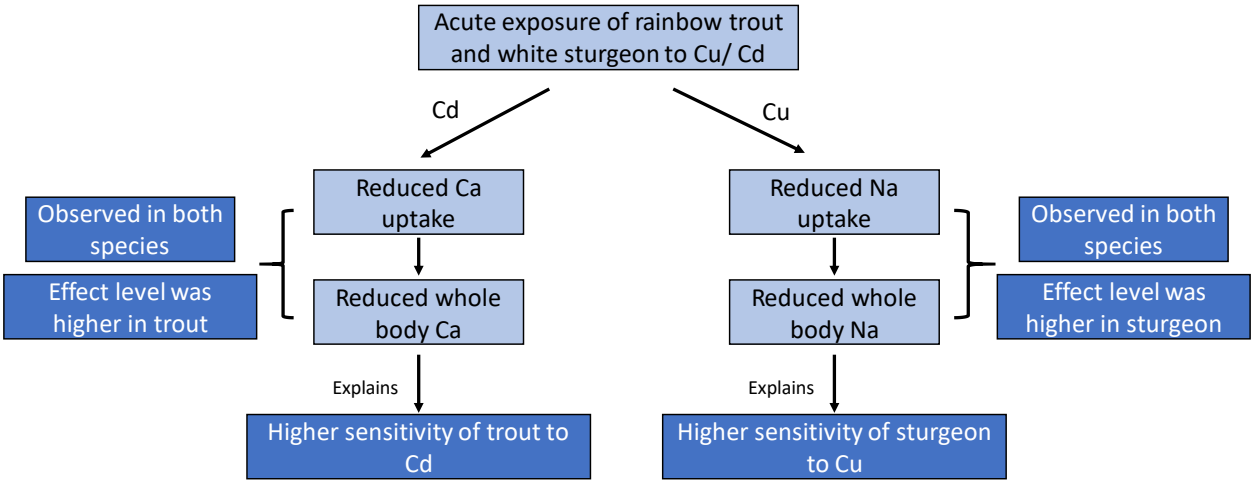

Figure 7. 1. Schematic representation of the role of ionoregulatory disruption in species-specific difference in the sensitivity to Cd and Cu

7.3 Biochemical and Molecular Basis of Sensitivity to Cadmium and Copper in Rainbow Trout and White Sturgeon

Oxidative stress plays an important role in mediating the toxicity of many metals (Wood, 2012). Both acute and long-term exposures to Cu and Cd induce oxidative stress response in fish (Cuypers et al., 2010; Paris-Palacios et al., 2000; Pedrajas et al., 1995; Sanchez et al., 2005). Hence, I presumed that a comparative study of metal induced oxidative stress response at biochemical and molecular levels in different species under the same water chemistry conditions is likely to provide valuable insights into the interrelationship between oxidative stress and apical toxicity endpoints such as mortality. Additionally, I also performed a comparative evaluation of other types of stress response and detoxification mechanisms for metals, such as heat shock protein and metallothionein responses at the gene expression level.

Rainbow trout and white sturgeon, at three different life-stages, were exposed to Cu or Cd for 96 hours at a concentration of 50% of the respective LC_{50} levels. For Cd, these exposure concentrations were 6.20, 1.06, and 0.97 µg/L for larval, swim-up and juvenile rainbow trout, respectively, and 8.33, 37.64, and 6.68 µg/L for larval, swim-up and juvenile white sturgeon. In other words, the exposure concentrations were lower in rainbow trout, the relatively more sensitive species; yet, Cd showed significantly greater LPO induction in larval and juvenile rainbow trout relative to the LPO levels in the respective life-stages of white sturgeon. During the swim-up life-stage, the data followed a similar trend, but the differences were not statistically significant (Chapter 5). For Cu, the 50% of respective LC_{50}, which was chosen as the exposure concentration, was consistently lower in white sturgeon, the more sensitive species. These concentrations were 18.18, 12.07, 17.75 µg/L for larval, swim-up and juvenile white sturgeon, respectively, and 30.28, 37.16, 41.55 µg/L for larval, swim-up and juvenile rainbow trout, respectively. Even though the absolute exposure concentration of Cu were lower in white sturgeon, this species consistently showed greater Cu-induced oxidative damage than rainbow trout during early life-stages, i.e. larval and swim-up (Chapter 6). Therefore, with both Cd and Cu, I observed that oxidative damage was greater in the more sensitive species even when the absolute exposure concentrations were lower, which explained the greater sensitivity of white sturgeon to Cu and the greater sensitivity of rainbow trout to Cd during early life-stages.

I attempted to explain species-specific differences in the LPO response with changes in the responses of enzymatic and non-enzymatic antioxidants at biochemical and gene expression levels. However, these parameters did not show a consistent response across all life-stages. Baseline GSH levels explained the sensitivity differences to Cu, but not to Cd. The level of baseline GSH was significantly higher in rainbow trout relative to white sturgeon, which explained the lower oxidative damage observed in rainbow trout following Cu exposure, since GSH is protective against oxidative damage (Birben et al., 2012). However, by the same rationale, higher GSH levels did not explain the higher oxidative damage in rainbow trout following Cd exposure. With Cd exposure, the HSP70 gene showed a significantly greater upregulation in all respective life-stages of rainbow trout relative to those in white sturgeon, which suggested that rainbow trout was under greater stress due to Cd exposure, and hence explained its higher sensitivity. On the other hand, with Cu exposure, the species with higher sensitivity i.e. white sturgeon did not show a significantly higher upregulation in HSP70 gene expression. MT gene upregulation, which is

considered a potential mechanism through which organisms cope with excess metal load, was significantly greater in rainbow trout during Cd exposure, which was unexpected because rainbow trout was shown to be more sensitive to Cd. With Cu, the data showed that as the fish aged, the reliance on GSH increased in rainbow trout, whereas white sturgeon appeared to recruit an increasing level of MT as the primary mechanism to neutralize oxidative stress and/or Cu loading. The differences in GSH and MT responses to Cu indicated that inherent biochemical mechanistic differences exist between these two species with regard to handling Cu exposure and such diversions in the response could lead to the species-specific differences in the sensitivity to Cu. It should be noted here that MT in my study was analysed only at the gene expression level. Information on post-transcriptional regulation and translation are critical for deriving definite conclusions about the relationship between amounts of mRNA and protein (Vogel and Marcotte, 2012). Nonetheless, a significantly positive correlation between abundances of MT mRNA transcript and protein in aquatic organisms exposed to metals have been shown previously (Knapen et al., 2007), and thus, it is hypothesized that the observed changes in MT mRNA observed in my study is also indicative of an increase in MT protein.

Irrespective of the mechanisms responsible, the species-specific differences in oxidative damage (LPO) explained the differences in sensitivity to Cd and Cu between rainbow trout and white sturgeon across multiple early life-stages. These observations are in agreement with a few other studies which have demonstrated that oxidative damage and associated antioxidant response could play important roles in life-stage and species-specific differences in the sensitivity to metals (Eyckmans et al., 2011; Tang et al., 2016). In my study, a lack of uniform mechanistic explanation of oxidative damage suggests that multiple mechanisms may interact in the process, which is not surprising given the complex nature of oxidative stress response pathways (Birben et al., 2012). Nonetheless, my study demonstrated that oxidative damage, which is the end result of oxidative stress, could be a good indicator, and hence a good surrogate, for testing species-specific differences in the sensitivity to metals. However, life-stage specific differences in oxidative damage need to be understood and considered if such testing is performed because we observed that during some life-stages oxidative damage may not predict species-specific differences.

Table 7. 1. Summary of the roles of various biochemical and molecular mechanisms in species-specific differences in the sensitivity to Cd

Parameter	Observation	Inference
Biochemical/molecular parameters which explain higher sensitivity of trout to Cd		
Oxidative damage (LPO)	In general, significantly higher LPO in trout than sturgeon	Higher oxidative damage in trout is expected to cause higher sensitivity to Cd
SOD and CAT enzyme gene expression	Lesser upregulation in trout	Explains the species-specific differences in LPO and hence sensitivity to Cd
HSP gene expression	Higher upregulation in trout	Indicates higher stress in trout and hence explain its higher sensitivity to Cd
Biochemical/molecular parameters which do not explain higher sensitivity of trout to Cd		
Whole body Cd accumulation	Significantly higher accumulation in sturgeon	Does not explain higher sensitivity of trout
MT gene expression	Higher upregulation in trout	Does not explain higher sensitivity of trout

Table 7. 2. Summary of the roles of various biochemical and molecular mechanisms in species-specific differences in the sensitivity to Cu

Parameter	Observation	Inference
Biochemical/molecular parameters which explain higher sensitivity of sturgeon to Cu		
Oxidative damage (LPO)	Significantly higher LPO in early life-stage sturgeon compared to trout (larval and swim-up)	Greater oxidative damage in sturgeon is expected to cause greater sensitivity to Cu
Baseline GSH	High in trout, which increases further with age. Suggests that trout depends more on GSH as it grows	Such diversion in response may lead to species-specific differences in apical endpoint (more research needed)
MT gene expression	Response to Cu increases with age in sturgeon but decreases in trout. Suggests that sturgeon depends more on MT as it grows	
Biochemical/molecular parameters which do not explain higher sensitivity of sturgeon to Cu		
Antioxidant enzymes activity	No consistent response between species across all life-stages	Cannot explain the species-specific differences in LPO and hence sensitivity to Cu
GSH/GSSG	No species-specific difference	Both species responded in a similar manner
HSP gene expression	No significant change in the expression	Cu has no measurable effect on HSP70 expression

7.4 Inconsistent Relationship Between Whole Body Accumulation of Metals and Sensitivity in Rainbow Trout and White Sturgeon

There was no consistent relationship between whole-body metal accumulation and observed differences in acute sensitivity to metals across life-stages and species. Rainbow trout, which are more sensitive to Cd, accumulated significantly less Cd relative to white sturgeon when exposed to the same Cd concentration for 24 h. Similarly, Cu accumulation results also did not demonstrate a consistent life-stage or species-specific pattern, as observed with other physiological and/or toxicological parameters examined in this study (e.g., 96h LC_{50}, and Cu-induced reduction in Na uptake and whole-body Na level). White sturgeon accumulated more Cu during the larval life-stage, whereas rainbow trout accumulated more Cu during the swim-up life-stage. Moreover, juvenile fish of both species showed similar levels of whole-body Cu burden during exposure to Cu. A similar lack of interrelationship between whole-body accumulation and sensitivity to metals has been reported in several other studies and literature reviews with different metals (Adams et al., 2011; Shaukat and Javed, 2013; US EPA, 2016). The poor correlation between metal accumulation and toxic effects of metals probably stems from the fact that the whole-body accumulation does not account for the distribution of metals to toxicologically critical tissues and cellular compartments as well as the inherent metal detoxification capacity of an organism (Rainbow, 2002). The development of a biotic ligand model (BLM) for various metals has accounted for some of these uncertainties between toxicity prediction and body burden. BLM modelling relies on the lethal accumulation (LA50) of a metal, which is defined as the short-term gill (biotic ligand) metal burden that is predictive of 96 hour LC_{50} (Niyogi and Wood, 2004b). The LA_{50} for a species is considered to be constant across different water chemistry. Usingthese constant LA_{50} values, BLM is able to predict the LC_{50} of a metal in different waters (Niyogi et al., 2008). However, it has been shown that the LA_{50} is not a constant even within a species, i.e. it may differ if the same members of same species are cultured in different water conditions (Vardy et al., 2014). Moreover, the LA_{50} is expected to differ among different species because the quantitative and possibly qualitative nature of metal binding sites may differ among different species. Nonetheless, these shortcomings do not prevent BLMs to be used in regulatory applications because in practice, BLM models are directly calibrated to toxicity and do not necessarily rely on measured LA_{50} values. However, when it comes to mechanistic understanding of the response of organisms to metals, the experimental observations discussed in this section suggest that metal

accumulation should only be evaluated along with the information on distribution of metals to critical organs and subcellular fractions within cells.

7.5 Overall Conclusion and Future Perspectives

In my study, differential changes in physiological parameters such as decreased ion uptake and whole-body ion levels (Na^+ and Ca^{2+}) following exposure to metals showed a remarkably similar pattern as observed with differences in sensitivity to metals across species and life-stages. Therefore, these findings suggest that ion physiology parameters could be good indicators of the life-stage- and species-specific differences in the sensitivity of fish to metals. Using different species than those investigated in this study, a few previous studies have also suggested that ionic turnover and ion uptake parameters could be good indicators of species-specific differences in sensitivity to metals such as Ag and Cu (Bianchini et al., 2002; Grosell et al., 2002). One possible reason for the species-specific differences in the vulnerability of Ca and Na uptake could be different binding properties of metals to the ionic transporters. In my study, I showed that the affinity of binding for Cd on biotic ligand was significantly greater in the more sensitive species rainbow trout, which explained the greater inhibition of Ca uptake by Cd in rainbow trout (Chapter 2). Theoretically, it is possible that the three-dimensional structures of ion transporters are different enough among species that it creates differences in the binding of metals. Such differences in three-dimensional structures of ion transporters are possible because of the differences in primary amino acid sequences among species. This hypothesis provides an interesting research avenue. An evolutionary relationship can be established among species based on the amino acid sequences of the ion transporters, and this relationship among species can then be compared against their respective sensitivities to metals. Such phylogenetic approaches could help us understand how molecular changes that emerged in response to challenges of the past environment may cause some species to be more sensitive than others when faced with environmental pollution. Phylogenetic approaches have shown promising results in explaining the differences in susceptibility to Cd among invertebrate species (Buchwalter et al., 2008).

LPO levels in the gills also generally explained the species-specific differences in sensitivity to Cd and Cu between rainbow trout and white sturgeon. In general, species with higher sensitivity to a metal showed a greater LPO induction. I made an attempt to understand the mechanisms underneath differing LPO patterns between species by evaluating the responses of

enzymatic and non-enzymatic antioxidants, metal accumulation and metallothionein induction. These parameters could only partially explain the LPO induction pattern, with no consistent response across all life-stages, which is not surprising given the complex nature of the interconnected pathways of the oxidative stress response (Birben et al., 2012). Regardless of the underlying mechanism, LPO seems to be a good indicator of species-specific differences in the sensitivity to Cd and Cu between rainbow trout and white sturgeon.

Some aspects that were not studied in my thesis were the subcellular distribution of metals, elimination of metals, and the effect of metals on the efflux of cations such as Na and Ca. The pattern of subcellular distribution of metals has been shown to play a significant role in determining toxicity in fish species (Eyckmans et al., 2012). Metals that are present in the metabolically available fractions are considered as toxic (Eyckmans et al., 2012). Based on what I observed in this thesis, it can be hypothesized that subcellular distribution of Cd and Cu differs between the two species in such a manner that it leads to different levels of accumulation in organelles, which is expected to cause different levels of oxidative stress and damage. Similarly, differential patterns of metal accumulation in the membranes would be expected to cause differential effects on ion transporters between species, thereby generating species-specific ionoregulatory effects. Indeed, in a recent study (not a part of the thesis), I exposed juvenile rainbow trout and white sturgeon to Cd (1.25 µg/L) for 7 days and evaluated the species-specific differences in the subcellular distribution of Cd in the liver. My study showed that rainbow trout accumulated 34.8% of total Cd in the organelles (ORG) fraction, which was significantly higher than only 7.7% accumulation in the ORG fraction of white sturgeon (unpublished data, Figure C7.S1). Similarly, Cd accumulation in the nuclei and cell debris (NCD) fraction of trout was 16.8% of total Cd, which was approximately two times higher than the accumulation in NCD of white sturgeon (8.5%) (unpublished data, Figure C7.S1). These preliminary findings suggest that the evaluation of interrelationships between subcellular distribution of metals and oxidative damage as well as ionoregulatory disruption could be an interesting prospect for future research. Cu has been shown to have significant impact on the efflux of Na (Grosell, 2012); therefore, the potential role of Na efflux in creating species-specific differences in the sensitivity to Cu cannot be ruled out. Although Na efflux was not measured in this study, efflux of Na appears to be less sensitive to Cu exposure as compared to influx, as demonstrated in two separate studies (Chowdhury et al., 2016; Lauren and McDonald, 1985). On the other hand, exposure to Cd has not been shown to

have any measurable effect on efflux of Ca in fishes. Finally, elimination of metals could differ between the species and hence, could play a role in species-specific differences in the sensitivity to metals. There are several routes of elimination in fish such as kidney, gill, and gut (Wood, 2012), and it is experimentally difficult to identify the most critical route of metal elimination that plays a role in species-specific differences in sensitivity. Hence, metal elimination was not compared between species in this study; however, considering the potential role of metal elimination in context with ion-regulatory processes, future studies should take these parameters into consideration.

A molecular initiating event (MIE) is defined as the first interaction between a chemical compound and a molecular target within an organism. These MIEs are considered the first key event post exposure, which initiates the toxicity response in organisms. If sufficiently perturbed, the MIE triggers a cascade of further downstream events (key events [KEs]) across different levels of organization (e.g. molecular, cellular, tissue, whole organism), which culminate in an adverse outcome (AO). This cascade of events is designated as an adverse outcome pathway (AOP) (Figure 7.2) (Allen et al., 2016; Hecker and LaLone, 2019).

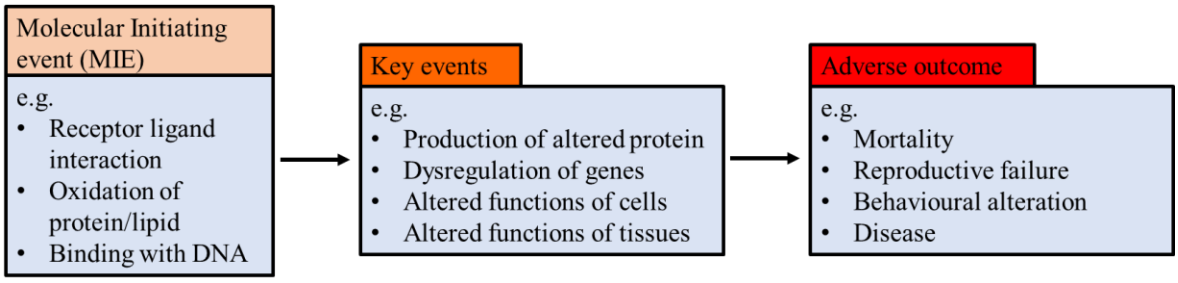

Figure 7. 2. Schematic representation of the steps involved in a typical Adverse Outcome Pathway (AOP)

I believe that studying the molecular and biochemical basis of the sensitivity of fishes to contaminants including metals is of great importance because such studies will help us in defining the MIEs, which in turn are crucial for developing well informed AOPs. The most recent advancement in the AOP framework is the development of quantitative adverse outcome pathways (qAOPs). A qAOP framework enables quantitative and dose-response prediction of an adverse effect (Conolly et al., 2017). Hence, development of novel qAOPs can be of tremendous support in regulatory decision-making, prioritizing toxicity testing of chemicals in animals, and reducing the number of animals used in research. For example, in a recent study, a simple linear regression based interspecific qAOP was built by utilizing a known quantitative link between sensitivity to activation of the aryl hydrocarbon receptor (AHR) and sensitivity to early life stage mortality (Doering et al., 2018). This qAOP model was able to predict the dose−response curves for early life-stage mortality for Dioxin-like compounds (DLC) in any fish and bird. The only information that is required to successfully predict the outcome is the molar mass of the DLC and the chemical- and species-specific EC50 for AHR for birds and fishes in an in-vitro AHR transactivation assay (Doering et al., 2018).

Similar qAOP based approaches can be developed for metals, which will enable the quantitative prediction of the probability of occurrence or severity of an adverse outcome. Well defined molecular and biochemical drivers of species-specific differences in the sensitivity to metals, such as those identified in this study (especially ion transport pathways) can be used to develop *in-vitro* assays for sensitivity prediction among species. EC_{50} values of metals from these assays can be determined and compared with apical endpoint values available from the literature (e.g., LC_{50}, EC_{50}) for the respective species. The relationships between *in-vitro* EC_{50s} and the dose response from apical endpoints (such as LC_{10}, LC_{25}, LC_{50}) can be incorporated into a model capable of predicting the dose-response patterns for data poor species. Once the model is fully established, the *in-vitro* reporter assays can be built from samples collected from even a single organism using non-invasive techniques; hence, these methods could be highly effective in prioritizing toxicity testing of metals in native, non-model species of regulatory concern, including critically endangered species.

In conclusion, my study showed that ion physiology parameters such as ion uptake and whole-body ion levels are good indicators of species-specific differences in the sensitivity of

rainbow trout and white sturgeon to Cd and Cu, and possibly other metals as well. In addition, LPO levels in the gills also generally explained the species-specific differences in sensitivity to Cd and Cu between rainbow trout and white sturgeon. The driving factors identified in this study are good candidates for developing qAOP based approaches for toxicity prediction of metals, after further characterization in more species.

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APPENDIX [1]

1 Supplementary data are included in this chapter. The figure or table number is presented as Cx.Sy, format, where ‘Cx’ indicates chapter number; ‘Sy’ indicates figure or table number.

Table C2.S 1. Water quality parameters for the water used in this study. Water of same composition was used throughout all exposures (life-stages and species). One water sample was randomly taken in the beginning of first exposure and one water sample at the final exposure

Samples	**Dissolved chloride**	**Dissolved organic carbon**	**Dissolved calcium**	**Dissolved magnesium**	**Dissolved potassium**	**Dissolved sodium**	**Dissolved sulfate**
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Water (beginning)	4	0.8	11	4.8	1.2	11	28
Water (end)	4	1.1	14	6.4	1.3	12	34
Average	4	0.95	12.5	5.6	1.25	11.5	31

Table C2.S 2. Water quality parameters analysed throughout the study (n = 174)

Date	**Metal**	**Temperature**	**pH**	**Dissolved oxygen (%)**	**Hardness (as CaCO3)**	**Alkalinity**
06-Jul-15	Cd	13.7	7.3	93.0	~60	~40
06-Jul-15	Cd	13.7	7.5	87.7	~60	~40
06-Jul-15	Cd	13.9	7.6	92.4	~60	~40
06-Jul-15	Cd	13.8	7.2	93.7	~60	~40
06-Jul-15	Cd	13.9	7.2	90.8	~60	~40
06-Jul-15	Cd	13.6	7.5	91.9	~60	~40
06-Jul-15	Cd	13.7	7.5	90.3	~60	~40
06-Jul-15	Cd	13.9	7.4	92.0	~60	~40
06-Jul-15	Cd	13.9	7.4	89.5	~60	~40
06-Jul-15	Cd	13.8	7.4	92.1	~60	~40
06-Jul-15	Cd	13.8	7.4	88.4	~60	~40
06-Jul-15	Cd	13.8	7.1	91.2	~60	~40
07-Jul-15	Cd	14.1	7.5	92.8	~60	~40
07-Jul-15	Cd	13.5	7.3	94.7	~60	~40
07-Jul-15	Cd	13.6	7.4	89.7	~60	~40
07-Jul-15	Cd	13.8	7.3	89.2	~60	~40

07-Jul-15	Cd	13.8	7.4	95.1	~60	~40
07-Jul-15	Cd	13.6	7.4	90.4	~60	~40
07-Jul-15	Cd	13.7	7.3	92.0	~60	~40
07-Jul-15	Cd	13.4	7.3	91.7	~60	~40
07-Jul-15	Cd	14.0	7.3	91.8	~60	~40
07-Jul-15	Cd	13.7	7.6	93.3	~60	~40
07-Jul-15	Cd	13.8	7.5	92.9	~60	~40
07-Jul-15	Cd	13.8	7.5	90.2	~60	~40
08-Jul-15	Cd	13.7	7.5	91.4	~60	~40
08-Jul-15	Cd	13.8	7.5	90.4	~60	~40
08-Jul-15	Cd	13.6	7.2	88.8	~60	~40
08-Jul-15	Cd	13.8	7.3	91.8	~60	~40
08-Jul-15	Cd	13.8	7.4	92.2	~60	~40
08-Jul-15	Cd	13.6	7.5	89.4	~60	~40
08-Jul-15	Cd	13.7	7.4	93.0	~60	~40
08-Jul-15	Cd	13.6	7.5	91.4	~60	~40
08-Jul-15	Cd	13.7	7.2	90.9	~60	~40
08-Jul-15	Cd	13.7	7.4	92.2	~60	~40
08-Jul-15	Cd	13.6	7.3	91.2	~60	~40
08-Jul-15	Cd	13.9	7.4	94.0	~60	~40
09-Jul-15	Cd	13.9	7.6	90.0	~60	~40
09-Jul-15	Cd	13.7	7.6	94.6	~60	~40
09-Jul-15	Cd	13.8	7.4	91.7	~60	~40
09-Jul-15	Cd	13.4	7.4	93.6	~60	~40
09-Jul-15	Cd	13.6	7.4	92.5	~60	~40
09-Jul-15	Cd	13.8	7.3	92.9	~60	~40
09-Jul-15	Cd	13.7	7.5	90.9	~60	~40
09-Jul-15	Cd	13.8	7.5	89.1	~60	~40
09-Jul-15	Cd	13.7	7.5	88.0	~60	~40
09-Jul-15	Cd	13.8	7.9	96.0	~60	~40
09-Jul-15	Cd	13.6	7.3	89.8	~60	~40
09-Jul-15	Cd	13.8	7.2	89.8	~60	~40
10-Jul-15	Cd	13.6	7.3	89.7	~60	~40
10-Jul-15	Cd	13.6	7.5	91.5	~60	~40
10-Jul-15	Cd	13.9	7.6	92.5	~60	~40
10-Jul-15	Cd	13.7	7.4	91.6	~60	~40
10-Jul-15	Cd	13.9	7.3	92.7	~60	~40
10-Jul-15	Cd	13.6	7.3	92.5	~60	~40
10-Jul-15	Cd	13.7	7.4	89.2	~60	~40
10-Jul-15	Cd	13.8	7.3	92.6	~60	~40
10-Jul-15	Cd	13.6	7.4	90.1	~60	~40
10-Jul-15	Cd	13.7	7.6	90.1	~60	~40
10-Jul-15	Cd	13.5	7.6	89.5	~60	~40

10-Jul-15	Cd	13.5	7.5	90.7	~60	~40
16-Jul-15	Cd	13.8	7.3	93.6	~60	~40
16-Jul-15	Cd	13.6	7.5	90.8	~60	~40
16-Jul-15	Cd	13.9	7.3	90.1	~60	~40
16-Jul-15	Cd	13.6	7.4	93.8	~60	~40
16-Jul-15	Cd	13.7	7.3	94.2	~60	~40
16-Jul-15	Cd	13.6	7.7	93.9	~60	~40
16-Jul-15	Cd	13.6	7.6	90.8	~60	~40
16-Jul-15	Cd	13.8	7.3	93.4	~60	~40
16-Jul-15	Cd	13.7	7.6	92.8	~60	~40
16-Jul-15	Cd	13.8	7.6	89.7	~60	~40
16-Jul-15	Cd	13.7	7.4	87.7	~60	~40
16-Jul-15	Cd	13.7	7.3	93.1	~60	~40
17-Jul-15	Cd	13.7	7.4	93.5	~60	~40
17-Jul-15	Cd	13.8	7.4	88.3	~60	~40
17-Jul-15	Cd	13.5	7.6	92.1	~60	~40
17-Jul-15	Cd	13.6	7.5	88.6	~60	~40
17-Jul-15	Cd	13.5	7.5	94.8	~60	~40
17-Jul-15	Cd	13.4	7.3	93.4	~60	~40
17-Jul-15	Cd	13.7	7.3	90.8	~60	~40
17-Jul-15	Cd	13.7	7.4	91.1	~60	~40
17-Jul-15	Cd	13.7	7.4	90.5	~60	~40
17-Jul-15	Cd	13.7	7.2	90.9	~60	~40
17-Jul-15	Cd	13.7	7.5	92.1	~60	~40
17-Jul-15	Cd	13.7	7.5	90.3	~60	~40
18-Jul-15	Cd	13.5	7.3	90.0	~60	~40
18-Jul-15	Cd	13.8	7.4	91.5	~60	~40
18-Jul-15	Cd	13.8	7.3	92.6	~60	~40
18-Jul-15	Cd	13.7	7.4	93.8	~60	~40
18-Jul-15	Cd	14.0	7.4	96.9	~60	~40
18-Jul-15	Cd	13.8	7.3	87.6	~60	~40
18-Jul-15	Cd	13.5	7.4	89.8	~60	~40
18-Jul-15	Cd	13.7	7.3	91.5	~60	~40
18-Jul-15	Cd	13.7	7.6	91.3	~60	~40
18-Jul-15	Cd	13.7	7.3	91.3	~60	~40
18-Jul-15	Cd	13.7	7.6	88.1	~60	~40
18-Jul-15	Cd	13.9	7.3	89.3	~60	~40
19-Jul-15	Cd	14.1	7.1	89.5	~60	~40
19-Jul-15	Cd	13.6	7.3	92.0	~60	~40
19-Jul-15	Cd	13.4	7.4	89.4	~60	~40
19-Jul-15	Cd	13.8	7.5	88.2	~60	~40
19-Jul-15	Cd	13.9	7.4	90.4	~60	~40
19-Jul-15	Cd	13.8	7.5	93.8	~60	~40

19-Jul-15	Cd	13.5	7.5	91.2	~60	~40
19-Jul-15	Cd	13.5	7.6	91.0	~60	~40
19-Jul-15	Cd	13.6	7.3	94.7	~60	~40
19-Jul-15	Cd	13.6	7.4	91.5	~60	~40
19-Jul-15	Cd	13.9	7.4	90.4	~60	~40
19-Jul-15	Cd	13.3	7.3	90.6	~60	~40
20-Jul-15	Cd	13.6	7.4	91.5	~60	~40
20-Jul-15	Cd	13.6	7.5	90.3	~60	~40
20-Jul-15	Cd	13.6	7.3	91.7	~60	~40
20-Jul-15	Cd	13.5	7.5	92.1	~60	~40
20-Jul-15	Cd	13.7	7.4	91.7	~60	~40
20-Jul-15	Cd	13.4	7.2	89.6	~60	~40
20-Jul-15	Cd	13.9	7.5	91.9	~60	~40
20-Jul-15	Cd	13.6	7.5	92.7	~60	~40
20-Jul-15	Cd	13.5	7.3	91.3	~60	~40
20-Jul-15	Cd	13.6	7.4	90.3	~60	~40
20-Jul-15	Cd	13.8	7.4	90.4	~60	~40
20-Jul-15	Cd	13.8	7.4	93.6	~60	~40
15-Aug-15	Cd	13.7	7.6	90.1	~60	~40
15-Aug-15	Cd	13.8	7.5	93.0	~60	~40
15-Aug-15	Cd	13.6	7.3	89.1	~60	~40
15-Aug-15	Cd	13.8	7.3	88.9	~60	~40
15-Aug-15	Cd	13.9	7.4	89.5	~60	~40
15-Aug-15	Cd	13.9	7.3	92.5	~60	~40
16-Aug-15	Cd	13.6	7.1	86.8	~60	~40
16-Aug-15	Cd	13.7	7.4	90.3	~60	~40
16-Aug-15	Cd	13.5	7.5	93.1	~60	~40
16-Aug-15	Cd	13.5	7.4	90.6	~60	~40
16-Aug-15	Cd	13.8	7.4	89.2	~60	~40
16-Aug-15	Cd	13.6	7.3	90.6	~60	~40
17-Aug-15	Cd	13.8	7.4	94.5	~60	~40
17-Aug-15	Cd	13.8	7.2	92.1	~60	~40
17-Aug-15	Cd	13.6	7.4	89.8	~60	~40
17-Aug-15	Cd	13.6	7.5	94.9	~60	~40
17-Aug-15	Cd	13.7	7.3	93.5	~60	~40
17-Aug-15	Cd	13.7	7.4	89.6	~60	~40
18-Aug-15	Cd	13.5	7.4	90.9	~60	~40
18-Aug-15	Cd	13.8	7.4	92.1	~60	~40
18-Aug-15	Cd	13.7	7.3	92.8	~60	~40
18-Aug-15	Cd	13.6	7.3	91.2	~60	~40
18-Aug-15	Cd	13.8	7.5	93.4	~60	~40
18-Aug-15	Cd	14.1	7.5	92.7	~60	~40
19-Aug-15	Cd	13.6	7.3	89.8	~60	~40

19-Aug-15	Cd	13.9	7.6	91.3	~60	~40
19-Aug-15	Cd	13.9	7.3	91.9	~60	~40
19-Aug-15	Cd	13.8	7.5	92.3	~60	~40
19-Aug-15	Cd	13.6	7.2	89.5	~60	~40
19-Aug-15	Cd	13.8	7.4	87.1	~60	~40
16-Sep-15	Cd	13.7	7.4	89.1	~60	~40
16-Sep-15	Cd	13.9	7.3	92.1	~60	~40
16-Sep-15	Cd	13.4	7.4	91.8	~60	~40
16-Sep-15	Cd	13.5	7.5	94.5	~60	~40
16-Sep-15	Cd	13.7	7.4	88.9	~60	~40
16-Sep-15	Cd	13.4	7.2	92.2	~60	~40
17-Sep-15	Cd	13.5	7.2	89.7	~60	~40
17-Sep-15	Cd	13.4	7.4	88.4	~60	~40
17-Sep-15	Cd	13.7	7.4	92.0	~60	~40
17-Sep-15	Cd	13.8	7.4	90.7	~60	~40
17-Sep-15	Cd	13.7	7.3	92.7	~60	~40
17-Sep-15	Cd	13.8	7.4	89.9	~60	~40
18-Sep-15	Cd	13.8	7.4	90.6	~60	~40
18-Sep-15	Cd	13.6	7.2	91.2	~60	~40
18-Sep-15	Cd	13.8	7.3	94.2	~60	~40
18-Sep-15	Cd	13.7	7.5	90.3	~60	~40
18-Sep-15	Cd	13.6	7.2	94.2	~60	~40
18-Sep-15	Cd	13.7	7.3	94.5	~60	~40
19-Sep-15	Cd	13.8	7.5	90.6	~60	~40
19-Sep-15	Cd	13.6	7.4	94.3	~60	~40
19-Sep-15	Cd	13.7	7.4	92.6	~60	~40
19-Sep-15	Cd	13.5	7.6	88.9	~60	~40
19-Sep-15	Cd	13.9	7.5	95.1	~60	~40
19-Sep-15	Cd	13.8	7.3	93.5	~60	~40

Table C2.S 3. Nominal and measured Cd concentrations in the exposure waters used for measuring toxicity (96h LC_{50}) and respective mortalities in different life-stages of white sturgeon and rainbow trout

Species	Life-stage	Nominal concentration (µg/L)	Measured Concentration (µg/L)	Concentration (nmol)	Mortality (out of 10)
Sturgeon	5 dph	Control	0.012	0.11	0
Sturgeon	5 dph	Control	0.012	0.11	0
Sturgeon	5 dph	Control	0.012	0.11	0
Sturgeon	5 dph	5	5.36	47.65	0
Sturgeon	5 dph	5	5.36	47.65	2
Sturgeon	5 dph	5	5.36	47.65	2
Sturgeon	5 dph	10	9.49	84.39	1
Sturgeon	5 dph	10	9.49	84.39	1
Sturgeon	5 dph	10	9.49	84.39	2
Sturgeon	5 dph	20	18.6	165.48	6
Sturgeon	5 dph	20	18.6	165.48	5
Sturgeon	5 dph	20	18.6	165.48	6
Sturgeon	5 dph	40	39.48	351.21	10
Sturgeon	5 dph	40	39.48	351.21	10
Sturgeon	5 dph	40	39.48	351.21	10
Sturgeon	5 dph	80	82.82	736.74	10
Sturgeon	5 dph	80	82.82	736.74	10
Sturgeon	5 dph	80	82.82	736.74	10
Sturgeon	5 dph	160	165.54	1472.65	10
Sturgeon	5 dph	160	165.54	1472.65	10
Sturgeon	5 dph	160	165.54	1472.65	10
Sturgeon	15 dph	Control	0.00	0	1
Sturgeon	15 dph	Control	0.00	0	2
Sturgeon	15 dph	Control	0.00	0	1
Sturgeon	15 dph	5	4.94	43.98	2
Sturgeon	15 dph	5	4.94	43.98	1
Sturgeon	15 dph	5	4.94	43.98	1
Sturgeon	15 dph	10	10.59	94.19	1
Sturgeon	15 dph	10	10.59	94.19	1
Sturgeon	15 dph	10	10.59	94.19	2
Sturgeon	15 dph	20	19.38	172.44	2
Sturgeon	15 dph	20	19.38	172.44	1
Sturgeon	15 dph	20	19.38	172.44	1
Sturgeon	15 dph	40	43.60	43.60	2
Sturgeon	15 dph	40	43.60	43.60	2

Sturgeon	15 dph	40	43.60	43.60	3
Sturgeon	15 dph	80	85.38	759.55	6
Sturgeon	15 dph	80	85.38	759.55	4
Sturgeon	15 dph	80	85.38	759.55	5
Sturgeon	15 dph	160	159.70	1420.65	9
Sturgeon	15 dph	160	159.70	1420.65	9
Sturgeon	15 dph	160	159.70	1420.65	10
Sturgeon	45 dph	Control	0.00	0.00	0
Sturgeon	45 dph	Control	0.00	0.00	0
Sturgeon	45 dph	Control	0.00	0.00	0
Sturgeon	45 dph	5	5.30	47.19	1
Sturgeon	45 dph	5	5.30	47.19	1
Sturgeon	45 dph	5	5.30	47.19	2
Sturgeon	45 dph	10	11.38	101.25	4
Sturgeon	45 dph	10	11.38	101.25	3
Sturgeon	45 dph	10	11.38	101.25	3
Sturgeon	45 dph	20	18.49	164.47	8
Sturgeon	45 dph	20	18.49	164.47	7
Sturgeon	45 dph	20	18.49	164.47	8
Sturgeon	45 dph	40	42.97	382.23	10
Sturgeon	45 dph	40	42.97	382.23	9
Sturgeon	45 dph	40	42.97	382.23	10
Sturgeon	45 dph	80	83.19	740.03	10
Sturgeon	45 dph	80	83.19	740.03	10
Sturgeon	45 dph	80	83.19	740.03	10
Sturgeon	45 dph	160	154.85	1377.54	10
Sturgeon	45 dph	160	154.85	1377.54	10
Sturgeon	45 dph	160	154.85	1377.54	10
Sturgeon	75 dph	Control	0.037	0.33	0
Sturgeon	75 dph	Control	0.037	0.33	0
Sturgeon	75 dph	Control	0.037	0.33	0
Sturgeon	75 dph	5	5.315	47.28	0
Sturgeon	75 dph	5	5.315	47.28	0
Sturgeon	75 dph	5	5.315	47.28	0
Sturgeon	75 dph	10	11.485	102.17	0
Sturgeon	75 dph	10	11.485	102.17	0
Sturgeon	75 dph	10	11.485	102.17	0
Sturgeon	75 dph	20	18.545	164.98	0
Sturgeon	75 dph	20	18.545	164.98	0
Sturgeon	75 dph	20	18.545	164.98	0
Sturgeon	75 dph	40	43.188	384.20	1
Sturgeon	75 dph	40	43.188	384.20	0
Sturgeon	75 dph	40	43.188	384.20	0

Sturgeon	75 dph	80	81.066	721.16	0
Sturgeon	75 dph	80	81.066	721.16	1
Sturgeon	75 dph	80	81.066	721.16	2
Trout	5 dph	Control	0.00	0	0
Trout	5 dph	Control	0.00	0.00	0
Trout	5 dph	Control	0.00	0.00	0
Trout	5 dph	0.312	0.33	2.90	0
Trout	5 dph	0.312	0.33	2.90	0
Trout	5 dph	0.312	0.33	2.90	0
Trout	5 dph	0.625	0.60	5.37	0
Trout	5 dph	0.625	0.60	5.37	0
Trout	5 dph	0.625	0.60	5.37	0
Trout	5 dph	1.25	1.18	10.53	0
Trout	5 dph	1.25	1.18	10.53	0
Trout	5 dph	1.25	1.18	10.53	0
Trout	5 dph	2.5	2.16	19.18	0
Trout	5 dph	2.5	2.16	19.18	0
Trout	5 dph	2.5	2.16	19.18	0
Trout	5 dph	5	4.39	39.07	0
Trout	5 dph	5	4.39	39.07	0
Trout	5 dph	5	4.39	39.07	1
Trout	5 dph	10	9.16	81.50	3
Trout	5 dph	10	9.16	81.50	2
Trout	5 dph	10	9.16	81.50	4
Trout	5 dph	20	18.82	167.39	8
Trout	5 dph	20	18.82	167.39	7
Trout	5 dph	20	18.82	167.39	8
Trout	15 dph	Control	0.00	0.00	0
Trout	15 dph	Control	0.00	0.00	0
Trout	15 dph	Control	0.00	0.00	0
Trout	15 dph	0.625	0.59	5.24	0
Trout	15 dph	0.625	0.59	5.24	0
Trout	15 dph	0.625	0.59	5.24	0
Trout	15 dph	1.25	1.15	10.22	0
Trout	15 dph	1.25	1.15	10.22	0
Trout	15 dph	1.25	1.15	10.22	0
Trout	15 dph	2.5	2.15	19.10	5
Trout	15 dph	2.5	2.15	19.10	5
Trout	15 dph	2.5	2.15	19.10	6
Trout	15 dph	5	4.32	38.42	10
Trout	15 dph	5	4.32	38.42	10
Trout	15 dph	5	4.32	38.42	10
Trout	15 dph	10	8.81	78.34	10

Trout	15 dph	10	8.81	78.34	10
Trout	15 dph	10	8.81	78.34	10
Trout	15 dph	20	18.38	163.49	10
Trout	15 dph	20	18.38	163.49	10
Trout	15 dph	20	18.38	163.49	10
Trout	45 dph	Control	0.00	0.00	0
Trout	45 dph	Control	0.00	0.00	0
Trout	45 dph	Control	0.00	0.00	0
Trout	45 dph	0.625	0.59	5.27	0
Trout	45 dph	0.625	0.59	5.27	0
Trout	45 dph	0.625	0.59	5.27	0
Trout	45 dph	1.25	1.21	10.80	1
Trout	45 dph	1.25	1.21	10.80	0
Trout	45 dph	1.25	1.21	10.80	1
Trout	45 dph	2.5	2.18	19.36	8
Trout	45 dph	2.5	2.18	19.36	9
Trout	45 dph	2.5	2.18	19.36	3
Trout	45 dph	5	4.87	43.29	10
Trout	45 dph	5	4.87	43.29	10
Trout	45 dph	5	4.87	43.29	10
Trout	45 dph	10	9.27	82.42	10
Trout	45 dph	10	9.27	82.42	10
Trout	45 dph	10	9.27	82.42	10
Trout	45 dph	20	19.58	174.18	10
Trout	45 dph	20	19.58	174.18	10
Trout	45 dph	20	19.58	174.18	10
Trout	75 dph	Control	0.037	0.33	0
Trout	75 dph	Control	0.037	0.33	0
Trout	75 dph	Control	0.037	0.33	0
Trout	75 dph	0.625	0.59	5.23	1
Trout	75 dph	0.625	0.59	5.23	0
Trout	75 dph	0.625	0.59	5.23	0
Trout	75 dph	1.25	0.96	8.57	1
Trout	75 dph	1.25	0.96	8.57	0
Trout	75 dph	1.25	0.96	8.57	0
Trout	75 dph	2.5	1.87	16.64	3
Trout	75 dph	2.5	1.87	16.64	2
Trout	75 dph	2.5	1.87	16.64	2
Trout	75 dph	5	4.53	40.33	8
Trout	75 dph	5	4.53	40.33	7
Trout	75 dph	5	4.53	40.33	8
Trout	75 dph	10	9.24	82.23	9
Trout	75 dph	10	9.24	82.23	9

Trout	75 dph	10	9.24	82.23	10
Trout	75 dph	20	17.36	154.41	10
Trout	75 dph	20	17.36	154.41	10
Trout	75 dph	20	17.36	154.41	10

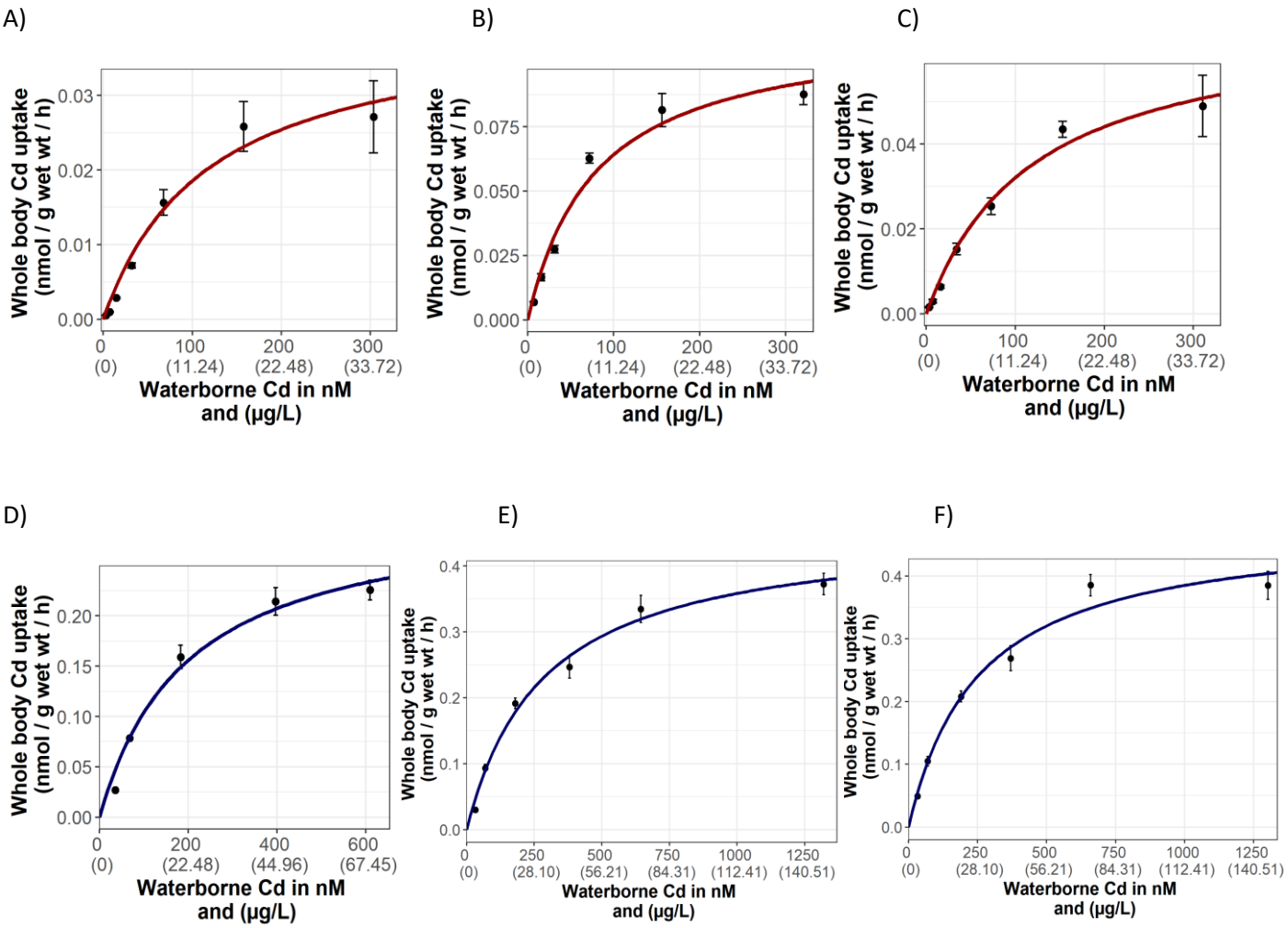

Figure C2.S 1. Concentration dependent whole-body Cd uptake at different waterborne Cd concentration in (A) 5 dph rainbow trout (B) 15 dph rainbow trout (C) 45 dph rainbow trout (D) 5 dph white sturgeon (E) 15 dph white sturgeon (F) 45 dph white sturgeon. Data is presented as mean ± SEM (n = 6). X-axis represents concentration of Cd in nM (values in parenthesis are concentration in µg/L). Calculations were performed on the basis of freely available waterborne Cd (as Cd^{2+}) which was estimated using Visual MINTEQ (version 3). Data was fitted using Michaelis-Menten model (MM.2) function in 'drc' package of R software version 3.1.2.

Table C3.S 1. Nominal and measured Cu concentrations in the exposure waters used for measuring toxicity (96h LC_{50}) and respective mortalities in different life-stages of white sturgeon and rainbow trout. Total number of fish per replicate were 10 in all groups with the exception of larval and swim-up rainbow trout, where the total number were 20.

Species	Life-stage	Nominal concentration (µg/L)	Measured concentration (µg/L)	Concentration (µmol)	Mortality
Sturgeon	Larval	Control	0.88	13.86	0
Sturgeon	Larval	Control	0.88	13.86	0
Sturgeon	Larval	Control	0.88	13.86	0
Sturgeon	Larval	2.5	2.91	45.83	0
Sturgeon	Larval	2.5	2.91	45.83	0
Sturgeon	Larval	2.5	2.91	45.83	0
Sturgeon	Larval	5	4.8	75.59	0
Sturgeon	Larval	5	4.8	75.59	0
Sturgeon	Larval	5	4.8	75.59	0
Sturgeon	Larval	10	10.67	168.03	2
Sturgeon	Larval	10	10.67	168.03	2
Sturgeon	Larval	10	10.67	168.03	1
Sturgeon	Larval	20	22.61	356.06	4
Sturgeon	Larval	20	22.61	356.06	1
Sturgeon	Larval	20	22.61	356.06	1
Sturgeon	Larval	40	38.84	611.65	6
Sturgeon	Larval	40	38.84	611.65	5
Sturgeon	Larval	40	38.84	611.65	6
Sturgeon	Swim-up	Control	0.25	3.94	0
Sturgeon	Swim-up	Control	0.25	3.94	1
Sturgeon	Swim-up	Control	0.25	3.94	1
Sturgeon	Swim-up	2.5	2.45	38.58	1
Sturgeon	Swim-up	2.5	2.45	38.58	0
Sturgeon	Swim-up	2.5	2.45	38.58	1
Sturgeon	Swim-up	5	6.87	108.19	2
Sturgeon	Swim-up	5	6.87	108.19	1
Sturgeon	Swim-up	5	6.87	108.19	2
Sturgeon	Swim-up	10	15.12	238.11	4
Sturgeon	Swim-up	10	15.12	238.11	2
Sturgeon	Swim-up	10	15.12	238.11	3
Sturgeon	Swim-up	20	24.15	380.31	3
Sturgeon	Swim-up	20	24.15	380.31	4
Sturgeon	Swim-up	20	24.15	380.31	4
Sturgeon	Swim-up	40	36.51	574.96	8

Sturgeon	Swim-up	40	36.51	574.96	7
Sturgeon	Swim-up	40	36.51	574.96	9
Sturgeon	Juvenile	Control	1.01	15.91	0
Sturgeon	Juvenile	Control	1.01	15.91	0
Sturgeon	Juvenile	Control	1.01	15.91	0
Sturgeon	Juvenile	2.5	3.54	55.75	0
Sturgeon	Juvenile	2.5	3.54	55.75	0
Sturgeon	Juvenile	2.5	3.54	55.75	0
Sturgeon	Juvenile	5	6.57	103.46	0
Sturgeon	Juvenile	5	6.57	103.46	0
Sturgeon	Juvenile	5	6.57	103.46	0
Sturgeon	Juvenile	10	13.15	207.09	1
Sturgeon	Juvenile	10	13.15	207.09	1
Sturgeon	Juvenile	10	13.15	207.09	0
Sturgeon	Juvenile	20	26.42	416.06	3
Sturgeon	Juvenile	20	26.42	416.06	1
Sturgeon	Juvenile	20	26.42	416.06	0
Sturgeon	Juvenile	40	45.84	721.89	10
Sturgeon	Juvenile	40	45.84	721.89	7
Sturgeon	Juvenile	40	45.84	721.89	8
Sturgeon	Juvenile	80	85.58	1347.72	10
Sturgeon	Juvenile	80	85.58	1347.72	10
Sturgeon	Juvenile	80	85.58	1347.72	10
Trout	Larval	Control	0.78	12.28	1
Trout	Larval	Control	0.78	12.28	0
Trout	Larval	Control	0.78	12.28	0
Trout	Larval	5	6.41	100.94	0
Trout	Larval	5	6.41	100.94	0
Trout	Larval	5	6.41	100.94	0
Trout	Larval	10	11.54	181.73	0
Trout	Larval	10	11.54	181.73	0
Trout	Larval	10	11.54	181.73	0
Trout	Larval	20	21.91	345.04	1
Trout	Larval	20	21.91	345.04	1
Trout	Larval	20	21.91	345.04	0
Trout	Larval	40	43.56	685.98	4
Trout	Larval	40	43.56	685.98	5
Trout	Larval	40	43.56	685.98	2
Trout	Larval	80	74.41	1171.81	13
Trout	Larval	80	74.41	1171.81	18
Trout	Larval	80	74.41	1171.81	12
Trout	Swim-up	Control	0.89	14.02	0
Trout	Swim-up	Control	0.89	14.02	0

Trout	Swim-up	Control	0.89	14.02	0
Trout	Swim-up	5	4.81	75.75	0
Trout	Swim-up	5	4.81	75.75	0
Trout	Swim-up	5	4.81	75.75	0
Trout	Swim-up	10	9.98	157.17	0
Trout	Swim-up	10	9.98	157.17	0
Trout	Swim-up	10	9.98	157.17	0
Trout	Swim-up	20	21.76	342.68	1
Trout	Swim-up	20	21.76	342.68	1
Trout	Swim-up	20	21.76	342.68	2
Trout	Swim-up	40	37.78	594.96	2
Trout	Swim-up	40	37.78	594.96	1
Trout	Swim-up	40	37.78	594.96	2
Trout	Swim-up	80	85.71	1349.76	8
Trout	Swim-up	80	85.71	1349.76	15
Trout	Swim-up	80	85.71	1349.76	14
Trout	Juvenile	Control	0.44	6.93	0
Trout	Juvenile	Control	0.44	6.93	0
Trout	Juvenile	Control	0.44	6.93	0
Trout	Juvenile	5	5.78	91.02	0
Trout	Juvenile	5	5.78	91.02	0
Trout	Juvenile	5	5.78	91.02	0
Trout	Juvenile	10	11.51	181.26	0
Trout	Juvenile	10	11.51	181.26	0
Trout	Juvenile	10	11.51	181.26	0
Trout	Juvenile	20	18.98	298.90	0
Trout	Juvenile	20	18.98	298.90	1
Trout	Juvenile	20	18.98	298.90	2
Trout	Juvenile	40	43.52	685.35	1
Trout	Juvenile	40	43.52	685.35	3
Trout	Juvenile	40	43.52	685.35	3
Trout	Juvenile	80	89.14	1403.78	6
Trout	Juvenile	80	89.14	1403.78	6
Trout	Juvenile	80	89.14	1403.78	4

Table C3.S 2. Water quality parameters analysed throughout the study (n = 180)

Date	Metal	Temperature	pH	Dissolved oxygen (%)	Hardness (as CaCO3)	Alkalinity
11-Jul-17	Cu	13.3	7.7	91.1	~60	~40
11-Jul-17	Cu	13.4	7.6	92.4	~60	~40
11-Jul-17	Cu	13.5	7.5	90.0	~60	~40
11-Jul-17	Cu	13.4	7.7	92.4	~60	~40
11-Jul-17	Cu	13.5	7.3	92.5	~60	~40
11-Jul-17	Cu	13.6	7.3	93.1	~60	~40
11-Jul-17	Cu	13.5	7.3	91.0	~60	~40
11-Jul-17	Cu	13.4	7.5	96.4	~60	~40
11-Jul-17	Cu	13.4	7.6	91.1	~60	~40
11-Jul-17	Cu	13.4	7.4	92.8	~60	~40
11-Jul-17	Cu	13.5	7.5	94.0	~60	~40
11-Jul-17	Cu	13.1	7.3	92.3	~60	~40
12-Jul-17	Cu	13.2	7.4	93.7	~60	~40
12-Jul-17	Cu	13.2	7.6	91.4	~60	~40
12-Jul-17	Cu	13.3	7.6	89.1	~60	~40
12-Jul-17	Cu	13.6	7.4	92.0	~60	~40
12-Jul-17	Cu	13.7	7.5	88.9	~60	~40
12-Jul-17	Cu	13.2	7.4	92.7	~60	~40
12-Jul-17	Cu	13.5	7.5	89.6	~60	~40
12-Jul-17	Cu	13.3	7.3	90.4	~60	~40
12-Jul-17	Cu	13.5	7.6	94.0	~60	~40
12-Jul-17	Cu	13.6	7.5	91.5	~60	~40
12-Jul-17	Cu	13.5	7.6	89.6	~60	~40
12-Jul-17	Cu	13.4	7.4	94.3	~60	~40
13-Jul-17	Cu	13.4	7.3	89.8	~60	~40
13-Jul-17	Cu	13.5	7.4	92.2	~60	~40
13-Jul-17	Cu	13.2	7.5	91.9	~60	~40
13-Jul-17	Cu	13.3	7.4	94.6	~60	~40
13-Jul-17	Cu	13.5	7.5	93.1	~60	~40
13-Jul-17	Cu	13.4	7.4	93.9	~60	~40
13-Jul-17	Cu	13.2	7.5	90.7	~60	~40
13-Jul-17	Cu	13.5	7.7	90.1	~60	~40
13-Jul-17	Cu	13.3	7.6	91.1	~60	~40
13-Jul-17	Cu	13.7	7.4	91.3	~60	~40
13-Jul-17	Cu	13.4	7.5	90.8	~60	~40
13-Jul-17	Cu	13.1	7.7	94.7	~60	~40
14-Jul-17	Cu	13.4	7.3	93.9	~60	~40
14-Jul-17	Cu	13.3	7.5	89.1	~60	~40

14-Jul-17	Cu	13.3	7.5	93.2	~60	~40
14-Jul-17	Cu	13.3	7.7	89.1	~60	~40
14-Jul-17	Cu	13.7	7.4	87.6	~60	~40
14-Jul-17	Cu	13.4	7.5	94.1	~60	~40
14-Jul-17	Cu	13.6	7.5	92.0	~60	~40
14-Jul-17	Cu	13.4	7.5	94.1	~60	~40
14-Jul-17	Cu	13.3	7.6	89.0	~60	~40
14-Jul-17	Cu	13.4	7.5	93.1	~60	~40
14-Jul-17	Cu	13.4	7.4	90.5	~60	~40
14-Jul-17	Cu	13.3	7.3	93.0	~60	~40
15-Jul-17	Cu	13.4	7.4	93.3	~60	~40
15-Jul-17	Cu	13.6	7.5	91.1	~60	~40
15-Jul-17	Cu	13.5	7.6	93.9	~60	~40
15-Jul-17	Cu	13.4	7.5	92.2	~60	~40
15-Jul-17	Cu	13.5	7.5	92.9	~60	~40
15-Jul-17	Cu	13.3	7.6	92.9	~60	~40
15-Jul-17	Cu	13.3	7.5	91.9	~60	~40
15-Jul-17	Cu	13.5	7.7	93.2	~60	~40
15-Jul-17	Cu	13.4	7.4	92.2	~60	~40
15-Jul-17	Cu	13.5	7.5	90.9	~60	~40
15-Jul-17	Cu	13.6	7.7	91.4	~60	~40
15-Jul-17	Cu	13.4	7.4	92.9	~60	~40
03-Aug-17	Cu	13.4	7.4	94.9	~60	~40
03-Aug-17	Cu	13.3	7.5	93.0	~60	~40
03-Aug-17	Cu	13.2	7.4	91.4	~60	~40
03-Aug-17	Cu	13.5	7.4	91.2	~60	~40
03-Aug-17	Cu	13.3	7.7	97.8	~60	~40
03-Aug-17	Cu	13.5	7.7	92.7	~60	~40
03-Aug-17	Cu	13.4	7.4	96.4	~60	~40
03-Aug-17	Cu	13.3	7.4	93.1	~60	~40
03-Aug-17	Cu	13.4	7.5	90.9	~60	~40
03-Aug-17	Cu	13.3	7.7	93.3	~60	~40
03-Aug-17	Cu	13.3	7.8	90.6	~60	~40
03-Aug-17	Cu	13.3	7.5	94.9	~60	~40
04-Aug-17	Cu	13.5	7.4	91.9	~60	~40
04-Aug-17	Cu	13.3	7.4	92.7	~60	~40
04-Aug-17	Cu	13.5	7.7	92.0	~60	~40
04-Aug-17	Cu	13.4	7.4	88.3	~60	~40
04-Aug-17	Cu	13.4	7.6	93.1	~60	~40
04-Aug-17	Cu	13.2	7.4	90.6	~60	~40
04-Aug-17	Cu	13.5	7.5	92.8	~60	~40
04-Aug-17	Cu	13.5	7.5	86.9	~60	~40
04-Aug-17	Cu	13.7	7.5	93.1	~60	~40

04-Aug-17	Cu	13.3	7.3	92.2	~60	~40
04-Aug-17	Cu	13.2	7.6	90.3	~60	~40
04-Aug-17	Cu	13.5	7.6	91.2	~60	~40
05-Aug-17	Cu	13.6	7.5	92.3	~60	~40
05-Aug-17	Cu	13.3	7.6	90.6	~60	~40
05-Aug-17	Cu	13.5	7.4	88.7	~60	~40
05-Aug-17	Cu	13.5	7.6	88.0	~60	~40
05-Aug-17	Cu	13.3	7.5	92.5	~60	~40
05-Aug-17	Cu	13.5	7.5	90.5	~60	~40
05-Aug-17	Cu	13.4	7.6	92.9	~60	~40
05-Aug-17	Cu	13.0	7.5	90.5	~60	~40
05-Aug-17	Cu	13.4	7.5	90.8	~60	~40
05-Aug-17	Cu	13.3	7.3	91.5	~60	~40
05-Aug-17	Cu	13.5	7.4	90.0	~60	~40
05-Aug-17	Cu	13.3	7.6	92.1	~60	~40
06-Aug-17	Cu	13.6	7.4	90.8	~60	~40
06-Aug-17	Cu	13.2	7.7	89.4	~60	~40
06-Aug-17	Cu	13.5	7.7	92.7	~60	~40
06-Aug-17	Cu	13.6	7.7	90.8	~60	~40
06-Aug-17	Cu	13.6	7.5	91.7	~60	~40
06-Aug-17	Cu	13.2	7.5	92.4	~60	~40
06-Aug-17	Cu	13.6	7.5	93.2	~60	~40
06-Aug-17	Cu	13.5	7.2	89.4	~60	~40
06-Aug-17	Cu	13.4	7.8	93.8	~60	~40
06-Aug-17	Cu	13.5	7.4	91.5	~60	~40
06-Aug-17	Cu	13.6	7.5	90.4	~60	~40
06-Aug-17	Cu	13.4	7.3	92.6	~60	~40
07-Aug-17	Cu	13.4	7.6	88.5	~60	~40
07-Aug-17	Cu	13.5	7.4	91.3	~60	~40
07-Aug-17	Cu	13.4	7.5	87.2	~60	~40
07-Aug-17	Cu	13.5	7.6	89.7	~60	~40
07-Aug-17	Cu	13.5	7.5	92.0	~60	~40
07-Aug-17	Cu	13.5	7.3	90.1	~60	~40
07-Aug-17	Cu	13.2	7.6	91.7	~60	~40
07-Aug-17	Cu	13.6	7.4	91.0	~60	~40
07-Aug-17	Cu	13.4	7.4	89.9	~60	~40
07-Aug-17	Cu	13.4	7.9	91.8	~60	~40
07-Aug-17	Cu	13.3	7.5	91.4	~60	~40
07-Aug-17	Cu	13.0	7.6	87.5	~60	~40
09-Sep-17	Cu	13.4	7.4	90.8	~60	~40
09-Sep-17	Cu	13.5	7.7	94.0	~60	~40
09-Sep-17	Cu	13.5	7.3	94.2	~60	~40
09-Sep-17	Cu	13.2	7.5	93.5	~60	~40

09-Sep-17	Cu	13.4	7.3	89.7	~60	~40
09-Sep-17	Cu	13.3	7.2	92.1	~60	~40
09-Sep-17	Cu	13.1	7.6	91.6	~60	~40
09-Sep-17	Cu	13.1	7.6	90.6	~60	~40
09-Sep-17	Cu	13.6	7.3	89.8	~60	~40
09-Sep-17	Cu	13.5	7.5	89.1	~60	~40
09-Sep-17	Cu	13.5	7.6	89.7	~60	~40
09-Sep-17	Cu	13.7	7.6	91.5	~60	~40
10-Sep-17	Cu	13.5	7.6	90.8	~60	~40
10-Sep-17	Cu	13.6	7.3	88.8	~60	~40
10-Sep-17	Cu	13.2	7.5	93.9	~60	~40
10-Sep-17	Cu	13.3	7.8	92.0	~60	~40
10-Sep-17	Cu	13.3	7.3	89.8	~60	~40
10-Sep-17	Cu	13.7	7.2	91.1	~60	~40
10-Sep-17	Cu	13.4	7.3	92.9	~60	~40
10-Sep-17	Cu	13.5	7.5	89.7	~60	~40
10-Sep-17	Cu	13.4	7.4	94.9	~60	~40
10-Sep-17	Cu	13.4	7.7	93.5	~60	~40
10-Sep-17	Cu	13.4	7.3	95.4	~60	~40
10-Sep-17	Cu	13.6	7.5	93.9	~60	~40
11-Sep-17	Cu	13.5	7.5	96.6	~60	~40
11-Sep-17	Cu	13.5	7.4	93.5	~60	~40
11-Sep-17	Cu	13.4	7.5	92.4	~60	~40
11-Sep-17	Cu	13.5	7.5	89.6	~60	~40
11-Sep-17	Cu	13.3	7.6	90.2	~60	~40
11-Sep-17	Cu	13.4	7.3	93.1	~60	~40
11-Sep-17	Cu	13.3	7.5	89.9	~60	~40
11-Sep-17	Cu	13.5	7.4	88.9	~60	~40
11-Sep-17	Cu	13.0	7.5	88.0	~60	~40
11-Sep-17	Cu	13.4	7.4	92.7	~60	~40
11-Sep-17	Cu	13.6	7.3	92.5	~60	~40
11-Sep-17	Cu	13.4	7.8	92.8	~60	~40
12-Sep-17	Cu	13.3	7.6	91.3	~60	~40
12-Sep-17	Cu	13.4	7.4	91.8	~60	~40
12-Sep-17	Cu	13.2	7.6	90.1	~60	~40
12-Sep-17	Cu	13.4	7.4	86.6	~60	~40
12-Sep-17	Cu	13.1	7.5	90.6	~60	~40
12-Sep-17	Cu	13.4	7.7	93.5	~60	~40
12-Sep-17	Cu	13.1	7.6	90.9	~60	~40
12-Sep-17	Cu	13.5	7.7	93.0	~60	~40
12-Sep-17	Cu	13.6	7.5	94.3	~60	~40
12-Sep-17	Cu	13.3	7.5	89.4	~60	~40
12-Sep-17	Cu	13.5	7.7	91.8	~60	~40

12-Sep-17	Cu	13.0	7.5	88.7	~60	~40
13-Sep-17	Cu	13.4	7.4	93.6	~60	~40
13-Sep-17	Cu	13.0	7.6	89.2	~60	~40
13-Sep-17	Cu	13.4	7.5	91.8	~60	~40
13-Sep-17	Cu	13.5	7.6	88.8	~60	~40
13-Sep-17	Cu	13.6	7.6	94.0	~60	~40
13-Sep-17	Cu	13.1	7.7	92.9	~60	~40
13-Sep-17	Cu	13.2	7.2	92.2	~60	~40
13-Sep-17	Cu	13.4	7.5	95.4	~60	~40
13-Sep-17	Cu	13.5	7.7	90.7	~60	~40
13-Sep-17	Cu	13.5	7.5	95.4	~60	~40
13-Sep-17	Cu	13.4	7.6	88.8	~60	~40
13-Sep-17	Cu	13.6	7.5	93.9	~60	~40

Table C3.S 3. Effect of 24-hour exposure to waterborne Cu concentrations on whole body Na levels (µmol/g wet weight) in rainbow trout and white sturgeon. Data are presented as mean ± SEM values (n = 5). Data were analysed using three-way ANOVA. Asterisks (*) demonstrate statistically significant differences in the exposure group as compared to respective control group ($p < 0.05$).

Whole body Na levels (µmol/g wet weight) after 24 h exposure to different Cu concentrations			
		Baseline (control)	**20 µg/L**
sturgeon larval	Mean ± SEM	45.36 ± 2.17	42.06 ± 2.73
sturgeon swim-up	Mean ± SEM	55.33 ± 3.8	52.20 ± 3.99
sturgeon juvenile	Mean ± SEM	45.15 ± 3.69	43.19 ± 3.04
trout larval	Mean ± SEM	23.84 ± 0.64	22.97 ± 1.53
trout swim-up	Mean ± SEM	37.32 ± 3.03	36.54 ± 0.65
trout juvenile	Mean ± SEM	29.32 ± 2.45	28.71 ± 1.27

Table C4.S 1. Information on the primers for selected candidate reference genes

Gene Name	Forward primer	Reverse primer	Product size (bp)
b-actin	GTCACCAACTGGGACGACAT	GTACATGGCAGGGGTGTTGA	174
DRP2	TCACCCATGAAGTTGATGAGCTGA	CCGTGCAGACATAGTACAGCCTCA	176
EF1a	AGCGCAATCAGCCTGAGAGGTA	GCTGGACAAGCTGAAGGCTGAG	160
GAPDH	ACGAAGACAAGTTCGACCCC	GGATGATGTTCTGGTGGGCA	219
G6PD	GGGAAGTACGCCGATGAGAG	TGGCTTCTCCACAATCACCC	189
HPRT	GCCTCAAGAGCTACTGCAATG	GTCTGGAACCTCAAATCCTATG	256
18S	GGCGCCCCCTCGATGCTCTTA	CCCCCGGCCGTCCCTCTTAAT	189
HSP70a	CGGGAGTTGTAGCGATGAGA	CTTCCTAAATAGCACTGAGCCATAA	140

Table C5.S 1. Sequences of rainbow trout and white sturgeon oligonucleotide primers used in qPCR

Species	Target gene	Primer sequence	Reference
White sturgeon	***β-actin***	F: ACTGCAAGTGCACAGACTG R: AGGAGCAGCAGCTTTTCTTG	(Tang et al., 2016)
White sturgeon	***CAT***	F: GAACGAAGAAGAGCGCCAG R: GATGCGGCTCCCATAGTCT	(Tang et al., 2016)
White sturgeon	***GPX***	F: AGTTGATGTGAACGGGAAGG R: ACTTGGGGTCAGTCATCAGG	(Tang et al., 2016)
White sturgeon	***SOD***	F: GCAGGTCCGTGGTGATTCAT R: TTCCGATGACACAGCAAGCT	(Tang et al., 2016)
White sturgeon	***MT***	F: CCGAGCACAATGAAAATGA R: ACATCTGCTGGAAGGTGGA	(Tang et al., 2016)
White sturgeon	***HSP70***	F: TGGGCCAGAAAGTGTCCAA R: GCCCAGGTCAAAGATCAGGA	in-house transcriptomics data
White sturgeon	***DMT1***	F: TGATCCCAATCCTCACCTTC R: ATACCCTACGAAGCCCAGGT	(Tang et al., 2016)
White sturgeon	***ECaC***	F: GTGCACCTGCTGATTGAAGA R: AACATCACCAAGTTGCCCTC	(Allen et al., 2011)
Rainbow trout	***β-actin***	F: TCCTTCCTCGGTATGGAGTCTT R: ACAGCACCGTGTTGGCGTACAG	(Aegerter et al., 2005)
Rainbow trout	***CAT***	F: TGATGTCACACAGGTGCGTA R: GTGGGCTCAGTGTTGTTGAG	(Fontagné-Dicharry et al., 2015)
Rainbow trout	***GPX1a***	F: AATGTGGCGTCACTCTGAGG R: CAATTCTCCTGATGGCCAAA	(Fontagné-Dicharry et al., 2015)
Rainbow trout	***GPX4a1***	F: GAAAGGCTTCCTGGGAAATG R: CTCCACCACACTGGGATCAT	(Fontagné-Dicharry et al., 2015)
Rainbow trout	***SOD1***	F: TGGTCCTGTGAAGCTGATTG R: TTGTCAGCTCCTGCAGTCAC	(Fontagné-Dicharry et al., 2015)
Rainbow trout	***SOD2***	F: TCCCTGACCTGACCTACGAC R: GGCCTCCTCCATTAAACCTC	(Fontagné-Dicharry et al., 2015)
Rainbow trout	***MTa***	F: CATGCACCAGTTGTAAGAAAGCA R: GCAGCCTGAGGCACACTTG	(Kwong et al., 2011)
Rainbow trout	***MTb***	F: TCAACAGTGAAATTAAGCTGAAATACTTC R: AAGAGCCAGTTTTAGAGCATTCACA	(Kwong et al., 2011)
Rainbow trout	***HSP70a***	F: CGGGAGTTGTAGCGATGAGA R: CTTCCTAAATAGCACTGAGCCATAA	(Kwong et al., 2011)
Rainbow trout	***HSP70b***	F: AGGCCCAACCATTGAAGAGA R: GCAATGTCCAGCAATGCAATA	(Kwong et al., 2011)
Rainbow trout	***ECaC***	F: GGACCCTTCCATGTCATTCTTATT R: ACAGCCATGACAACTGTTTCC	(Shahsavarani et al., 2006)

Rainbow trout	***DMT1 (NRAMPb)***	F: CCTCCCCTCCGGCTTCAGAC R: GGTCCCGTAAAGGCCCAGAGTT	(Kwong et al., 2011)
Rainbow trout	***DMT1 (NRAMPg)***	F: ACCCGCTCCATCGCCATCTT R: ACCCCTCCGCCTATCTTCCACA	(Kwong et al., 2011)

Table C6.S 1. HSP70 gene expression in rainbow trout and white sturgeon across different life-stages following 96 h exposure to Cu. Data is presented as mean ± SD of fold change in gene expression (n= 6). There was no statistically significant difference between the control and exposure group in any life-stage and species. Multiple forms of HSP are not known in sturgeon but are known in trout (HSP70a and HSP70b)

Species	**Life-stage**	**Gene**	**Mean fold change (range) (in exposure group, with respect to control)**
white sturgeon	larval	*HSP70*	0.6 (0.4 – 0.8)
white sturgeon	swim-up	*HSP70*	1.8 (1.3 – 2.7)
white sturgeon	juvenile	*HSP70*	1.04 (0.8 – 1.4)
rainbow trout	larval	*HSP70a*	1.8 (1.3 – 2.4)
rainbow trout	swim-up	*HSP70a*	1.4 (0.8 – 2.5)
rainbow trout	juvenile	*HSP70a*	0.7 (0.4 – 1.3)
rainbow trout	larval	*HSP70b*	1.1 (0.5 – 2.6)
rainbow trout	swim-up	*HSP70b*	0.9 (0.5 – 1.8)
rainbow trout	juvenile	*HSP70b*	1.1 (0.7 – 1.6)

Table C6.S 2. Sequences of rainbow trout and white sturgeon oligonucleotide primers used in qPCR

Species	**Target gene**	**Primer sequence**	**Reference**
white sturgeon	***EF1a***	F: ACTGCAAGTGCACAGACTG R: AGGAGCAGCAGCTTTTCTTG	(Akbarzadeh et al., 2011)
white sturgeon	***β-actin***	F: ACTGCAAGTGCACAGACTG R: AGGAGCAGCAGCTTTTCTTG	(Tang et al., 2016)
white sturgeon	***MT***	F: CCGAGCACAATGAAAATGA R: ACATCTGCTGGAAGGTGGA	(Tang et al., 2016)

white sturgeon	***HSP70***	F: TGGGCCAGAAAGTGTCCAA R: GCCCAGGTCAAAGATCAGGA	in-house transcriptomics data
rainbow trout	***ß-actin***	F: TCCTTCCTCGGTATGGAGTCTT R: ACAGCACCGTGTTGGCGTACAG	(Aegerter et al., 2005)
rainbow trout	***EF1a***	F: AGCGCAATCAGCCTGAGAGGTA R: GCTGGACAAGCTGAAGGCTGAG	(Bobe et al., 2006)
rainbow trout	***MTa***	F: CATGCACCAGTTGTAAGAAAGCA R: GCAGCCTGAGGCACACTTG	(Kwong et al., 2011)
rainbow trout	***MTb***	F: TCAACAGTGAAATTAAGCTGAAATACTTC R: AAGAGCCAGTTTTAGAGCATTCACA	(Kwong et al., 2011)
rainbow trout	***HSP70a***	F: CGGGAGTTGTAGCGATGAGA R: CTTCCTAAATAGCACTGAGCCATAA	(Kwong et al., 2011)
rainbow trout	***HSP70b***	F: AGGCCCAACCATTGAAGAGA R: GCAATGTCCAGCAATGCAATA	(Kwong et al., 2011)

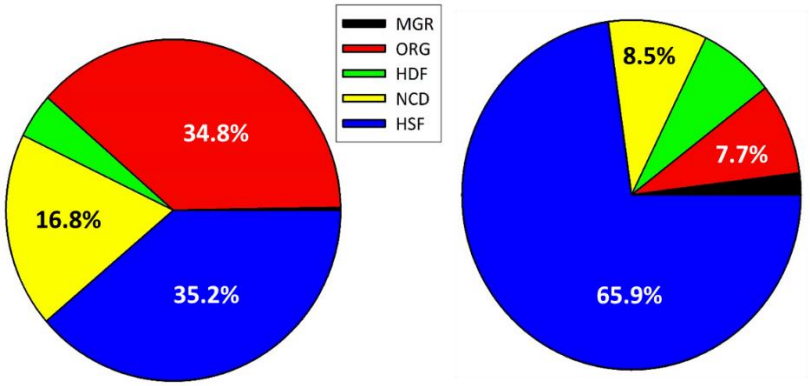

Figure C7.S 1. Relative contribution of different subcellular fractions in total Cd accumulation in rainbow trout (left) and white sturgeon (right). MGR: metal rich granules; ORG: organelles; HDF: heat denatured fraction; NCD: nuclei and cell debris; HSF: heat stable fraction