PHYSIOLOGICAL AND TOXICOLOGICAL EFFECTS OF SELENIUM AND MERCURY MIXTURES IN BROOK TROUT (*SALVELINUS FONTINALIS*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Dedication

To my Parents: Thank you for all your support and encouragement.

Abstract

Addition of Se to systems impacted by Hg has been considered for a remediation strategy because there is evidence that Se decreases the accumulation and toxicity of Hg. However the current understanding of the potential adverse impacts of this strategy is limited. The present study investigated the species differences in the effects of Se/Hg mixtures in brook trout and rainbow trout. Fish maintained in the laboratory were fed Se-enriched diets, received a single injection of $HgCl_2$ and were sampled on Day 28 of the exposure. Species differences in tissue levels of Se and Hg were detected, along with Se preventing the adverse effects of Hg on oxidative stress and thyroid function. Differences in Se and Hg compartmentalization and feeding behaviour in the laboratory may have caused the differences in the responses to the Se/Hg mixtures. Data on Se and Hg interactions and species differences are important for water quality management.

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List of Symbols and Abbreviations

Symbol	Definition
AChE	acetylcholinesterase
ANOVA	analysis of variance
BIA	biological impedance analysis
BT	brook trout
bw	body weight
GSH	reduced glutathione
Hg	mercury
HSI	hepatosomatic index
ICP-MS	inductively coupled plasma mass spectrometry
ip	intraperitoneal
LPO	lipid peroxidation
MDA	malondialdehyde
med	medium
MeHg	methylmercury
RT	rainbow trout
Se	selenium
SeMet	selenomethionine
SGR	specific growth rate
T3	triiodothyronine
T4	thyroxine
THg	total mercury
ww	wet weight

CHAPTER 1: Mercury and Selenium Interactions in Fresh Water Organisms:

A Literature Review

Introduction

Pollution from anthropogenic sources and its potential impact, particularly in freshwater systems, is a worldwide concern. Resource development and fossil fuel powered industries emit a wide range of contaminants into the environment, and coal mining and coal burning have been shown to release the essential trace element selenium (Se) into the aquatic environment (Hu et al. 2009). The recent energy crisis led to a substantial increase in coal mining activities. Even though Se is essential, an increase in concentrations of Se slightly above the homeostatic range can result in toxic effects in organisms. Fossil fuel powered industries can also increase the concentration of Hg in aquatic systems, contaminating fish with Hg levels above those appropriate for humans to consume (Bjerregaard et al. 1999). Aquatic systems may therefore be contaminated with both Se and Hg, as has been documented in the region of Sudbury, Ontario Canada, where years of smelting and mining activities affected lakes (Keller et al. 1992), resulting in increased concentrations of Se and Hg (Chen et al. 2001, Pyle et al. 2005, Belzile et al. 2006).

The co-occurrence of Se and Hg in many systems impacted by anthropogenic activities led to studies of the potential interaction between these two elements. An early study by Parizek and Ostadalo (1967) reported Se to have an antagonistic, or protective, effect on the mortality in rats caused by exposure to Hg. Subsequent studies reported Se exposure to decrease Hg accumulation in different aquatic organisms and suggested that increasing Se concentrations in aquatic systems could be a possible remediation approach

to decrease elevated concentrations of Hg (Rudd et al. 1980, Turner and Rudd 1983, Paulsson and Lundbergh 1991). Several studies have also reported Se to decrease the toxic effects of Hg in fish (Arabi and Alaeddini 2005, Cogun et al. 2012, Mulder et al. 2012).

Numerous studies investigated the effects of the interaction between Se and Hg in different organisms and the growing literature on this topic has been reviewed by Pelletier (1986), Cuvinaralar and Furness (1991), and Yang et al. (2008). Yang et al. (2008) discussed the interactions between Se and different molecular forms of Hg and suggested mechanisms of antagonism in mammals, birds and fish. All of the studies reviewed by Yang et al. (2008) on birds and fish provided evidence that suggests an antagonistic interaction of Se on Hg. However, there are some studies that suggest that the co-exposure of Se and Hg can cause adverse effects. Also, Yang et al. (2008) reviewed studies in the laboratory and field for mammals and birds but did not include laboratory studies with fish. The objective of the current review is to provide an update on the literature investigating the interaction between Se and Hg in aquatic species and identify the current knowledge gaps.

Selenium

Sources

Selenium (Se) is an essential element that has antioxidant health benefits, however, slightly above homeostatic concentrations, Se can become toxic (Chapman et al. 2010). Main sources of Se contamination are processes associated with mining, agriculture and industry (Hamilton 2004, Lemly 2004, Hu et al. 2009). Due to the

growing demand for energy, mining activities have increased, subsequently increasing the loading of Se to the aquatic environment (Miller et al. 2009a).

Coal mining is a major source of Se contamination as many coal mining related activities including removing and processing coal, can result in the leaching of Se into aquatic environments (Lemly 2004). Another source of Se in the aquatic environments is the disposal of Se rich slurried fly ash generated by coal burning powerplants (Southworth et al. 1994, Southworth et al. 2000).

Agricultural processes such as irrigation and subsurface drainage are also a large contributor to the release of Se to the environment in areas underlain with seleniferous shale (Lemly 1993, Hu et al. 2009). Regions that have high salt content in soils need subsurface drainage systems to remove salts after the water evaporates; therefore, drainage water may become contaminated with high levels of Se which can be deposited into aquatic systems (Presser and Ohlendorf 1987, Lemly 1993, Presser 1994, Lemly 2004).

Additionally, metal smelters such as copper smelters, can increase Se pollution (Nriagu and Wong 1983). Selenium can be released through disposal of wastes or emitted into the air which can result in contamination of nearby and distant aquatic systems (Small et al. 1981, Nriagu and Wong 1983, Lemly 2004). Due to the many anthropogenic sources of Se and an increase in Se pollution, there is an urgent need for increased monitoring, revision of regulations and more effective technologies in order to minimize the long lasting impacts of this contaminant on the aquatic environment.

Environmental Fate

Selenium cycles in aquatic systems by several different processes (Lemly 1999). Selenium is present in water mostly as selenite (SeO_3^{2-}) or selenate (SeO_4^{2-}) (Cooke and Bruland 1987, Presser and Ohlendorf 1987, Fan et al. 2002) and becomes available to the biota as Se species are oxidized or methylated (Lemly 1999). Selenium in sediments can enter the food chain through uptake up by plants and other organisms (Lemly 1999). Selenomethionine (SeMet), a seleno amino acid, is the dominant form of Se in foods and can bioaccumulate in the food chain (Cooke and Bruland 1987, Fan et al. 2002, Ralston and Raymond 2010). Lower level organisms can consume Se containing particles and Se can transfer up the food chain to higher level consumers (Fan et al. 2002, Chapman et al. 2010), causing toxicity in more sensitive higher level species (Lemly 1999, Chapman et al. 2010). Even if the input of Se to the aquatic system is discontinued, toxicity may persist because of the high levels in food chains and the cycling of Se from the sediment and detritus (Lemly 1997a, 1999; Chapman et al. 2010).

Metabolism

The uptake of Se by fish occurs primarily through diet and to some extent from the water (Dallinger et al. 1987, Hamilton 2004). The absorption of Se in the intestines occurs at a high rate (Hilton et al. 1982) and different chemical forms of Se have been suggested to be absorbed by different transport systems (Ralston and Raymond 2010). For example, SeMet may be absorbed by an amino acid transporter in the intestines of fish (Bakke et al. 2010). Selenium is distributed throughout the body via the blood to different organs (Hilton et al. 1982). In the liver, Se may be metabolized, stored, eliminated (Hilton et al. 1982) or incorporated into proteins. Selenomethionine is

metabolized by a process similar to methionine and can be assimilated into proteins as SeMet or metabolized to selenocysteine which can also be incorporated into proteins (Kajander et al. 1991, Misra et al. 2012c). Elimination of Se can occur via fecal and urinary excretion (Hilton et al. 1982); however, increased exposure to Se may overburden elimination mechanisms and result in accumulation of Se in organs such as the liver (Hilton et al. 1982).

Mechanism of Toxicity

The toxicity of Se may occur as a result of different mechanisms. Substitution of Se for sulphur (S), resulting in improper synthesis and folding of proteins, has been hypothesized as the cause of teratogenic deformities in egg-laying vertebrates including fish (Lemly 1997b, Chapman et al. 2010). However, more recent evidence suggested oxidative stress as an important mechanism of toxicity (Palace et al. 2004, Spallholz et al. 2004, Misra and Niyogi 2009). Selenomethionine itself does not directly elicit oxidative stress, however, in the presence of the enzyme methioninase, SeMet is converted into methylselenol which redox cycles with glutathione and generates toxic superoxide radicals (Palace et al. 2004, Spallholz et al. 2004). Fish are among the organisms most vulnerable to the toxicity of Se (Lemly 2004).

Toxic Effects

In fish the main toxic effect and biological indicator of Se toxicity is teratogenicity (Chapman et al. 2010), evident by deformities at early life stages (Lemly 1997b, Holm et al. 2005). In fish and other egg-laying vertebrates, Se can be maternally transferred during vitellogenesis, stored in the yolk until hatching and then taken up by the developing embryo via yolk absorption (Lemly 2002, Holm et al. 2005). The

deformities that subsequently occur in fish include irregular curvature of the spine, abnormalities in the fins and gills, and craniofacial malformations (Lemly 1997b, Pilgrim 2012). In severe and chronic cases of Se exposure, such as in Belews Lake, North Carolina, even adult fish exhibited a range of toxic effects including gill swelling, altered hematology, presence of cataracts, alterations in the liver, kidney, heart and ovaries, and reproductive failure (Lemly 2002). Other effects have been observed in adult and juvenile fish in recent studies. In a laboratory study by Miller et al. (2007), acute exposure to selenite caused a stress response indicated by an increase in plasma cortisol and plasma glucose in juvenile rainbow trout (*Oncorhynchus mykiss*). Similarly, adult zebrafish (*Danio rerio*) fed high concentrations of SeMet had elevated cortisol, higher whole body triglycerides, increased glycogen levels and lower swimming speed (Thomas and Janz 2011). Species-specific effects also occur, and rainbow trout are more sensitive to Se-induced cortisol impairment than brook trout (*Salvelinus fontinalis)* (Miller and Hontela 2011). There is extensive evidence for the toxic effects of Se at concentrations above the homeostatic requirements. However, most of the studies investigated the effects of Se as a single toxicant, even though in many impacted aquatic systems Se co-occurs with other pollutants, especially Hg (Yang et al. 2008).

Mercury

Sources

Mercury (Hg), a nonessential element, is a pollutant of great concern (Harada 1995). The knowledge gained from the Minamata Bay, Japan disaster has led to greatly diminished point source emissions; however, Hg is still being released into the environment by many sources (Ditri 1991).

Current sources of Hg emission are a result of natural processes and anthropogenic activities. Natural releases of mercury include degassing of the earth's crust and mantle, wild fires and eruptions from geysers and volcanoes (Ditri 1991, Schroeder and Munthe 1998). Anthropogenic releases of Hg into the environment include fossil fuel combustion, the production of mercury, cement and caustic soda, and waste disposal (Pirrone et al. 2009). Once released Hg can persist in the environment, be transported long distances, cycle in different environmental compartments and go through multiple transformations (Schroeder and Munthe 1998).

Environmental Fate

The emission of Hg into the atmosphere is a global issue as this contaminant can be readily transported (Pirrone et al. 2010). Different forms of Hg are present in the atmosphere and can vary in their distribution (Schroeder and Munthe 1998). The removal of different chemical forms of Hg from the atmosphere may occur via wet and dry deposition, and atmosphere and surface exchanges (Schroeder and Munthe 1998). The removal is affected by the concentration of Hg, the type of surfaces it exchanges with and the chemical reactions it may undergo (Schroeder and Munthe 1998, Vette et al. 2002). The emission of Hg to the atmosphere and the deposition can impact both neighbouring and remote aquatic systems.

Once in the aquatic environment, Hg may undergo different transformations including its conversion to the highly toxic methylmercury CH_3Hg^+ (MeHg) (Ullrich et al. 2001). Methylation of Hg occurs in sediments and to some degree in the water column (Furutani and Rudd 1980, Korthals and Winfrey 1987, Hintelmann et al. 1995). Sulphate reducing bacteria are thought to be the main methylators of Hg in sediments (Compeau

and Bartha 1985, Gilmour et al. 1992, Ullrich et al. 2001). Methyl Hg can be taken up by aquatic organisms and transferred up trophic levels (Watras et al. 1998, Kim and Burggraaf 1999, Kidd et al. 2012), therefore predatory fish, a component in the diets of animals, including humans, may accumulate Hg and increase the risk of exposure to potentially harmful levels of Hg. The risk of Hg exposure to humans through the consumption of fish has resulted in consumption guidelines set at 0.5 mg/kg (Schroeder and Munthe 1998).

Metabolism

Aquatic organisms are exposed to Hg mostly through consumption of contaminated prey species and, to a lesser extent, contaminated water (Trudel and Rasmussen 2006, Bjerregaard et al. 2011). After ingestion, MeHg is readily absorbed in the gut, where it can enter the blood, bind and diffuse into cells and be distributed throughout the organism (Boudou and Ribeyre 1985, Boudou et al. 1991, Handy 1996, Bridges and Zalups 2005, Hoyle and Handy 2005). It has also been suggested that inorganic and organic forms of Hg mimic amino acids, enabling them to cross cell membranes (Bridges and Zalups 2005, Broniatowski and Dynarowicz-Latka 2009). The molecular mimicry of inorganic and organic Hg, and the subsequent transport to tissues such as the kidney, intestine, liver, and placenta has been reviewed by Bridges and Zalups (2005). Natural or artificial exposure to Hg can result in accumulation in the muscle, liver (Bjerregaard et al. 1999, Bjerregaard et al. 2011), brain (Branco et al. 2011), gills, kidneys, spleen, and blood of different fish (Boudou and Ribeyre 1985). The elimination of Hg can occur via slow elimination and longer half-life when stored in the muscle and fast elimination and shorter half-life in the liver and kidneys (Trudel and

Rasmussen 1997, Van Walleghem et al. 2007). Furthermore, the total accumulation of Hg in an organism is a result of the total uptake and elimination (Reinfelder et al. 1998), therefore, increased or prolonged exposure to Hg may result in greater accumulation and subsequent toxic effects.

Mechanism of Toxicity

Mercury can cause toxicity to most organisms but the severity depends on the chemical form of Hg, and the length and route of exposure (Bridges and Zalups 2005). Mercury causes a range of cell injury and cell death; it can bind to and decrease the availability of sulfhydryl groups, disrupting key biochemical pathways, which may lead to oxidative stress (MonnetTschudi et al. 1996, Son et al. 2001, Gopal 2003, Broniatowski and Dynarowicz-Latka 2009)

Mercury is a well-known neuro-toxicant and as discussed in a review by Aschner and Aschner (1990), MeHg can cross the blood brain barrier and cause toxic effects. Methylmercury can also transfer across the placental barrier and accumulate in a developing brain resulting in toxicity (Wolfe et al. 1998). In multiple studies conducted on rats, the suggested mechanism for toxicity of MeHg in neurons and cerebellar granule cells was oxidative stress, impaired neurotransmission (Godefroy et al. 2012), and the disruption of calcium channels leading to cell death (Sarafian and Verity 1991, Busselberg 1995, Limke and Atchison 2002). Mercury is also a reproductive toxicant (Crump and Trudeau 2009). Effects on reproduction may be caused by Hg-induced disruption of the hypothalamic-pituitary-gonadal axis, altered developmental and cellular division, and cell death (Crump and Trudeau 2009).

Toxic Effects

Methylmercury is considered the most toxic form of Hg and the most commonly detected form in fish tissues (Cappon and Smith 1981). Aquatic organisms are exposed to MeHg mostly through contaminated diet which can result in increased accumulation in aquatic organisms at higher trophic levels (Watras et al. 1998, Kim and Burggraaf 1999, Bjerregaard et al. 2011, Kidd et al. 2012). Numerous laboratory studies examined the toxicity of MeHg in different fish species and documented a range of damaging effects. Laboratory studies with rainbow trout (*Oncorhynchus mykiss*) exposed to MeHg reported a decrease in fertilizing ability of sperm (Billard and Roubaud 1985), immune cell impairment (Voccia et al. 1994), an increase in plasma cortisol during acute exposure and an impairment of the stress response during chronic exposure, an increase in plasma thyroxine (Bleau et al. 1996), and an increase in oxidative stress in gill tissue (Arabi and Alaeddini 2005). Fathead minnow (*Pimephales promelas*) fed MeHg in laboratory studies had lower plasma testosterone and estradiol, a decrease in the development of gonads in females, reduced spawning and altered spawning behaviour (Drevnick and Sandheinrich 2003, Sandheinrich and Miller 2006). In a field study, high concentrations of Hg were linked to lower plasma thyroid hormones in brown trout (*Salmo trutta*) (Mulder et al. 2012). Interestingly, some adverse effects of Hg exposure were alleviated by co-exposure to Se (Arabi and Alaeddini 2005, Mulder et al. 2012).

Mercury and Selenium Interaction

Hg-Se Interactions

Selenium interacts with other trace elements including arsenic, cadmium, copper, lead and mercury (Chapman et al. 2010). The most investigated at this time and potentially most environmentally relevant interaction is between Se and Hg (Yang et al. 2008). As early as 1967, Parizek and Ostadalo (1967) reported lower mortality in rats that were injected with selenite and mercuric chloride, compared to rats injected with mercuric chloride and mercuric chloride and sulphur (Parizek and Ostadalo 1967). There have been many subsequent studies investigating the interaction between Se and Hg in terrestrial, marine and freshwater organisms (reviewed by Cuvinaralar and Furness, 1991; Yang et al. 2008).

In the review by Cuvinaralar and Furness (1991), key concepts of Se and Hg interactions were discussed, including the interactions between different forms of Se and Hg, the proposed mechanisms for the antagonistic effects, and the evidence for synergistic effects. Five mechanisms that may explain the antagonistic interaction between Se and Hg were proposed based on evidence in the literature: 1) excretion and redistribution of Hg in the presence of Se, 2) competition between Se and Hg for binding sites, 3) complex formation between the two elements, 4) Se-induced conversion of Hg from a highly toxic to less toxic species, and 5) the prevention of oxidative damage (Cuvinaralar and Furness 1991). More recently, a novel mechanism for the protective effect of Se was proposed by Ralston and Raymond (2010). Sequestration of Se by Hg, due to the high chemical affinity between Se and Hg, decreases the levels of available Se and the production of selenoproteins. Therefore, Ralston and Raymond (2010) suggest

that the protective effect of supplemental Se against Hg toxicity is to provide adequate Se levels to maintain normal function of the selenoproteins.

Yang et al. (2008) discussed the antagonism of different species of Se and Hg in different organisms and presented the results of numerous studies investigating the antagonistic effect of Se on Hg in mammals, birds, and aquatic organisms. They also discussed the mechanisms that may explain an antagonistic interaction in the aquatic environment, including the formation of Hg-Se complexes that may prevent or limit Hg from being methylated within the aquatic system, Se binding to methylated Hg and promoting demethylation, and limiting retention of MeHg due to higher Se accumulation (Yang et al. 2008). All of the studies reviewed by Yang et al. (2008) on birds and fish provided evidence that suggests an antagonistic interaction of Se on Hg. However, there are some studies that suggest that the co-exposure of Se and Hg can cause adverse effects. It has been suggested that Se could be used as a remediation treatment of Hg contaminated waters (Paulsson and Lundbergh 1991). However, addition of Se to Hg-contaminated lakes as a remediation measure is a highly controversial topic. Moreover, the research on Se and Hg interactions is vast and ongoing and the results of numerous recent studies have not been examined by earlier reviews. Therefore, a current review is needed to present recent field and laboratory studies that provide evidence for the presence or absence of antagonism between Se and Hg.

Recent Studies and Toxic Effects

A summary of recent studies on fish and aquatic birds is provided in Table 1.1. Important aspects such as the chemical form of Se and Hg investigated in the study, distinction between laboratory or field study, and the interactions and the effects of the

co-exposure to the two elements are presented. The aim of Table 1.1 is to provide an overview of the Se and Hg interactions under different exposure conditions and draw attention to the outcome of the interaction. The accumulation or elimination of Hg in tissues of the exposed organisms in the presence of Se (Table 1) has been investigated in fish and birds, with varying results, depending on factors such as the chemical form of Hg and the animal species investigated (Paulsson and Lundbergh 1991, Scheuhammer et al. 1998, Bjerregaard et al. 1999, Southworth et al. 2000, Scheuhammer et al. 2001). Tissue Hg concentrations are important to consider in assessment of habitat health and potential risks to human health, since humans may be exposed to Hg through consumption of contaminated fish. It is also important to present the differences between the response to Se and to Hg. Many studies suggest an antagonistic interaction between Se and Hg, with Se counteracting the effects of Hg; however, adverse effects have also been reported (Heinz and Hoffman 1998). Due to environmental relevance of the Se and Hg interactions, many recent Hg toxicity studies (Table 1.1) manipulate experimentally the Se concentrations to gain a better understanding of the toxic effects of Hg and the relationship with Se.

Table 1.1- Studies investigating the interaction between Se and Hg in aquatic organisms exposed to Se and Hg in the laboratory or in the field (see earlier studies on the interaction in reviews by Cuvinaralar and Furness (1991) and Yang et al. (2008)).

Field Studies					
Species	**Exposure to Se and Hg**	**Tissue**	**Evidence of Interaction and Results**	**Antagonism or No Antagonism**	**Reference**
Common loons (*Gavia immer*) and bald eagles (*Haliaeetus leucocephallus*)	Lifelong, Canadian lakes and rivers	Liver and brain	Positive correlation between liver Se and THg indicating complex formation. Lower MeHg and higher Se in brain of eagle compared to loons.	Antagonism	Scheuhammer et al. (2008)
Largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*)	Lifelong, 7 lakes near and 7 lakes far from coal-fired power plants in N. Carolina	Muscle	↓ Hg concentration and ↑Se concentration in fish near power plants.	Antagonism	Sackett et al. (2010)
Brown Trout (*Salmo trutta*)	Lifelong, in Lake Mjøsa, Norway	Liver	Se ↓the induction of metallothionein caused by Hg	Antagonism	Sormo et al. (2011)
Brown Trout	Lifelong, in Se-deprived Lake reference Lake	Muscle, liver, and plasma	Hg-induced ↓ in plasma thyroid hormones was less severe in the presence of adequate concentrations of Se	Antagonism	Mulder et al. (2012)
Sunfish (*Lepomis sp*)	Lifelong, in streams exposed to fly ash	Whole Body	Negative correlation between Se and Hg concentrations in the body	Antagonism	Reash (2012)

Laboratory Studies

Species	Exposure to Se and Hg	Tissue/System	Results	Antagonism or No Antagonism	Reference
Mallards (*Anas platyrhynchos*)	10ppm CH_3HgCl and 10 ppm SeMet diet for ~ 3 months	Health and reproduction	Combination diets prevented mortality and paralysis in adults reported in Hg only diets. Combination diets ↓ number of young produced and ↑ teratogenic effects.	Antagonism in Adults No Antagonism on reproduction or offspring	Heinz and Hoffman (1998)
Rainbow trout (*Oncorhynchus mykiss*)	Eight week exposure to 10μg/g (SeO_2) diets. Single ip injection (1 mg Hg + 1 mg CH_3Hg-Hg/kg bw).	Multiple organs	Se diets ↑ elimination of organic Hg from muscle, liver, and kidneys and inorganic Hg from kidneys only.	Antagonism	Bjerregaard et al. (1999)
Rainbow trout	60 min 0-1000 μM $HgCl_2$ + 0.1 mM Na_2SeO_3	Gill homogenates	Se alleviated Hg-induced oxidative stress by ↓ lipid peroxidation, ↓GST activity, and ↑ GSH levels.	Antagonism	Arabi and Alaeddini (2005)

Laboratory Studies Species	Exposure to Se and Hg	Tissue/System	Results	Antagonism or No Antagonism	Reference
Sacramento split tail (*Pogonichthys macrolepidotus*)	4 weeks of SeMet (0.64, 8.2, 35.0 µg/g) and MeHg diet (0.01, 0.13, 4.7, 11.7 µg/g)	Whole body and multiple organs from larvae	Se ↓ whole body Hg accumulation. Pathological alterations to the liver, kidney and gill tissue and deformities were reported in Hg and high Se combination diets.	Antagonism	Deng et al. (2008)
Goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*)	Multiple chemical forms of Se diets Se diet fed over a range of exposure lengths. Radioactively labelled $HgCl_2$ and/or CH_3HgCl exposed via ip injection or diets	Whole body	Se ↓ elimination of organic Hg but not inorganic Hg in goldfish and zebrafish.	Antagonism	Bjerregaard et al. (2011)

Laboratory Studies Species	Exposure to Se and Hg	Tissue/System	Results	Antagonism or No Antagonism	Reference
Tilapia (*Oreochromis niloticus*)	7 and 14 day water exposure to Na_2SeO_3 (0.1-2.0 mg/L) and $Hg(NO_3)_2$ (0.01-0.1mg/L)	Hematological and biochemical parameters	Se alleviated Hg-induced ↓ in hemoglobin content, red blood and white blood cell counts at 14 days	Antagonism	Cogun et al. (2012)
Mallard, chicken (*Gallus gallus*), and double-crested cormorant (*Phalacrocorax auritus*)	Single injection into the egg of SeMet (0-0.6 µg/g) and/or CH_3HgCl (0-1.6)	Embryos (7-day stage)	Co-exposure caused ↑ survival but also ↑ incidence and variety of deformities	Antagonism in survival No Antagonism in teratogenicity	Heinz et al. (2012)
White Sturgeon (*Acipenser transmontanus*)	48 hours after a single oral intubation of SeMet (500µg/kg bw) and MeHg (850 µg/kg bw)	Multiple organs	Co-exposure ↓ accumulation of Hg in the kidneys and blood and ↓Se accumulation in the kidneys, liver, brain, and muscle.	Antagonism	Huang et al. (2013)

Laboratory Studies					
Species	**Exposure to Se and Hg**	**Tissue/System**	**Results**	**Antagonism or No Antagonism**	**Reference**
Yellow Perch (*Perca flavescens*)	5-8 weeks mesocosm, Na_2SeO_3 (0-1.6 µg/ L)and $HgCl_2$ (0.3 µg/L)	Muscle and liver	Se ↓ spike muscle THg and background liver THg	Antagonism	Mailman et al. (2014)
Zebrafish (*Danio rerio*)	50 and 153 days diet SeMet (0.7mg/kg, 10 mg/kg) and CH_3HgCl (0.05 mg/kg, 12 mg/kg)	Whole body and eggs. Growth, survival, and reproduction	SeMet ↓ Hg concentrations and alleviated the effect of Hg on condition factor and survival. However, embryo survival and reproduction ↓ in SeMet and Hg groups.	Antagonism in Hg accumulation and survival. No Antagonism in reproduction	Penglase et al. (2014)
Zebrafish (*Danio rerio*)	8 weeks with a 4 week depuration period, diet SeMet (5 µg/g) and MeHg (5 and 10 µg/g)	Muscle	SeMet ↓ Hg concentrations after 8 week co exposure and after the 4 week depuration period	Antagonism	Amlund et al. (2015)

Although numerous studies investigated the Se and Hg interactions, more research is needed to elucidate areas that remain uncertain. As outlined in Table 1.1, there are some recent studies on fish and aquatic birds that provide evidence for an antagonistic interaction between Se and Hg, with Se exerting a protective (antagonistic) effect on either accumulation of Hg in the body or the toxicity of Hg. The studies by Scheuhammer et al. (2008), Sackett et al. (2010), Sormo et al. (2011), and Cogun et al. (2012) provided support for such an interaction. Other studies, however, reported an additive or synergistic interaction between Se and Hg, including effects on reproduction (Heinz and Hoffman 1998, Deng et al. 2008, Heinz et al. 2012). Therefore, additional research is needed both on multiple systems in the body and multiple life stages in order to understand the full effect Se and Hg exposure may have on an organism throughout its life.

The review of current literature (Table 1.1) also identified species differences as a topic that requires more research. Recent studies have investigated species differences in response to the exposure of Se and Hg (Scheuhammer et al. 2008, Sackett et al. 2010, Heinz et al. 2012). However, further research is needed in order to increase the understanding of Se-Hg interactions and how they affect different organisms. More importantly, species sensitivity data will provide vital information to ensure proper management and water quality guidelines, which are essential to the long term sustainability of aquatic health. Species sensitivity data will also provide useful information for the consideration of Se treatment in Hg contaminated aquatic systems where the lack of understanding may lead to severe consequences such as Se toxicity.

Thesis Project

The critical review of studies investigating the Se-Hg interaction in fish and identification of knowledge gaps led to the research project proposed in this thesis. The thesis project will investigate the Se-Hg interaction in brook trout and rainbow trout exposed to dietary selenium (SeMet) and injected Hg ($HgCl_2$) in the laboratory. A laboratory approach was chosen in order to control exposures to Se and Hg, and the size and age of the fish. Brook trout and rainbow trout were chosen for this study because they are two closely related species that inhabit aquatic systems contaminated with Se and Hg. Rainbow trout are a commonly used species in toxicology and are sensitive to many contaminants. Also, previous studies have indicated that rainbow trout are more sensitive to Se than brook trout. Fish will be exposed to Se and Hg mixtures and tissue concentrations of Se and Hg, oxidative stress and hormonal, growth and energetic status of the fish will be assessed.

The specific objectives of my research are:

1) To characterize and compare the tissue concentrations of Se and Hg in rainbow trout and brook trout exposed to Se, Hg or mixtures of Se and Hg

2) To characterize and compare the physiological and toxicological effects of Se, Hg and mixtures of Se and Hg in rainbow trout and brook trout

In order to complete these objectives I will test three predictions:

    i)   Exposure to supplemental Se will decrease Hg accumulation in muscle and liver

    ii)   Exposure to supplemental Se will alleviate the effects of Hg on stress, and hormonal, growth, and energetic status

iii)   There will be differences in the response to Se and Hg between rainbow

trout and brook trout

Two experiments will be carried out to test these predictions. First, a preliminary study

will be completed in order to investigate an appropriate, non-lethal dose of Se and Hg for

both species. Second, a follow-up experiment will be completed to investigate the

physiological and toxicological effects, as well as species differences in the response to

Se and Hg mixtures.

CHAPTER 2: Physiological and Toxicological Effects of Selenium and Mercury Mixtures in Two Salmonid Species: Rainbow Trout and Brook Trout

Introduction

Mercury (Hg), a nonessential trace element, is a well characterized pollutant (Harada 1995) released into the environment through natural processes and anthropogenic activities. Natural sources of Hg include degassing of the earth's crust and mantle, wildfires and eruptions from geysers and volcanoes (Ditri 1991, Schroeder and Munthe 1998) while anthropogenic releases include fossil fuel combustion, production of mercury, cement and caustic soda, and waste disposal (Pirrone et al. 2009). Mercury can be dispersed long distances through the atmosphere and affect remote and vulnerable ecosystems (Travnikov 2005), increasing the concentration of Hg in aquatic systems and contaminating fish with Hg levels above those appropriate for humans to consume (Bjerregaard et al. 1999).

The toxic effects of inorganic and organic Hg on aquatic organisms are well documented, (for review see Wolfe et al. 1998). In fish, exposure to Hg causes visual impairment (Hawryshyn et al. 1982), gill lesions (Daoust et al. 1984), and reduced viability of immune cells (Voccia et al. 1994). Exposure to Hg also elicits reproductive toxicity in fish, including lower sperm motility (Dietrich et al. 2010), a decrease in plasma testosterone and estradiol (Drevnick and Sandheinrich 2003), lower gonadal somatic indices, reduced spawning and altered spawning behaviour (Sandheinrich and Miller 2006); for review see Crump and Trudeau (2009). Mercury pollution and atmospheric dispersal can result in the co-occurrence of Hg with other contaminants such as Se in aquatic systems.

Unlike Hg, a metal with no biological function, selenium (Se) is an essential micronutrient, as a constituent of glutathione peroxidase, thioredoxin reductase and iodothyronine deiodinases (Ralston and Raymond 2010). However, a small increase in Se concentration above homeostatic levels leads to toxic symptoms (Misra et al. 2012a). Selenium reaches aquatic systems through the disruption of Se-rich soil, disturbance of sedimentary rock through mining coal, phosphorus and uranium and by burning fossil fuels (Hu et al. 2009). Fish and other egg laying vertebrates are among the organisms most vulnerable to Se, as it accumulates in their tissues, reaching potentially toxic concentrations (Lemly 2004). In fish the main toxic effect and biological indicator of Se toxicity is teratogenicity (Chapman et al. 2010), evident by deformities at early life stages (Lemly 1997b, Holm et al. 2005). Studies on rainbow trout have also shown effects caused by excess dietary or waterborne Se including lower growth rate and feeding, mortality, decreased cell viability (Hilton et al. 1980), increased oxidative stress in hepatocytes (Misra et al. 2012a), increased plasma glucose and cortisol, and other stress-related responses (Miller et al. 2007). Differences among fish species in sensitivity to the teratogenic effects of Se have been reported, with rainbow trout as being more sensitive compared to brook trout (Holm et al. 2005).

Even though Se can be toxic, there is evidence that Se also has a protective effect against the toxicity of Hg in fish. Arabi and Alaeddini (2005) reported that exposure of rainbow trout gill homogenates to $HgCl_2$ decreased GSH and increased malondialdehyde (MDA) concentrations, indicators of oxidative stress, but these effects were alleviated by co-exposure with Se. Similarly, Hg-induced decrease in plasma thyroid hormone levels was less severe in the presence of adequate concentrations of Se (Mulder et al. 2012).

Furthermore, a recent study with tilapia (*Oreochromis niloticus*) reported that Se decreased hematological damage caused by Hg exposure (Cogun et al. 2012).

Along with a protective effect against Hg toxicity, Se has also been documented to decrease tissue concentrations of Hg in laboratory studies. For example, rainbow trout exposed to an intraperitoneal (ip) injection of organic and inorganic Hg and administered a selenite enriched diet for 8 weeks showed a significant decrease of MeHg concentration in the muscle, liver, and kidney, compared to fish fed a normal diet (Bjerregaard et al. 1999). However, a significant decrease of inorganic Hg was only reported in the kidney compared to fish fed a normal diet (Bjerregaard et al. 1999). Similarly, Se enriched diets also decreased the retention of MeHg in goldfish (*Carassius auratus*); however, inorganic Hg was unaffected (Bjerregaard et al. 2011). In contrast, evidence for increased accumulation of inorganic Hg in the presence of Se has also been reported. For example, waterborne selenite and selenate increased the accumulation of inorganic Hg in the muscle of plaice (*Pleuronectes platessa*) (Davies and Russell 1988). Also, waterborne sodium selenate increased the uptake of mercuric chloride in minnows (*Phoxinus phoxinus*) (Cuvin and Furness 1988).

There is also evidence for decreased tissue concentrations of Hg with co-exposure to Se in field studies. A study in Tennessee, USA reported that ceasing disposal of Se rich fly ash into a quarry caused an increase in concentration of Hg in largemouth bass (Southworth et al. 1994, Southworth et al. 2000). Also, a controlled release of Se into 11 different lakes in Sweden led to a decrease in Hg concentration in perch and northern pike (Paulsson and Lundbergh 1991). Multiple studies have investigated the co-occurrence of Se and Hg near a metal smelter in Sudbury Ontario, Canada and reported

that concentrations of Hg in tissues of aquatic organisms were negatively correlated with water and tissue concentrations of Se (Chen et al. 2001, Belzile et al. 2006, Yang et al. 2010).

Different mechanisms for the antagonistic interaction between Hg and Se have been suggested. A review by Cuvinaralar and Furness (1991) outlined different mechanisms that may explain the antagonistic relationship including: 1) Se induced Hg redistribution, 2) a competition between Se and Hg for binding sites or receptors, 3) the formation of Hg-Se complexes, 4) a conversion of highly toxic MeHg to less toxic inorganic Hg, and 5) decreased oxidative damage. In a recent comprehensive review, Yang et al. (2008) examined different protective mechanisms for multiple species of Se and Hg in both mammals and aquatic organisms, reviewed studies with mammals, birds, and aquatic organisms, and discussed Se addition to lakes as a method to reduce Hg accumulation. Despite studies executed in the laboratory, under controlled exposures to both Se and Hg, and studies executed in the field, uncertainties remain about the interactions between Se and Hg in different fish species.

The purpose of this study was therefore to investigate the Se-Hg interaction in two species of salmonids, brook trout (BT) and rainbow trout (RT) exposed to Se and Hg in the laboratory under controlled conditions. The fish were subjected to a co-exposure of a single ip injection of inorganic Hg and fed an enriched diet of selenomethionine (SeMet) for two weeks, to examine multiple physiological and toxicological endpoints under exposure to Se, Hg or mixtures of Se and Hg. Tissue burdens of Se and Hg were measured to investigate the effects the interaction may have on accumulation of the two elements. Multiple growth metrics, including specific growth rate (SGR) (($\ln \text{Weight}_2 -$

ln Weight$_1$)t^{-1} x 100), hepatosomatic index (liver weight/body weight), and condition factor (weight/length^3) were analyzed to test the prediction that negative effects on growth occur as a result of exposure to Se or Hg only and are alleviated by the co-exposure to Se and Hg. Acetylcholine esterase activity was measured to investigate the potential of Se to alleviate Hg neurotoxicity. Due to the ability of Se to protect against oxidative stress and potentially cause oxidative stress at high concentrations, oxidative stress parameters (reduced glutathione GSH and lipid peroxidation LPO) were measured. Also, because Se is an essential component in iodothyronine deiodinases (Kohrle 1999) plasma thyroid hormones triiodothyronine (T3) and thyroxine (T4) levels were measured.

Materials and Methods

Experimental Fish

Juvenile BT (n=89, mean $\pm$ SE body weight 57.9 $\pm$ 10.8 g, fork length 17.7 $\pm$ 0.93 cm) and RT (n=86, body weight 81.0 $\pm$ 14.0 g, fork length 19.2 $\pm$ 1.2 cm) were acquired from Allison Creek Trout Brood Station (Coleman, Alberta, Canada). Fish were held in acclimation tanks (336 L-754 L) and fed control diets for two weeks before transfer to the experimental units. Tank water quality parameters were monitored and maintained at 12 $\pm$ 0.3 ºC, pH of 7.9-8.9, water hardness of 180-204 mg/L and 8.5 $\pm$ 0.40 mg/L of dissolved oxygen.

Pilot Study for Hg Exposure

A pilot study was conducted on BT to select the concentrations of HgCl$_2$ for the ip injection which would result in a muscle and liver tissue concentration near the human consumption guideline of 0.5 µg Hg/g. Fish (n=7) were injected with either saline (n=1) or 0.1, 0.3, and 0.5 mg Hg/g body weight (n=2). Fish were fed control diet for one week

and on Day 7 fish were euthanized with MS-222 (Sigma Aldrich, Catalogue #E10521).

Dorsal muscle sections and livers were removed, frozen at $-80\ °C$, and analyzed for Hg

(See Tissue Se and Hg Concentration).

Hg injection

Experimental fish were subjected to a single ip injection of either saline or $HgCl_2$

(Sigma-Aldrich) on Day 1. A stock solution of Hg (74.07 mg/mL) was diluted to 5

mg/mL and 3 mg/mL and injected, based on the weight of each fish, resulting in a dose of

0.5 and 0.3 mg Hg/g body weight for brook trout and rainbow trout, respectively. The

injected dose (0.5 mg/kg) was selected based on the pilot study; a lower dose (0.3 mg/kg)

was used in the RT experiment to avoid mortalities.

Experimental Se-enriched Diet Preparation

Selenium enriched diets were prepared from EWOS commercial trout chow

pellets (Micro Complete Feed for Salmonids, product 501666, EWOS Canada, Surrey,

BC) at the Food Science and Technology Labs (Crop Diversification Centre South,

Brooks, Alberta) in December 2012. The pellets were fed into an electric hammer mill

and ground into flour. The flour was mixed with distilled water and gelatin (1.8 kg of

flour, 0.9 L of distilled water, 36 g of gelatin) in a Hobart N-50 (Hobart Canada, Toronto,

ON). To prepare the Se enriched diets, 2.7 and 7.2 mL of 25 mg/mL stock SeMet (Sigma

Aldrich, Catalogue # S3132) solution was added to the mixture to create medium (Med)

(14.5 µg Se/g) and high (37.0 µg Se/g) concentration diets. To prepare the control diet,

water and gelatin only were added to the mixture. The paste was passed through a 1/8-

inch round meat grinder attachment to create long thin noodles. The noodles were placed

on long trays and dried in a Harvest Saver R-5 tray dryer (Commercial Dehydrator

Systems, Eugene, OR) at 35 ºC with maximum airflow. The dried noodles were processed into small pellets by a custom made electric noodle breaker and stored at -20ºC.

Exposure to Selenium and Mercury

Fish were injected ip with inorganic Hg or saline on Day 1 of the exposure and fed twice a day, ad libitum, standard or SeMet-enriched diet (0, 15 and 45 µg/g) for 14 days (See Se diet preparation and Hg injection). Fish were euthanized with MS-222 (Sigma Aldrich, Catalogue #E10521) on Day 14 and body length, weight, and biological impedance (See BIA) were measured. A blood sample was taken from the caudal vasculature, centrifuged at 13 000 rpm and plasma was collected and frozen in liquid nitrogen. The brain, a dorsal muscle section, and liver were removed and frozen at –80 ºC until analysis.

Analyses

Tissue Selenium and Mercury Concentration

Portions of muscle and liver samples were dried in a 60ºC oven, ground to powder (0.100g $\pm$ 0.005) and placed into 50 mL borosilicate tubes with 2 mL of concentrated, trace metal grade HNO_3 (Omnitrace ® NX0407-4) added. Tubes were placed into a dry aluminum hot block at 75ºC for 16 hours and allowed to cool to room temperature. The samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) on an Elan DRC-E. The detection limit for Se was 0.09 µg Se/g and the detection limit for Hg was 0.03 µg Hg/g. Duplicates, blanks and TORT-2 standards (National Research Council Canada) were used for quality control.

Water Hg Analysis

Tank water samples were collected in triplicate and analyzed for total Hg at the Centre for Earth Observation Science, University of Manitoba, using the EPA 1631 method and a Cold Vapor Atomic Fluorescence Spectrometry on a Tekran 2600.

Oxidative Stress Analysis

Reduced glutathione (GSH) and lipid peroxidation (LPO) were measured as indicators of oxidative stress. Livers were homogenized in phosphate buffer and separated into aliquots for analysis of GSH, LPO and protein. A Bioxytech GSH 400 kit (Catalogue #21011) was used to measure GSH (µmol GSH/mg Protein) and Bioxytech LPO-596 kit (Catalogue #21012) was used to measure LPO (µmol MDA/mg Protein), as described by Miller et al. (2007). Protein concentration was measured by a spectrophotometric assay (595 nm) using Bradford reagent (Sigma-Aldrich, Catalogue B6916).

BIA

Biological impedance analysis was used as a simple, nonlethal method to estimate condition and body lipid and water composition (Rasmussen et al. 2012), as previously described by Cox and Hartman (2005). Resistance and reactance were measured in series and used to calculate the phase angle φ (tan $\varphi = \chi cR^{-1}$) (Rasmussen et al. 2012).

Plasma Hormone Analysis

Plasma T3 (Catalogue #06b-254215), T4 (Catalogue #06b-254011) and cortisol (#07-221102) were measured using radioimmunoassay kits purchased from Medicorp (Montreal, Quebec). Assay characteristics including intra- and inter-assay variability were assessed using internal standards, as described by Hontela et al. (1995).

Acetylcholinesterase

Acetylcholinesterase (AChE) activity was measured using a spectrophotometric assay as previously described by Miller et al. (2009b). In summary, brain tissue was homogenized in phosphate buffer in a ratio of 1:3. The homogenate was centrifuged at 13 000 rpm at 5ºC for four minutes and the supernatant was collected. Following three centrifugations the remaining supernatant was diluted with phosphate buffer. The diluted supernatant and Tris Buffer (Tris Buffered Saline Tablets, Sigma T-5030) was pipetted into a 96 well microplate (Costar, 3596) in triplicate and gently agitated at room temperature for 10 minutes. DTNB (Sigma 5,5' Dithiobis (2-nitro-benzoic acid), D8130) and acethylthiochlorine iodide (AChI, Sigma minimum 98% TLC, A5751) were added and a second agitation at room temperature for 10 minutes was completed. Change in absorbance was measured at 405 nm every 2 minutes for 10 min. Assay characteristics have been previously described by Miller et al. (2009b).

Statistical Analysis

All statistical analyses was completed using R (R Development Core Team, 2012), and R studio (Version 0.98.953). A two-way analysis of variance (ANOVA) was used to compare effects and interactions of Se and Hg exposure. Analysis of Hg in tissues was compared using a one-way ANOVA among the different exposure groups. Analyses were adjusted using a Bonferroni correction to allow for multiple comparison tests.

Results

Pilot Study

The concentration of Hg in brook trout muscle and liver tissue was measured in the pilot study (Table 2.1). The concentration of Hg in both tissues on Day 7 post injection increased with the dose of Hg injected. At the highest injected dose, 0.5 mg Hg/g body weight, the concentration of Hg was measured at a concentration slightly below the consumption guideline of 0.05 ppm in the muscle tissue, and was therefore chosen as the injection dose for the main experiment. However, during the main experiment, BT injected at 0.5 mg Hg/g body weight had mortalities and therefore the dose was reduced to 0.3 mg Hg/ g body weight for RT to limit mortalities.

Brook Trout

Se Tissue Concentrations

The concentrations of Se in muscle of BT exposed to Se and Hg for 14 days are presented in Fig. 2.1A. The figure presents the results from all the treatment groups and the inset graph present the results of the Se exposure main effect. The Se exposure main effect inset graph combines all the fish exposed to each Se diet group without or with Hg. The concentration of Se in muscle increased significantly with increasing concentrations of Se enrichment in the diets (see inset graph in Fig. 2.1 A, F= 21.8, $p<0.0001$). However, Hg exposure did not have a significant effect on the concentration of Se (F=1.86, $p>0.05$), and there was no interaction between Se exposure and Hg exposure (F=5.46, $p>0.05$).

The concentrations of Se in livers of BT (n =4) exposed to Se and Hg for 14 days are presented in Fig. 2.1B. Liver Se was significantly higher in the Se Med and Se High

diet groups compared to the control (see inset graph in Fig. 2.1 B, F=13.2, $p<0.001$) Also, as observed for muscle Se, Hg exposure did not have an effect on the concentration of liver Se (F=3.45, $p>0.05$) and there was no interaction between Se exposure and Hg exposure (F=1.63, $p>0.05$).

Hg Tissue Concentrations

The concentrations of Hg in BT muscle on Day 14 are presented in Fig. 2.2A. Two weeks after the single ip injection, Hg had accumulated in the muscle of exposed BT. Since muscle Hg concentrations in treatment groups not exposed to Hg had concentrations near background, they were not included in the statistical analysis. In Hg exposed BT, Se diet exposure did not affect (F=2.23, $p>0.05$) the concentration of Hg in muscle (Fig. 2.2 A). The muscle Hg : Se molar ratios were calculated in the Control + Hg (0.76 $\pm$ 0.11), Se Med + Hg (0.60 $\pm$ 0.30), and Se High + Hg (0.69 $\pm$ 0.16) exposure groups. The muscle Hg : Se molar ratios were slightly less than 1.0 and there was no significant effect of Se exposure (One-Way ANOVA, $F_{2,41}$=3.0, $p>0.05$).

In Hg exposed BT, Se diet exposure did not affect (F= 0.21, $p>0.05$) the concentration of Hg in liver tissue (Fig. 2.2B). Since liver Hg concentrations in treatment groups not exposed to Hg were near background levels, they were not included in the statistical analysis. The liver Hg : Se molar ratios were calculated in the Control + Hg (10.6 $\pm$ 0.67), Se Med + Hg (8.59 $\pm$ 3.43), and Se High + Hg (7.14 $\pm$ 2.41) exposure groups. The liver Hg : Se molar ratios for Hg exposure groups were greater than 1.0 and there was no significant effect of Se exposure (One-Way ANOVA, $F_{2,9}$=0.51, $p>0.05$).

Oxidative Stress

Liver GSH concentrations in BT, measured as an indicator of oxidative damage, are presented in Fig.2.3. Levels of GSH were significantly higher in the Hg exposed treatment group (see inset graph Fig 2.3, F=18.1, $p<0.0001$), however, Se exposure did not affect GSH levels in BT (F=0.406, $p>0.05$) and there was no interaction between Se exposure and Hg exposure (F=1.63, $p>0.05$). Lipid peroxidation was also measured in BT as an indicator of oxidative damage in the liver; however levels of LPO were near background levels or below detection limit (data not shown).

AChE

Acetylcholine esterase activity was measured in the brain tissue of BT. Exposure to Se and Hg alone or in mixtures did not affect AChE activity (all F<2.42, all $p>0.05$), data not shown).

Thyroid Hormones

Plasma T3 and T4 concentrations were measured in BT and are presented in Fig. 2.4. Plasma T3 concentrations (Fig. 2.4A) were significantly lower in groups exposed to Hg compared to control and groups exposed to Se only (see inset graph Fig. 2.4A, F= 23.1, $p<0.0001$). Exposure to Se did not have an effect (F=2.23, $p>0.05$) and there was no interaction between Se and Hg exposure (F=2.79, $p>0.05$). However, when only control and Se exposed groups (without Hg) were compared, plasma T3 in Se High was significantly higher than the Control and Se Med exposures (One-way ANOVA $F_{2, 40}$=7.9, $p<0.01$). There was no interaction effect (F=0.474, $p>0.05$) or main effects of Se (F=0.551, $p>0.05$) or Hg exposure (F=5.16, $p>0.05$) on the concentration of plasma T4 in BT (Fig. 2.4B).

Growth

Indicators of growth, including SGR, condition factor, phase angle, (Table 2.2) and HSI (Fig. 2.5) were measured in BT. There were no main effects or interaction effects of Se and Hg exposure on BT SGR, condition factor, or phase angle (all F<3.96, all $p>0.05$). However, BT exposed to Hg had significantly higher HSI compared to control and groups exposed to Se only (see inset graph Fig. 2.5, F=42.8, $p<0.0001$). Selenium exposure alone did not have an effect on HSI (F=2.90, $p>0.05$) and there was no interaction between Se exposure and Hg exposure (F=0.348, $p>0.05$).

Rainbow Trout

Se Tissue Concentrations

The concentration of Se in muscle and liver of RT exposed to Se and Hg are presented in Fig. 2.6. The concentration of Se in muscle (Fig. 2.6A) increased significantly with increasing concentrations of Se enrichment in the diets (see inset graph Fig. 2.6A, F=42.4, $p<0.0001$). However, Hg exposure did not have a significant effect on Se levels (F=8.20, $p>0.05$) and there was no interaction between Se exposure and Hg exposure (F= 2.58, $p>0.05$).

The concentration of Se in livers of RT (n =4) exposed to Se and Hg are presented in Fig. 2.6B. Liver Se was significantly higher in the Se Med and Se High exposure groups compared to the control (see inset graph Fig. 2.6B, F=10.1, $p<0.05$). Also, as observed for muscle Se, Hg exposure did not have an effect on the concentration of liver Se (F=8.74, $p>0.05$) and there was no interaction between Se exposure and Hg exposure (F=3.26, $p>0.05$).

Hg Tissue Concentrations

The concentrations of Hg in RT muscle are presented in Fig. 2.7A. Two weeks after the single ip injection, Hg had accumulated in the muscle of exposed RT. Since muscle Hg concentrations in treatment groups not exposed to Hg had concentrations near background levels, they were not included in the statistical analysis. In Hg exposed RT, the Se diet exposure did not affect the concentration of Hg in muscle tissue (Fig. 2.7A) (F=0.368, $p > 0.05$). The muscle Hg : Se molar ratios were calculated in the Control + Hg (0.55 $\pm$ 0.07), and Se Med + Hg (0.29 $\pm$ 0.06), and Se High + Hg (0.25 $\pm$ 0.07) exposure groups. The muscle Hg : Se molar ratios for Hg exposure groups were less than 1.0 and there was no significant effect of Se exposure (One-Way ANOVA, $F_{2,35}=6.1$, $p > 0.05$).

In RT exposed to Hg, the Se diet exposure did not affect the concentration of Hg in the liver (F=0.323, $p > 0.05$). Since liver Hg concentrations in treatment groups not exposed to Hg had concentrations near background levels, they were not included in the statistical analysis (Fig. 2.7B). The liver Hg : Se molar ratios were calculated in the Control + Hg (1.2 $\pm$ 0.10), Se Med + Hg (1.2 $\pm$ 0.46), and Se High + Hg (0.85 $\pm$ 0.38) exposure groups. The liver Hg : Se molar ratios for Hg exposure groups were near 1.0 and there was no significant effect of Se exposure (One-Way ANOVA, $F_{2,9}=0.35$, $p > 0.05$).

Oxidative Stress

Reduced glutathione (GSH) was measured in RT as an indicator of oxidative damage in the liver and results are presented in Fig. 2.8. Levels of GSH were significantly higher in the Hg exposed treatment group (see inset graph Fig 2.8, F=17.7,

$p<0.05$). However, Se exposure did not affect GSH levels in RT (F=2.00, $p>0.05$) and there was no interaction between Se and Hg exposure (F=1.04, $p>0.05$).

Lipid peroxidation was also measured in RT as an indicator of oxidative damage in the liver. Levels of LPO were near background levels or below detection limit (data not shown).

AChE

Acetylcholine esterase activity was measured in RT brain tissue. Exposure to Se and Hg alone or in mixtures did not affect AChE activity (all F<1.04, all $p>0.05$, data not shown).

Thyroid Hormones

Plasma T3 and T4 concentrations were measured in RT and are presented in Fig. 2.9. Plasma T3 concentrations (Fig. 2.9A) were significantly lower in groups exposed to Hg compared to control and groups exposed to Se only (see inset graph Fig. 2.9A, F=17.5, $p<0.0001$). Exposure to Se, however, did not have an effect (F=0.282, $p>0.05$) and there was no interaction between Se and Hg exposure on plasma T3 concentrations (F=0.215, $p>0.05$). Similarly, plasma T4 concentrations (Fig. 2.9B) were significantly lower in groups exposed to Hg compared to control and groups exposed to Se only (see inset graph Fig. 2.9B, F=10.0, $p<0.01$). Exposure to Se however, did not have an effect (F=1.05, $p>0.05$) and there was no significant interaction between Se and Hg (F=0.97, $p>0.05$).

Growth

Indicators of growth were measured in RT including SGR and condition factor presented in Table 2.3 and phase angle and HSI presented in Fig. 2.10. There was no

significant interaction effect or main effects of Se exposure and Hg exposure on RT SGR or condition factor (all F<3.02, all p>0.05). However, HSI was significantly higher (F=32.7, p<0.0001) in groups exposed to Hg compared to control and groups exposed to Se only (see inset graph Fig. 2.10A). Also, phase angle or lipid content was significantly lower in groups exposed to Hg compared to control and groups exposed to Se only (see inset graph Fig. 2.10B, F=9.46, p<0.01).

Discussion

The present study was designed to investigate and test the hypothesis that Se interacts with Hg and that there are species differences in response to the interaction. Brook trout and RT were fed diets enriched with Se concentrations known to accumulate and lead to environmentally relevant Se tissue burdens (Lemly 1996, Luoma 2006, Misra et al. 2012c). The single ip injection of Hg was administered on Day 1 and the Se enriched diets were given on Day 1-14. The dose of the ip injected $HgCl_2$ was chosen based on tissue burden results from the pilot study and fish consumption guidelines. However, mortality of some BT required lowering the dose for RT. Both the Se diet and inorganic Hg ip injection resulted in dose-dependent Se and Hg tissue burdens, representative of environmental exposures in a contaminated system (Southworth et al. 2000, Sormo et al. 2011).

In the present study, increasing Se diet concentrations did not decrease Hg concentrations in the muscle or liver of BT or RT. Correspondingly, Hg exposure did not diminish Se concentrations in the muscle or liver of either species. Previous studies of Se and inorganic Hg interactions in fish provide varying and conflicting results (Bjerregaard et al. 1999). For example, in a study with caged pike exposed to Se and Hg in food and/or

water, selenite in food decreased Hg accumulation in tissues (Turner and Swick 1983). In contrast, a study on plaice reported increased accumulation of inorganic Hg in fish exposed to waterborne selenate and selenite (Davies and Russell 1988) and exposure to waterborne sodium selenate and mercuric chloride increased uptake of Hg in minnows (Cuvin and Furness 1988). Results similar to the present study were reported for rainbow trout exposed to a Se diet and an ip injection of MeHg-Cl and $HgCl_2$ (Bjerregaard et al. 1999). Inorganic Hg accumulated in the muscle and liver; however, no significant decrease of inorganic Hg was reported in the muscle and liver after 8 weeks of exposure to 1.5 ± 0.3 and 9.8 ± 1.7 µg Se/g diets (Bjerregaard et al. 1999).

The Hg : Se molar ratio was previously used to compare levels of Se and Hg in aquatic organisms. In fish, Se protects against Hg when Se is in molar excess, because there is enough Se to maintain normal functions after Se is sequestered by Hg (Ralston 2008, Peterson et al. 2009). The Hg : Se molar ratio in BT suggests that Se and Hg were near equal molar concentrations in the muscle but Hg was in molar excess of Se in the liver. In RT, the Hg : Se molar ratio suggests Se to be in molar excess of Hg in the muscle but was near equal molar concentrations in the liver. The absence of an interaction between Se and Hg on tissue burdens may have been a result of a low Se exposure in comparison to Hg exposure. However, more research is required to determine the protective ratio in different species and different tissues (Burger 2012). To further characterize the effects of co-exposure to Se and Hg in BT and RT, in addition to the concentrations of Se and Hg in muscle and liver, multiple physiological and toxicological endpoints were investigated

Liver GSH concentrations were measured, along with LPO, in BT and RT to assess oxidative stress. Mercury is a pro-oxidant and can decrease concentrations of GSH and cause oxidative stress (Chan et al. 1982, Arabi 2004, Arabi and Alaeddini 2005). In contrast, Se is a known antioxidant and is a key component in the glutathione peroxidases (GPx) antioxidant systems (Branco et al. 2011). In the present study, exposure to Hg significantly increased GSH levels in BT and RT. Similarly, a study by Atli and Canli (2008) reported an increase in GSH concentrations in tilapia exposed to Cd, Zn, and Cu. The results of the present study suggest a response to Hg was produced, but was not severe enough after two weeks to cause high levels of oxidative stress and depletion of GSH. Additionally, the LPO levels were measured near background or below detection limit, which also suggests that although an increase in GSH was detected in Hg treated groups, the defensive response (Atli and Canli 2008) was not overwhelmed by the exposures. There was no protective effect of Se observed on the Hg-induced increase in GSH. The molar ratios in liver for BT and RT suggest that Se was not in molar excess of Hg and therefore the concentration of Se may not have been high enough to provide a protective effect. In contrast, Arabi and Alaeddini (2005) reported Hg exposure decreased the concentration of GSH and increased LPO in rainbow trout gill homogenate, and co-exposure to Se protected against Hg induced oxidative stress. Selenium may protect against oxidative stress by the action of glutathione peroxidase (Rotruck et al. 1973, Sun et al. 1995, Arabi and Alaeddini 2005) which eliminates hydrogen peroxide and lipid hydroperoxides (Cohen and Hochstein 1963, Behne et al. 1996). Additional research is warranted to investigate whether higher concentration or increased exposure to Se may prevent the increase in GSH and the potential defensive response to Hg.

Mercury is a potent neurotoxicant (Aschner and Aschner 1990) and inorganic Hg has been reported to inhibit AChE activity in electric ray (*Torpedo californica*) (Frasco et al. 2007). The present study investigated Hg toxicity on AChE activity and examined whether exposure to Se would alleviate the toxic effects. However, no significant effects were observed for BT and RT brain AChE activity after exposure to Se, Hg and Se-Hg mixtures. Acetylcholinesterase in fish is more commonly used as an indicator of organophosphate contamination (Sturm et al. 1999, Sturm et al. 2007, Miller et al. 2009b, Quinn et al. 2010). Moreover, inorganic Hg is known to interact in Ellman's reaction, a common method to measure AChE activity, and may interfere with AChE measurements (Frasco et al. 2005). More research is needed to investigate the effects of Hg on AChE activity, and the development of protocols to measure AChE activity in organisms exposed to metals including Hg.

Selenium is a component in iodothyronine deiodinases which regulate the synthesis and activation of thyroid hormones (Kohrle 1999, Ralston and Raymond 2010). Brook trout and RT exposed to Hg had significantly lower plasma thyroid hormone concentrations and co-exposure to Se did not alleviate this effect of Hg. Other studies reported that exposure to Hg caused histopathological alterations to the thyroid gland and interfered with the uptake of iodide (Kirubagaran and Joy 1989, 1994), anomalies which would lead to low plasma T3 and T4. The mechanisms responsible for the low plasma thyroid hormone levels in fish exposed to Hg are not known at present, however the high affinity of Hg for Se and the formation of Hg-Se complexes could decrease Se available for the iodothyronine deiodinases (Kohrle 1999, Ralston and Raymond 2010). Similar effects of Hg on thyroid hormones were observed in a recent field study of brown trout

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by Mulder et al. (2012) where fish sampled in a lake with low Se concentrations and Hg in molar excess of Se had lower plasma thyroid hormone compared to a reference lake with adequate Se concentration. Increasing the concentration or exposure to Se may ultimately alleviate the effect of Hg on thyroid hormones regulation, however more research is required.

In both BT and RT there was no effect of Se exposure or Hg exposure on SGR and condition factor. However, while there were no effects observed in BT, Hg exposure in RT did induce a significant decrease in phase angle suggesting lower overall condition and lower lipid content in the muscle tissue (Rasmussen et al. 2012). Metabolism, and consequently lipid content, may be altered due to increased stress which may activate energy reserves in response to a stressor (Vijayan and Moon 1992, Bleau et al. 1996). However, plasma cortisol measurements were at or near background levels for both BT and RT (<10 ng/mL, data not shown). In both BT and RT, Hg induced a significant increase in HSI. In contrast to the results of the present study, a study on largemouth bass reported significantly lower HSI in fish sampled from a site highly contaminated with Hg (Friedmann et al. 2002). Furthermore, a study by Larose et al. (2008) reported a negative relationship between HSI and liver Hg of walleye. Exposure to Hg induces hyperplasia in gill epithelial cells of RT fingerlings and fry (Wobeser 1975). In the present study, exposure of juvenile trout to inorganic Hg may have caused increased liver size and Se enriched diets did not alleviate this effect. The molar ratio in liver for BT and RT indicated that Se was not in molar excess of Hg which may explain the absence of antagonism. More research is needed to determine if an even greater concentration or exposure to Se would prevent this response to Hg exposure.

Conclusion

   The present study with BT and RT was designed to investigate the interaction between Se and Hg under controlled laboratory conditions. Co-exposure to two weeks of Se enriched diets and a single ip injection of inorganic Hg led to an accumulation of Se and Hg in the muscle and liver of both species. However, increased concentrations of Se in the diet did not decrease Hg accumulation in the muscle or liver. Increased exposure to Se has been reported to decrease concentrations of organic Hg in several studies with fish (Paulsson and Lundbergh 1991, Bjerregaard et al. 1999, Belzile et al. 2006, Bjerregaard et al. 2011); however, the interaction between Se and inorganic Hg is not as clear (Turner and Swick 1983, Bjerregaard et al. 1999, Bjerregaard et al. 2011). In the present study, Se enriched diets did not alleviate the effects of Hg on HSI, GSH, and plasma thyroid hormones. The lack of an antagonistic effect of Se enrichment on Hg accumulation and toxicity may be a result of the chemical forms of Se and Hg used, the route and timing of exposure, different fish species, and the Hg : Se molar ratio (Bjerregaard et al. 2011). In fish, excess Se may protect against the toxicity of Hg (Ralston 2008, Peterson et al. 2009), however, the protective ratio for different species and tissues remains unclear (Burger 2012). In order to further investigate the potential protective effect of dietary SeMet on ip injected $HgCl_2$, an increase in the concentration of Se in tissues may be needed. This may be accomplished by increasing the concentration of Se in the diets, or by a pre-exposure to Se to increase the tissue Se concentration prior to Hg injection.

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Table 2.1 –
Muscle and liver Hg concentrations (mean $\pm$ SE) on Day 7 of a pilot study in brook trout (n=2) injected ip on Day 1.

Hg injected Dose (mg Hg/g bw)	Muscle Concentration (µg/g)	Liver Concentration (µg/g)
0.1	0.07 ± 0.008	2.21 ± 0.09
0.3	0.20 ± 0.07	12.73 ± 5.7
0.5	0.39 ± 0.08	15.26 ± 1.1
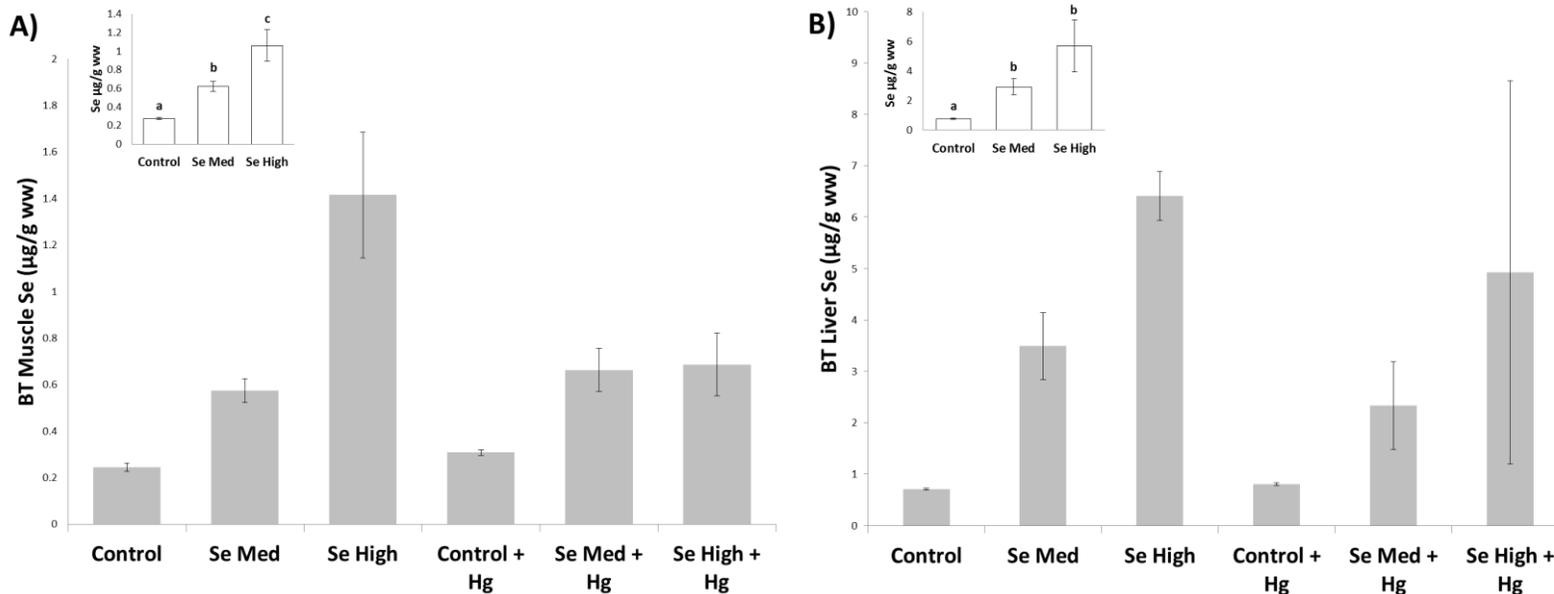
Fig. 2.1-

Concentrations of Se (mean $\pm$ SE) in A) Muscle (n=14-16) and B) Liver (n=4) on Day 14 in BT exposed to a single ip injection of HgCl$_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant interaction effect between Se exposure and Hg exposure for muscle (Two-way ANOVA, $F_{2, 83}=5.46$, $p>0.05$) or liver Se concentration (Two-way ANOVA, $F_{2, 18}=1.63$, $p>0.05$). There was a significant Se exposure effect on muscle Se (see inset graph Fig. 2.1 A) (Two-Way ANOVA, $F_{2,83}=21.8$, $p<0.0001$) and liver Se (see inset graph Fig. 1 B) (Two-Way ANOVA, $F_{2, 18}=13.2$, $p<0.001$). Letters represent significant differences between Se treatment groups. Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests.

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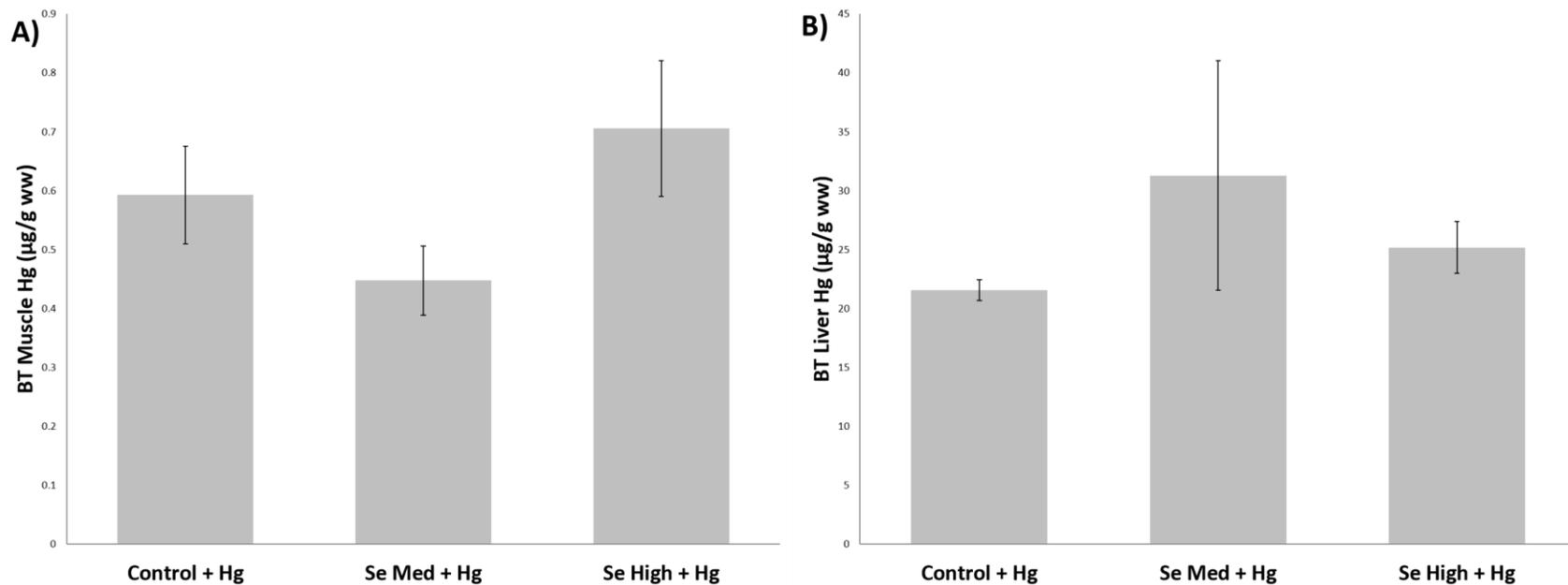
<u>Fig. 2.2 –</u>
Concentrations of Hg (mean $\pm$ SE) in A) Muscle (n=14-16) and B) Liver (n=4) on Day 14 in BT exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant Se exposure effect on muscle (One-way ANOVA, $F_{2, 41}=2.23$, $p>0.05$) or liver Hg concentrations (One-way ANOVA, $F_{2, 9}=0.21$ $p>0.05$). Analyses were adjusted using a Bonferroni correction to allow for multiple comparison tests. Concentrations of Hg in muscle and liver of BT not injected with Hg (Control, Se Med and Se High groups) were at or near background levels – data not shown.
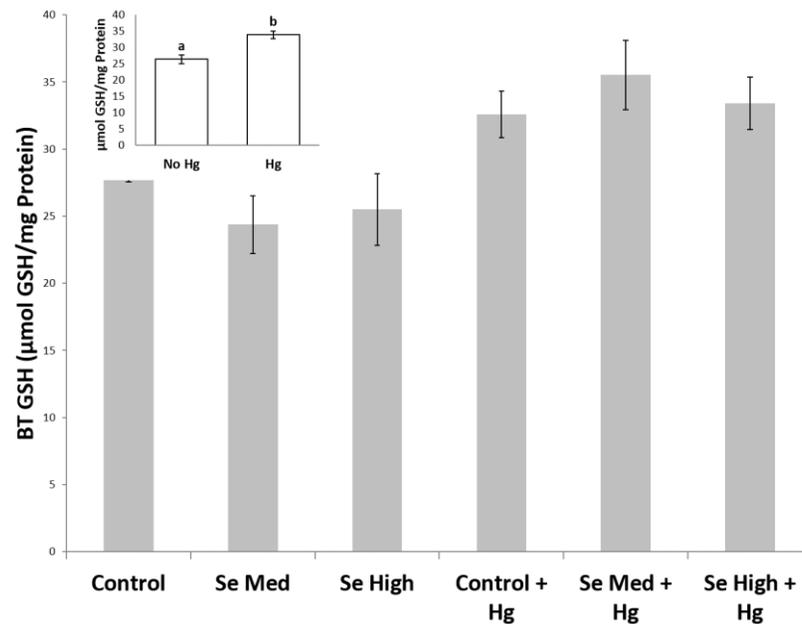
Fig. 2.3-

Liver GSH (mean $\pm$ SE) concentrations on Day 14 in BT (n=13-15) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant Se exposure effect (Two-way ANOVA, $F_{2, 79}=0.406$, $p>0.05$) or interaction effect between Se exposure and Hg exposure (Two-way ANOVA, $F_{2, 79}=1.63$, $p>0.05$). There was a significant Hg exposure effect (see inset graph, Two-Way ANOVA, $F_{2, 79}=18.1$, $p<0.0001$). Letters represent significant differences between No Hg and Hg exposure groups. Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests.
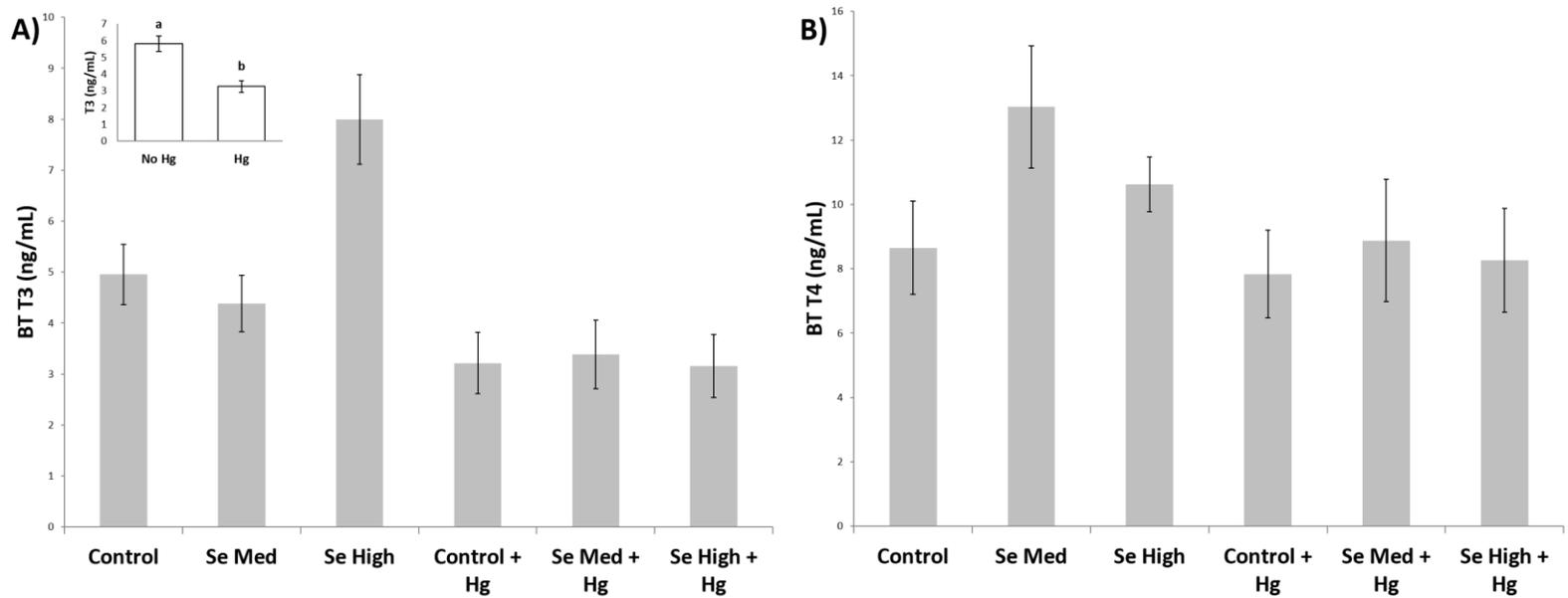
Fig. 2.4-

Plasma thyroid hormone (mean $\pm$ SE) concentrations on Day 14 in BT (n=13-16) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant interaction effect between Se exposure and Hg exposure for T3 (Two-way ANOVA, $F_{2, 81}=2.79$, $p>0.05$) and T4 (Two-way ANOVA, $F_{2, 80}=0.474$, $p>0.05$). There was a significant Hg exposure effect on T3 (see inset graph Fig. 2.4A, Two-Way ANOVA, $F_{1, 81}=23.1$, $p<0.0001$) but not T4 (Two-Way ANOVA, $F_{1, 80}=5.16$, $p>0.05$). Letters represent significant differences between No Hg and Hg exposure groups. Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests. In the T3 Se only data group, Se High is significantly different from the Control and Se Med (One-way ANOVA $F_{2, 40}=7.9$, $p<0.01$).

Table 2.2 –
Growth measured as SGR, condition factor, and phase angle (mean $\pm$ SE) on Day 14 in BT (n=13-16) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant Se or Hg exposure main effect, and there was no significant interaction effect between Se and Hg exposure (Two-way ANOVA, all F<3.96, all p>0.05). Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests.

Treatment	SGR (%/Day)	Condition Factor	Phase Angle
Control	0.49 $\pm$ 0.10	1.01 $\pm$ 0.02	17.3 $\pm$ 0.27
Se Med	0.34 $\pm$ 0.09	1.07 $\pm$ 0.02	17.5 $\pm$ 0.44
Se High	0.42 $\pm$ 0.15	1.07 $\pm$ 0.02	16.8 $\pm$ 0.43
Control + Hg	0.45 $\pm$ 0.14	1.08 $\pm$ 0.02	16.5 $\pm$ 0.46
Se Med + Hg	0.46 $\pm$ 0.15	1.05 $\pm$ 0.02	17.7 $\pm$ 0.60
Se High + Hg	0.32 $\pm$ 0.19	1.01 $\pm$ 0.03	17.9 $\pm$ 0.35
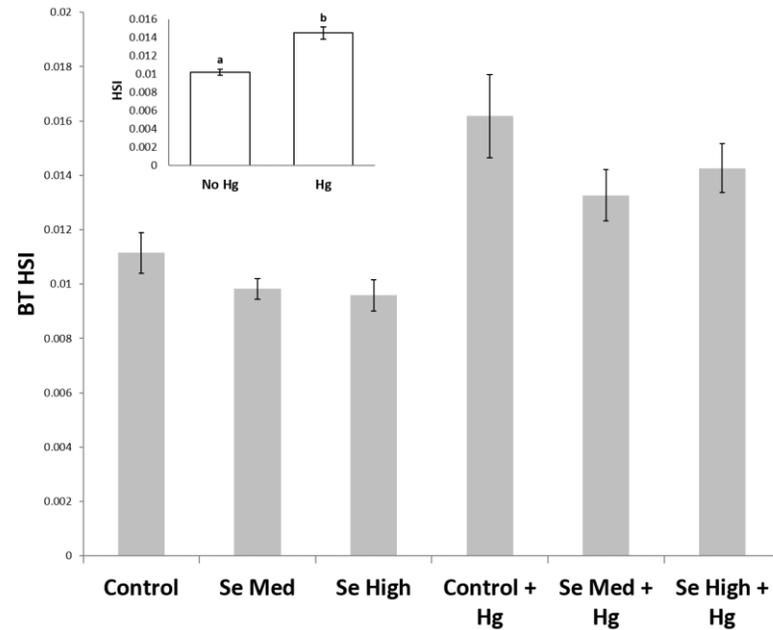
Fig. 2.5–

Hepatosomatic Index (mean $\pm$ SE) on Day 14 in BT (n=14-16) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant interaction effect between Se and Hg (Two-way ANOVA, $F_{2, 83}=0.348$, $p>0.05$). There was a significant Hg exposure effect (see inset graph, Two-Way ANOVA, $F_{1, 83}=42.8$, $p<0.0001$). Letters represent significant differences between No Hg and Hg exposure groups. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
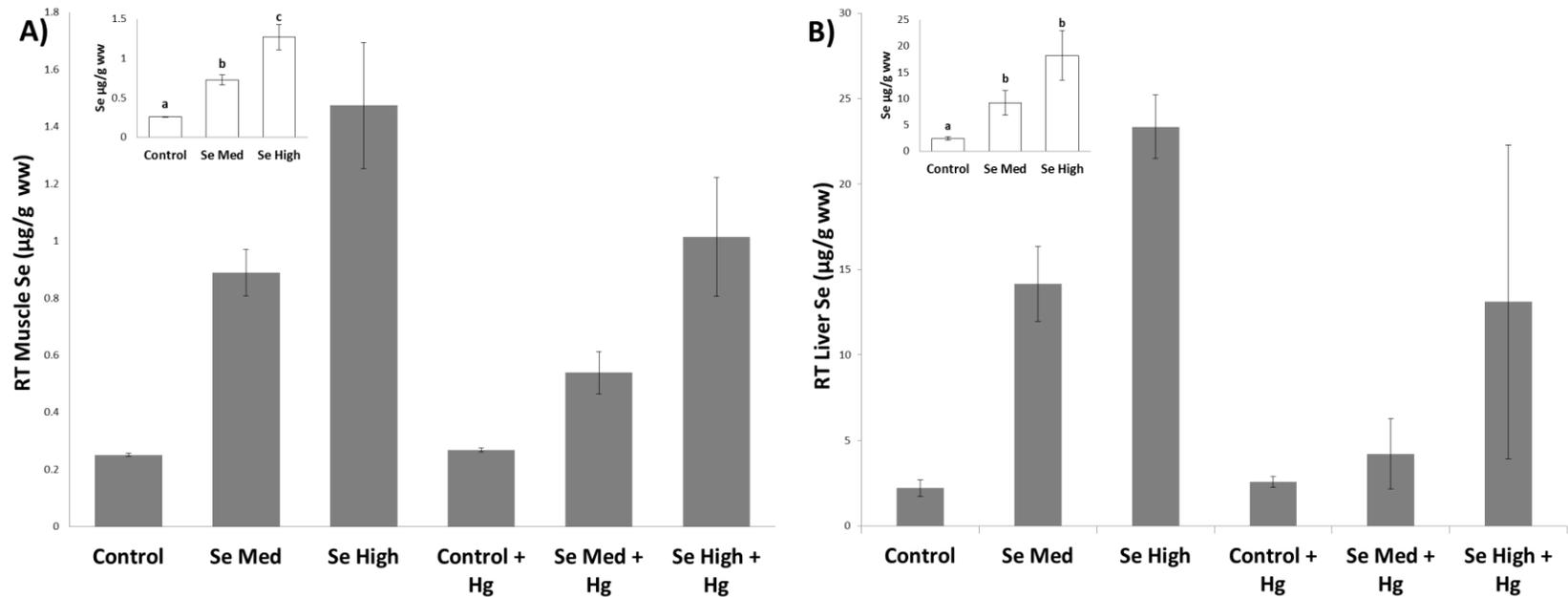
Fig. 2.6 –

Concentrations of Se (mean $\pm$ SE) in A) Muscle (n=12-15) and B) Liver (n=4) of RT exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant interaction effect between Se exposure and Hg exposure on muscle (Two-way ANOVA, $F_{2, 79}=2.58$, $p>0.05$) or liver Se concentration (Two-way ANOVA, $F_{2, 18}=3.26$, $p>0.05$). There was a significant Se exposure effect on both muscle (see inset graph Fig. 6 A, Two-Way ANOVA, $F_{2, 79}=42.4$, $p<0.0001$) and liver (see inset graph Fig. 6 B, Two-Way ANOVA, $F_{2, 18}=10.1$, $p<0.05$). Letters represent significant differences between Se treatment groups. Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests.

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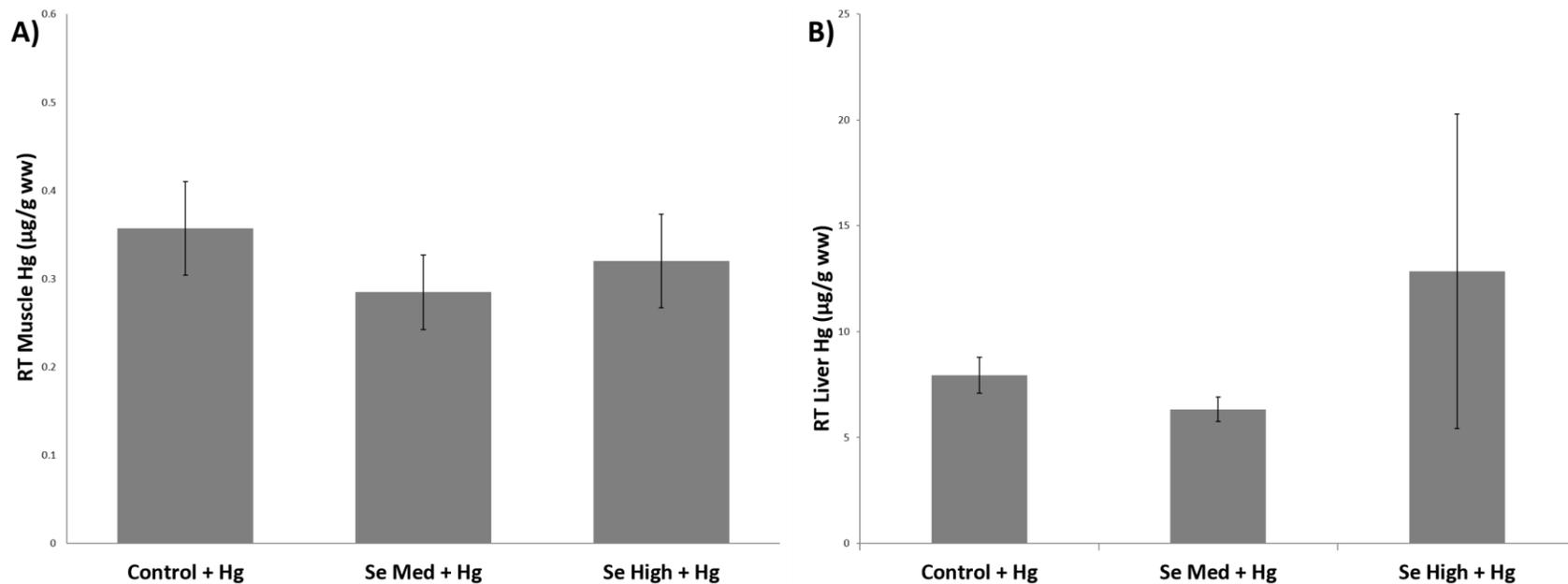
Fig. 2.7 –

Concentrations of Hg (mean $\pm$ SE) in A) Muscle (n=13-15) and B) Liver (n=4) on Day 14 in RT exposed to a single ip injection of HgCl$_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant Se exposure effect on muscle (One-way ANOVA, $F_{2, 38}$=0.368, $p>0.05$) and liver Hg concentration (One-way ANOVA, $F_{2, 9}$=0.323, $p>0.05$). Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests. Concentrations of Hg in muscle and liver of BT not injected with Hg (Control, Se Med and Se High groups) were at or near background levels – data not shown.
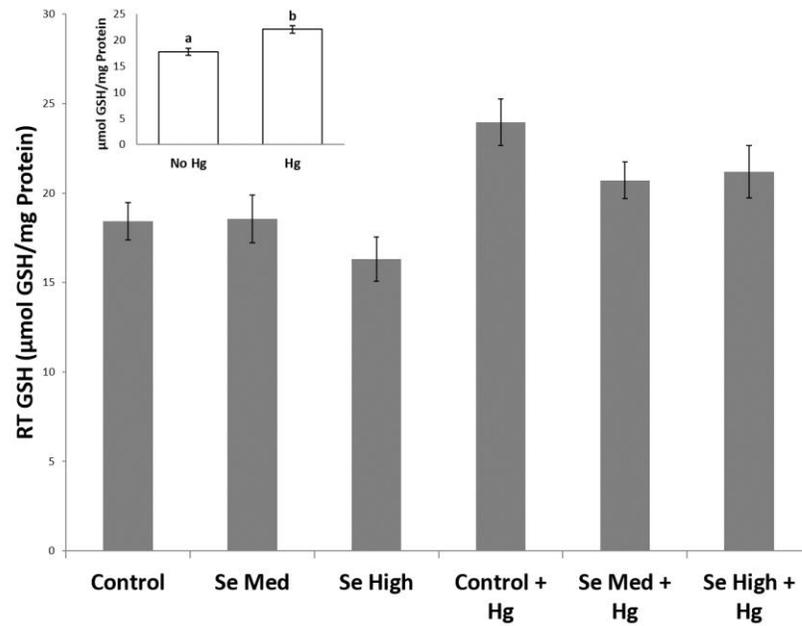
Fig. 2.8-

Liver GSH (mean $\pm$ SE) concentrations on Day 14 in RT (n=12-15) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant interaction effect between Se exposure and Hg exposure (Two-way ANOVA, $F_{2, 78}=1.04$, $p>0.05$). There was a significant Hg exposure effect (see inset graph, Two-Way ANOVA, $F_{2,78}=17.7$, $p<0.05$). Letters represent significant differences between No Hg and Hg exposure groups. Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests.
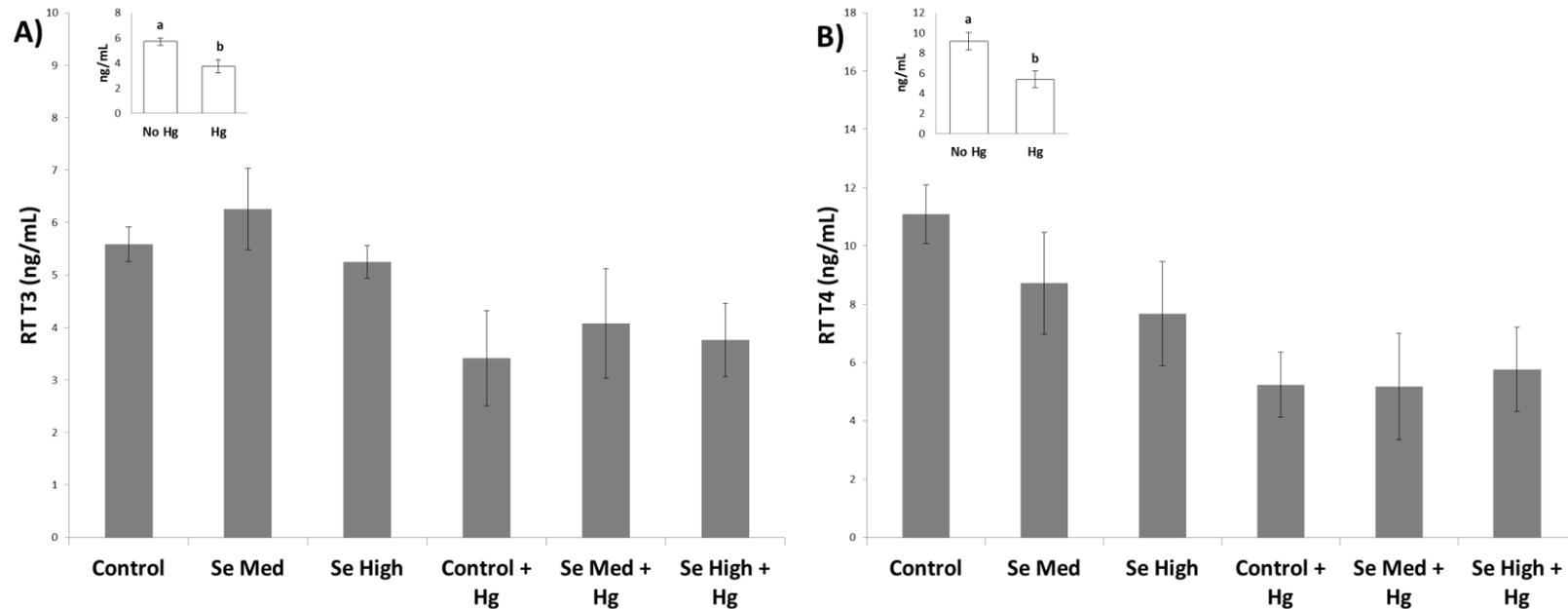
Fig. 2.9-

Plasma thyroid hormone (mean $\pm$ SE) concentrations on Day 14 in RT (n=12-15) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant interaction effect between Se exposure and Hg exposure for T3 (Two-Way ANOVA, $F_{2, 79}=0.215$, $p>0.05$) or T4 (Two-Way ANOVA, $F_{2, 76}=0.968$, $p>0.05$). There was a significant Hg exposure effect for T3 (see inset graph Fig. 9 A, Two-Way ANOVA, $F_{1, 79}=17.5$, $p<0.0001$) and T4 (see inset graph Fig. 9 B, Two-Way ANOVA, $F_{1, 76}=10.0$, $p<0.05$). Letters represent significant differences between No Hg and Hg exposure groups. Analyses have been adjusted using a Bonferroni correction to allow for multiple comparison tests.

Table 2.3 −
Growth measured as SGR, condition factor, and phase angle (mean $\pm$ SE) on Day 14 in RT (n=12-15) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant Se or Hg exposure effect or interaction effect between Se and Hg exposure (Two-way ANOVA, all F<3.02, all p>0.05). Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.

Treatment	SGR (%/Day)	Condition Factor
Control	0.55 $\pm$ 0.15	1.20 $\pm$ 0.04
Se Med	0.48 $\pm$ 0.11	1.16 $\pm$ 0.02
Se High	0.42 $\pm$ 0.17	1.14 $\pm$ 0.03
Control + Hg	0.37 $\pm$ 0.16	1.18 $\pm$ 0.03
Se Med + Hg	0.31 $\pm$ 0.22	1.16 $\pm$ 0.03
Se High + Hg	0.31 $\pm$ 0.17	1.09 $\pm$ 0.03
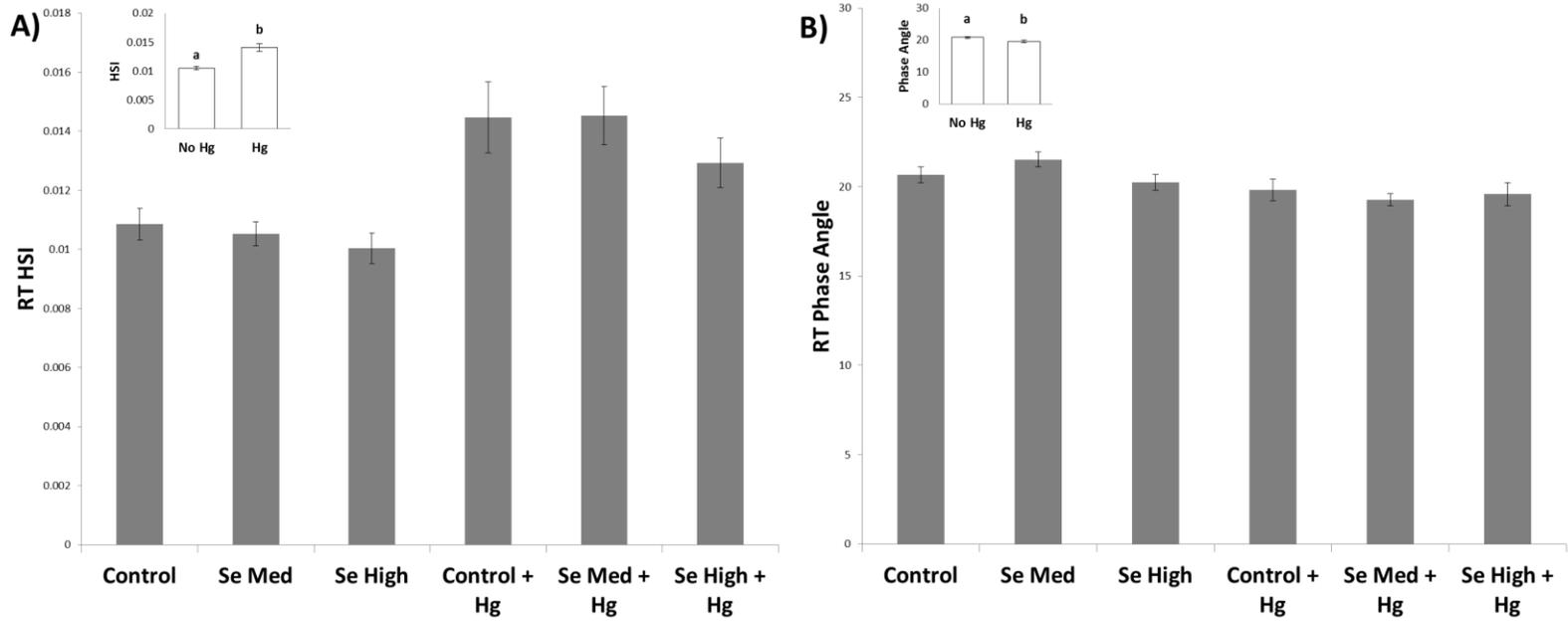
Fig. 2.10–

Hepatosomatic index and phase angle (mean $\pm$ SE) on Day 14 in RT (n=14-16) exposed to a single ip injection of $HgCl_2$ on Day 1 and fed different concentrations of Se through diets on Day 1-14. There was no significant interaction effect between Se and Hg for HSI (Two-Way ANOVA, $F_{2, 79}$=0.121, $p>0.05$) or Phase angle (Two-Way ANOVA, $F_{2, 80}$=1.48, $p>0.05$). There was a significant Hg exposure effect on HSI (see inset graph Fig. 10 A, Two-Way ANOVA, $F_{1, 83}$=32.7, $p<0.0001$) and phase angle (see inset graph Fig. 10 B, Two-Way ANOVA, $F_{1, 80}$=9.5, $p<0.05$). Letters represent significant differences between No Hg and Hg exposure groups. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.

CHAPTER 3: Does Selenium Protect Against Toxicity of Mercury: A Comparative Study with Brook Trout and Rainbow Trout

Introduction

Selenium (Se) is an essential element that is beneficial for organisms to consume as a micronutrient. Selenium is incorporated into proteins vital for biological functions including selenoenzymes such as glutathione peroxidases essential for oxidative damage prevention, iodothyronine deiodinases which regulate synthesis and activation of thyroid hormones, and thioredoxin reductases which maintain the cellular redox status (Kohrle 1999, Ralston and Raymond 2010).

Selenium concentrations above those required to maintain normal functions can result in toxicity (Hamilton 2004). Anthropogenic processes have increased the release of Se and increased the risk of exposure in fish and wildlife (Hamilton 2004). Fish and other egg laying vertebrates are among the organisms most vulnerable to Se, as it accumulates in their tissues, reaching potentially toxic concentrations (Lemly 2004). The substitution of Se for sulphur during protein synthesis was suggested as the main mechanism for toxicity (Lemly 1997b). More recently, studies have also provided evidence for oxidative stress as another key mechanism of Se toxicity (Palace et al. 2004). In fish, the main toxic effect and biological indicator of Se toxicity is teratogenicity (Chapman et al. 2010), evident by deformities at early life stages (Lemly 1997b, Holm et al. 2005). In severe and chronic cases of Se contamination, fish have exhibited toxic effects such as gill swelling, altered hematology, alterations in the liver, kidney, heart and ovaries and reproductive failure (Lemly 2002). Laboratory studies with fish exposed to elevated Se reported

elevated plasma cortisol and glucose (Miller et al. 2007), as well as reduced swimming speeds (Thomas and Janz 2011).

Unlike the essential element Se, Mercury (Hg) is a nonessential trace element. Mercury is also a dangerous anthropogenic pollutant that can enter the environment as a result of fossil fuel combustion, production of mercury, cement and caustic soda, and disposing of wastes (Pirrone et al. 2009). Mercury can be dispersed long distances through the atmosphere and can affect remote and vulnerable ecosystems (Travnikov 2005), increasing the concentration of Hg in aquatic systems and contaminating fish with Hg levels above those appropriate for humans to consume (Bjerregaard et al. 1999). The mechanism of Hg toxicity is suggested to be a result of Hg binding to sulfhydryl groups and disrupting essential processes, however, mechanisms like oxidative stress may elicit toxicity in specific organs (Son et al. 2001, Broniatowski and Dynarowicz-Latka 2009). Mercury has a high affinity for Se and may irreversibly bind to Se, preventing the normal functions and processes of key selenoenzymes (Ralston and Raymond 2010).

The toxic effects of inorganic and organic Hg on aquatic organisms are well documented, for review see Wolfe et al. (1998). In fish, exposure to Hg has caused visual impairment (Hawryshyn et al. 1982), gill lesions (Daoust et al. 1984), and reduced viability of immune cells (Voccia et al. 1994). Exposure to Hg also elicits reproductive toxicity in fish, including lower sperm motility (Dietrich et al. 2010), a decrease in plasma testosterone and estradiol (Drevnick and Sandheinrich 2003), lower gonadal somatic indices, reduced spawning and altered spawning behaviour (Sandheinrich and Miller 2006); for review see Crump and Trudeau (2009). The effects of Hg on Se dependent processes have also been investigated. In a study by Mulder et al. (2012),

brown trout with tissue Hg in molar excess of Se had lower plasma T3 levels. In a study by Branco et al. (2011), exposure to waterborne MeHg inhibited thioredoxin and thioredoxin reductase in the brain and liver of zebra-seabreams. In a follow up study, exposure to waterborne inorganic Hg caused an inhibition of thioredoxin reductase in the liver; however, the inhibition was alleviated by exposure to Se (Branco et al. 2012).

Selenium can act as an antagonist against the toxic effects of MeHg and inorganic Hg (Ralston and Raymond 2010). There is evidence that Se has a protective effect against the toxicity of Hg in fish. Arabi and Alaeddini (2005) reported that gill homogenates of rainbow trout exposed to $HgCl_2$ had decreased GSH and increased malondialdehyde (MDA) concentrations which were alleviated by co-exposure with Se. Furthermore, a recent study reported that Se decreased hematological damage in tilapia caused by Hg exposure (Cogun et al. 2012).

Along with a protective effect against Hg toxicity, Se decreases tissue concentrations of Hg. For example, rainbow trout exposed to an intraperitoneal (ip) injection of organic and inorganic Hg and administered a selenite enriched diet for 8 weeks showed a significant decrease of MeHg concentration in the muscle, liver, and kidney, compared to fish fed a normal diet (Bjerregaard et al. 1999). However, a significant decrease of inorganic Hg was only reported in the kidney compared to fish fed a normal diet (Bjerregaard et al. 1999). Similarly, Se enriched diets also decreased the retention of MeHg in goldfish (*Carassius auratus*); however, inorganic Hg was unaffected (Bjerregaard et al. 2011). In contrast, evidence for increased accumulation of inorganic Hg in the presence of Se has also been reported. For example, waterborne selenite and selenate increased the accumulation of inorganic Hg in the muscle of plaice

(*Pleuronectes platessa*) (Davies and Russell 1988) and waterborne sodium selenate increased the uptake of mercuric chloride in minnows (*Phoxinus phoxinus*) (Cuvin and Furness 1988).

The mechanisms for the antagonistic interaction between Se and Hg reviewed by Cuvinaralar and Furness (1991) include: 1) Se induced Hg redistribution, 2) a competition between Se and Hg for binding sites or receptors, 3) the formation of Hg-Se complexes, 4) a conversion of highly toxic MeHg to less toxic inorganic Hg, and 5) decreased oxidative damage. Yang et al. (2008) reviewed protective mechanisms for multiple species of Se and Hg in mammals, birds, and aquatic organisms, and discussed Se addition to lakes as a method to reduce Hg accumulation. However, despite the evidence available in the literature, uncertainties remain about the interactions between Se and Hg in different fish species.

Field studies provided some evidence for decreased tissue concentrations of Hg with co-exposure to Se. A study in Tennessee, USA reported that the removal of Se rich deposits into a quarry lake caused an increase in concentration of Hg in largemouth bass (Southworth et al. 1994, Southworth et al. 2000). Multiple studies have investigated the co-occurrence of Se and Hg near a metal smelter in Sudbury Ontario, Canada; concentrations of Hg in tissues of aquatic organisms were negatively correlated with water and tissue concentrations of Se (Chen et al. 2001, Belzile et al. 2006, Yang et al. 2010). Also, a controlled release of Se into 11 different lakes in Sweden led to a decrease in Hg concentration in perch and northern pike, and concentrations of Se and Hg differed between species before and after treatment (Paulsson and Lundbergh 1991). More research is required to compare the effect of Se on Hg contamination in multiple species

to establish with confidence that addition of Se into aquatic systems lowers Hg contamination of fish tissues.

Species differences in response to pollutants are an important consideration for water quality management. Differences in vulnerability can exist among closely related species which may affect their responses to contaminants (Miller and Hontela 2011). For example, in a study by Holm et al. (2005), RT had higher concentrations of Se in eggs than BT at similar concentrations of Se in muscle tissue. Also, RT had higher incidence of Se induced larval malformations than BT (Holm et al. 2005). A field study by Miller et al. (2009a) provided additional evidence of species differences to Se exposure between RT and BT. The authors reported that increased Se exposure elicited a greater decrease in vitamin A in RT compared to BT. The authors suggest that RT exposed to increasing levels of Se may not have sufficient vitamin A available to their eggs to limit oxidative stress in developing embryos (Miller et al. 2009a). Additionally, Miller and Hontela (2011) reported that cortisol secretion was impaired by sodium selenite exposure in both RT and BT; however, the cells of RT were more sensitive, with a lower EC_{50}.

The present study tested the hypothesis that Se acts antagonistically against Hg accumulation and toxicity and that there will be species differences between BT and RT, in response to Se and Hg exposure. The fish were fed an enriched diet of SeMet from Day 1 to Day 28 and received a single intraperitoneal (ip) injection of $HgCl_2$ on Day 14, to examine multiple physiological and toxicological endpoints under exposure to Se, Hg, or mixtures of Se and Hg. Tissue burdens of Se and Hg were measured to characterize the interaction on accumulation of Se and Hg. Multiple growth metrics, including specific growth rate (SGR) ((ln Weight$_2$ – ln Weight$_1$)t^{-1} x 100), hepatosomatic index (liver

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weight/body weight), and condition factor (weight/length^3) were analyzed to test the

prediction that negative effects on growth occur as a result of exposure to Se or Hg only,

and are alleviated by co-exposure to Se and Hg. Acetylcholine esterase activity was

measured to investigate the potential of Se to alleviate Hg neurotoxicity. Oxidative stress

parameters (reduced glutathione GSH and lipid peroxidation LPO) were measured

because Se protects against oxidative stress and potentially causes oxidative stress at high

concentrations (Miller et al. 2007). Also, because Se is an essential component in

iodothyronine deiodinases (Kohrle 1999), plasma thyroid hormones triiodothyronine (T3)

and thyroxine (T4) were measured.

Materials and Methods

Experimental Fish

Juvenile brook trout (n=99, mean $\pm$ SE, body weight 18.0 $\pm$ 5.34 g, fork length

12.1 $\pm$ 1.1 cm) and rainbow trout (n=91, body weight 25.7 $\pm$ 4.8 g, fork length 12.9 $\pm$

0.71 cm) were acquired from Allison Creek Trout Brood Station (Coleman, Alberta

Canada). Fish were held in acclimation tanks (336 L-754 L) and fed control diets for two

weeks before transfer to experimental units. Tank water quality parameters were

monitored and maintained at 12 $\pm$ 0.07 ºC, pH of 7.8-7.9, water hardness of 180-204

mg/L and dissolved oxygen of 7.69 $\pm$ 0.50 mg/L.

Experimental Se-enriched Diet Preparation

Selenium enriched diets were prepared from EWOS commercial trout chow

pellets (Micro Complete Feed for Salmonids, product 501666, EWOS Canada, Surrey,

BC) at the Food Science and Technology Labs (Crop Diversification Centre South,

Brooks, Alberta) in December 2012. The pellets were fed into an electric hammer mill

and ground into flour. The flour was mixed with distilled water and gelatin (1.8 kg of flour, 0.9 L of distilled water, 36 g of gelatin) in a Hobart N-50 (Hobart Canada, Toronto, ON). To prepare the Se enriched diets, 2.7 and 7.2 mL of 25 mg/mL stock SeMet (Sigma Aldrich, Catalogue # S3132) solution was added to the mixture to create Medium (Med) (14.5 µg Se/g) and High (37.0 µg Se/g) concentration diets. To prepare the control diet, water and gelatin only was added to the mixture. The paste was passed through a 1/8-inch round meat grinder attachment to create long thin noodles. The noodles were placed on long trays and dried in a Harvest Saver R-5 tray dryer (Commercial Dehydrator Systems, Eugene, OR) at 35 ºC with maximum airflow. The dried noodles were processed into small pellets by a custom made electric noodle breaker and stored at -20 ºC .

Hg injection

Experimental fish were subjected to a single intraperitoneal (ip) injection of either saline or $HgCl_2$ (Sigma-Aldrich) on Day 14. A stock solution of Hg (74.07 mg/mL) was diluted to 3 mg/mL and injected, based on the weight of each fish, resulting in a dose of 0.3 mg Hg/g body weight.

Selenium Pre-Treatment and Mercury exposure

Fish were fed twice a day, ad libitum, standard or SeMet-enriched diet (0, 15 and 45 µg/g) from Day 1 to Day 28 and were injected with inorganic Hg or saline on Day 14 (see Se diet preparation and Hg injection). Fish were euthanized with MS-222 (Sigma Aldrich, Catalogue #E10521) on Day 28 and body length, weight, and biological impedance (see BIA) were measured. A blood sample was taken from the caudal vasculature, centrifuged at 13 000 rpm and plasma was collected and frozen in liquid

nitrogen. The brain, dorsal muscle section, and liver were removed and frozen at $-80\,°C$ until analysis.

Analyses

Tissue Selenium and Mercury Concentration

Portions of muscle and liver samples were dried in a 60ºC oven and ground to powder ($0.100g \pm 0.005$) and placed into 50 mL borosilicate tubes with 2 mL of concentrated, trace metal grade HNO_3 (Omnitrace ® NX0407-4) added. Tubes were placed into a dry aluminum hot block at 75ºC for 16 hours and allowed to cool to room temperature. The samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) on an Elan DRC-E. The detection limit for Se was 0.09 µg Se/g and the detection limit for Hg was 0.03 µg Hg/g. Duplicates, blanks and TORT-2 standards (National Research Council Canada) were used for quality control.

Water Hg Analysis

Tank water samples were collected in triplicate and analyzed for Hg at the Centre for Earth Observation Science, University of Manitoba, using the EPA 1631 method and a cold vapor atomic fluorescence spectrometry on a Tekran 2600.

Oxidative Stress Analysis

Reduced glutathione (GSH) and lipid peroxidation (LPO) were measured as indicators of oxidative stress. Livers were homogenized in phosphate buffer and separated into aliquots for analysis of GSH, LPO and protein. A Bioxytech GSH 400 kit (Catalogue #21011) was used to measure GSH (µmol GSH/mg Protein) and Bioxytech LPO-596 kit (Catalogue #21012) was used to measure LPO (µmol MDA/mg Protein), as described by Miller et al. (2007). Protein concentration was measured by a

spectrophotometric assay (595 nm) using Bradford reagent (Sigma-Aldrich, Catalogue B6916).

BIA

Biological impedance analysis was used as a simple, nonlethal method to estimate body lipid and water content (Rasmussen et al. 2012), as previously described by Cox and Hartman (2005). Resistance and reactance was measured in series and used to calculate phase angle φ (tan $φ = χcR^{-1}$) (Rasmussen et al. 2012).

Plasma Hormone Analysis

Plasma T3 (Catalogue #06b-254215), and T4 (Catalogue #06b-254011) were measured using radioimmunoassay kits purchased from Medicorp (Montreal, Quebec). Assay characteristics including intra- and inter-assay variability were assessed using internal standards, as described by Hontela et al. (1995).

Acetylcholinesterase

Acetylcholinesterase (AChE) activity was measured using a spectrophotometric assay as previously described by Miller et al. (2009b). In summary, brain tissue was homogenized in phosphate buffer in a ratio of 1:3. The homogenate was centrifuged at 13 000 rpm at 5°C for four minutes and the supernatant was collected. Following three centrifugations the remaining supernatant was diluted with phosphate buffer. The diluted supernatant and Tris buffer (Tris- buffered saline tablets, Sigma T-5030) was pipetted into a 96 well microplate (Costar, 3596) in triplicate and gently agitated at room temperature for 10 minutes. DTNB (Sigma 5,5' Dithiobis (2-nitro-benzoic acid), D8130) and acethylthiochlorine iodide (AChI, Sigma minimum 98% TLC, A5751) were added and a second agitation at room temperature for 10 minutes was completed. Change in

absorbance was measured at 405 nm every 2 minutes for 10 min. Assay characteristics have been previously described by Miller et al. (2009b).

Statistical Analysis

All statistical analyses were completed using R (R Development Core Team, 2012), and Rstudio (Version 0.98.953). A three-way analysis of variance (ANOVA) was used to compare the effects between species, Se exposure and Hg exposure and differences were determined by a Tukey post hoc. A two-way ANOVA was used to compare species and Se diet treatment effects within the Hg exposure group for Hg concentrations in muscle and liver tissue. All *p*-values were adjusted using a Bonferroni Correction to allow for multiple comparison tests.

Results

Selenium and Mercury Concentrations

The concentration of Se in BT and RT muscle are presented in Fig. 3.1. The figure presents the results from both species for all treatment groups and the inset graph presents the results of the interaction effect between Se exposure and species. The inset graph combines all the fish exposed to each Se diet group for each species without or with Hg. There was a main interaction effect between Se exposure and species (see inset graph in Fig 3.1, F=7.0, *p*<0.01) as the concentration of Se in BT and RT was significantly higher in the Se High exposure groups compared to the Controls and Se Med exposure groups. However, the concentrations in Se Med exposure groups were not significantly different from the Controls for both species. Also, BT had significantly lower concentrations of Se in the Se Med and Se High exposure groups compared to RT (see inset graph). There was no main effect of Hg exposure on Se concentration in muscle

tissue and there was no other two or three way interaction effects between species, Se exposure, or Hg exposure (all F<3.11, all $p>0.05$).

The concentrations of Se in BT and RT liver are presented in Fig. 3.2. There was an interaction effect between Se exposure and species (see inset graph in Fig. 3.2 A, F=31.4, $p<0.00001$).The concentration of Se in BT and RT increased significantly with increasing concentration of Se in the diets. Also, BT had significantly lower concentrations of Se in the Se Med and Se High exposure groups compared to RT. There was also an interaction between Se exposure and Hg exposure (see inset graph 3.2 B, F=11.4, $p<0.0001$). The concentration of Se in trout liver increased significantly with increasing Se concentration in the diet in both the presence and absence of Hg. However, when exposed to Hg in combination with Se enriched diets, the concentration of Se was significantly lower compared to the same Se exposure groups in the absence of Hg. There was no 3 way interaction between species, Se exposure, and Hg exposure (F=0.874, $p>0.05$).

The concentrations of Hg in BT and RT muscle are presented in Fig. 3.3. Two weeks after the single ip injection, Hg was detected in the muscle of exposed BT and RT. Since muscle Hg concentrations in groups not exposed to Hg had concentrations near background levels, they were not included in the statistical analysis. There was an interaction effect between Se exposure and species for muscle Hg concentration (F=10.3, $p<0.001$). The concentration of Hg in BT muscle was not affected by exposure to increasing concentrations of Se in the diet. In RT muscle, the concentration of Hg was significantly higher when fed Se High diets compared to the Hg Control and Se Med exposure groups. Furthermore, BT had significantly higher Hg concentrations in the Hg

Control than RT and had significantly lower Hg concentrations in the Se High exposure group.

The concentrations of Hg measured in BT and RT liver are presented in <u>Fig. 3.4</u>. Two weeks after the single ip injection, Hg was detected in the liver of exposed BT and RT. Since liver Hg concentrations in treatment groups not exposed to Hg had concentrations near background, they were not included in the statistical analysis. There was a significant species effect (see inset graph, F=43.5, p<0.00001) as brook trout had significantly higher concentration of Hg than RT in liver. There was no main effect of Se treatment (F=0.025, p>0.05) and there was no interaction between species and Se exposure (F=1.85, p>0.05).

The Hg : Se molar ratios for Hg exposure groups in muscle and liver for both species are shown in <u>Table 3.1</u>. In muscle, there was a species main effect as BT had a significantly higher molar ratio than RT (F=13.0, p<0.001). There was no Se exposure effect (F=3.49, p>0.05) or interaction effect between Se exposure and species (F=0.085, p>0.05). All ratios in muscle were less than 1.0. In liver, there was also a species main effect as BT had a significantly higher molar ratio than RT (F=78.1, p<0.0001). Also, there was a Se exposure main effect as the liver molar ratio in trout decreased significantly with increasing Se exposure (F=19.6, p<0.00001). However, there was no interaction effect between Se exposure and species (F=2.81, p>0.05). In the liver, only RT exposed to a Se Med and Se High diets had molar ratios less than 1.0.

The concentration of Hg in tank water was measured as an indication of Hg elimination and is presented in Table 3.2. Higher levels of Hg were detected in the water

of tanks that contained trout exposed to Hg. Water in tanks holding rainbow trout exposed to Hg and fed control diets had a particularly high concentration of Hg.

Oxidative Stress

Reduced glutathione (GSH) was measured as an indicator of oxidative status in the liver and is presented in Fig. 3.5. There was an interaction between species, Se exposure and Hg exposure (F=15.7, $p<0.00001$). In BT, increasing Se concentration in diets did not affect GSH levels in the absence of Hg. Although, exposure to Hg and exposure to Hg in combination with Se Med diet resulted in a significant increase in BT GSH levels compared to Control and Se only exposure groups, this effect was not observed when BT were exposed to Hg in combination with Se High diets. In RT, exposure to Se Med and Se High diets significantly lowered GSH levels in the absence of Hg compared to the Control; however, exposure to Hg and Hg in combination with Se diets was not significantly different than the Control. In comparison, BT had significantly lower GSH levels than RT in the Control, and significantly higher levels when exposed to Hg and Hg in combination with Se Med diets.

Lipid peroxidation was also measured as an indicator of oxidative damage in the liver and is presented in Fig. 3.6. There was a species main effect as levels of LPO were significantly lower in BT compared to RT (see inset graph Fig. 3.6 A, F=14.6, $p<0.0001$). Also, there was a Hg exposure main effect as levels of LPO were significantly higher in Hg exposed trout (see inset graph Fig. 3.6 B, F=21.2, $p<0.0001$). There was no selenium exposure main effect and there was no 2 or 3 way interaction effects between species, Se exposure, and Hg exposure (all F<2.25, all $p>0.05$).

AChE

Acetylcholine esterase activity was measured in the brain tissue of BT and RT and is presented in Fig. 3.7. There was an interaction between species, Se exposure and Hg exposure in AChE activity (F=26.4, $p<0.00001$). In BT, AChE activity decreased significantly after exposure to Se High diet, Hg and Hg in combination with Se Med diets compared to the Control. However, this effect was not observed when BT were exposed to Hg in combination with Se High diets. In RT, AChE activity increased significantly after exposure to Hg and Hg in combination with Se Med diets compared to the Control, however, this effect was not observed when exposed to Hg in combination with Se High diets. BT had significantly higher AChE activity in the Control, Se Med, and Hg in combination with Se High exposure groups and had lower AChE activity after exposure to Hg in combination with Se Med diets compared to RT.

Thyroid Hormones

Thyroid hormone triiodothyronine (T3) concentrations measured in the plasma of BT and RT are presented in Fig. 3.8. There was a main effect of Hg exposure as the concentration of T3 was significantly lower in trout exposed to Hg (see inset graph, F=36.3, $p<0.0001$). There was no Se exposure main effect and there was no 2 or 3 way interaction effects between species, Se exposure, or Hg exposure (all F<3.63, all $p>0.05$).

Plasma thyroid hormone thyroxine (T4) concentrations of BT and RT are presented in Fig. 3.9. There was a species main effect as the concentrations of T4 were significantly lower in BT compared to RT (see inset graph Fig. 3.9 A, F=10.5, $p<0.01$). Also, there was a Se exposure main effect as the concentrations of T4 were significantly higher in the Se High exposure group compared to the Control (see inset graph Fig. 3.9

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B, F=7.40, $p<0.001$); however, this effect was not observed when exposed to Se Med exposure group. Furthermore, there was a Hg exposure main effect as the plasma T4 concentrations were significantly lower in Hg exposed trout compared to trout not exposed to Hg (see inset graph 3.9 C, F=34.4, $p<0.00001$). There were no 2 or 3 way interactions between species, Se exposure, or Hg exposure (all F<1.03, $p>0.05$).

Growth

For specific growth rate, presented in Fig. 3.10, there was a species main effect as BT had significantly lower specific growth rate compared to RT (see inset graph Fig. 3.10 A, F=114.5, $p<0.00001$). Also, there was a Hg exposure main effect as Hg exposed trout had significantly lower specific growth rate compared to trout not exposed to Hg (see inset graph Fig. 3.10 B, F=38.5, $p<0.00001$). Selenium enriched diets did not have an effect and there was no 2 or 3 way interaction effects between species, Se exposure, or Hg exposure (all F<3.46, all $p>0.05$).

For Condition factor, presented in Fig. 3.11, there was a species main effect as BT condition factor was significantly lower compared to RT (see inset graph Fig. 3.11 A, F=292.6, $p<0.00001$). Also, there was a Hg exposure main effect as Hg exposed trout had a significantly lower condition factor than trout not exposed to Hg (see inset graph Fig. 3.11 B, F=20.6, $p<0.00001$). Selenium enriched diets did not have an effect and there were no 2 or 3 way interaction effects between species, Se exposure, or Hg exposure (all F<3.26, $p>0.05$).

For Phase angle, presented in Fig. 3.12, there was a species main effect as BT phase angle was significantly lower compared to RT (see inset graph Fig. 3.12 A, F=69.7, $p<0.00001$). There was also an interaction between Se exposure and Hg exposure (see

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inset graph Fig. 3.12 B, F=6.45, $p<0.01$). Phase angle increased significantly in trout fed Se Med and Se High diets; however, this effect was not observed when exposed to Hg and Hg in combination with Se. There were no other interaction effects between species, Se exposure, or Hg exposure (all F<3.91, all $p>0.05$).

For Hepatosomatic index, presented in Fig. 3.13, there was a species main effect as BT HSI significantly lower compared to RT (see inset graph Fig. 3.13 A, F=13.2, $p<0.001$). Also, there was a Se exposure main effect, as the Se Med diet group had significantly higher HSI levels compared to the Control, however, Se High diet was not significantly different from the Control or Se Med diet group (see inset graph Fig. 3.13 B, F= 6.32, $p<0.05$). There was also a Hg exposure main effect, as Hg exposed trout had significantly higher HSI levels compared to trout not exposed to Hg (see inset graph Fig. 3.13 C, F=77.6, $p<0.00001$).

Discussion

Species specific responses to contaminants have been documented in numerous systems, even among closely related species, and are relevant to both fundamental research and management (Miller and Hontela 2011). These differences, and the mechanisms responsible for them, are an important topic in toxicology. There is some evidence of the antagonistic effect of Se on Hg toxicity and accumulation (Bjerregaard et al. 1999, Cogun et al. 2012). The aim of this study was to investigate the interaction between Se and Hg, and the potential species differences between BT and RT after co-exposure to Se and Hg.

In the present study, concentrations of Se in the muscle and liver increased after exposure to Se enriched diets. Brook trout however, accumulated less Se through diet

exposures compared to RT. In contrast, a study on Se in wild BT and RT reported BT had a higher muscle burden and a lower egg burden of Se compared to RT (Holm et al. 2005). Species differences in the tissue compartmentalization of Se may exist, but the mechanisms remain unclear (Holm et al. 2005). The concentration of Se in muscle tissue of trout was not affected by Hg exposure; however, it was lower in the liver of trout when exposed to both Se diets and the Hg injection. This may be a result of Se redistribution in the presence of Hg to other Se dependent organs (Huang et al. 2013); however, it may also be a result of a Hg-induced decrease in feeding on Se enriched diets. This conclusion is supported by lower specific growth rate and condition in Hg exposed trout.

Mercury accumulated in muscle and liver of BT and RT after a single ip injection of $HgCl_2$. Numerous studies with different fish species reported lower concentrations of MeHg in fish exposed to Se (Southworth et al. 1994, Bjerregaard et al. 1999, Southworth et al. 2000, Chen et al. 2001, Belzile et al. 2006, Yang et al. 2010, Bjerregaard et al. 2011). However, the effects of Se on inorganic Hg accumulation are not as conclusive. Bjerregaard et al. (1999, 2011) observed no significant decrease in muscle or liver Hg of rainbow trout and goldfish injected ip with inorganic Hg and fed selenite enriched diets. In contrast, minnows accumulated more inorganic Hg from water when also exposed to selenite or selenate (Cuvin and Furness 1988, 1990). In the present study, Se enriched diets had no significant effect on the concentration of Hg in muscle or liver of BT. In RT, while the Se Med diet did not affect muscle Hg concentration, the Se High diet increased it. This result suggests an interaction between Se and Hg, possibly leading to the formation of a complex between Se and Hg that is not readily excreted. However, Se enriched diets had no effect on the concentration of Hg in the liver. Concentrations of Hg

in muscle and liver differed between species. Brook trout had higher muscle Hg concentrations than RT when fed control diets, and higher concentrations of Hg in the liver for all Hg exposure groups. This may be a result of a higher elimination rate of Hg in RT than BT, which is supported by the higher concentrations of Hg in the RT tank water compared BT tank water. Liu et al. (2013) reported lower whole body Hg concentrations in RT compared to zebrafish, suggesting a higher rate of Hg excretion in RT. Interestingly, BT in the present study had lower muscle Hg concentrations than RT when exposed to Se High diets. This may be a result of an interaction between Se and Hg in RT, such as the formation of a Hg-Se complex, with inorganic Hg complexed with selenolcysteine and Hg/Se groups bound to selenoprotein P (Khan and Wang 2009), molecules not readily eliminated from the body. This Se and Hg interaction, however, was not observed in the liver. Consequently, the tissue distribution of Se and Hg and the subsequent interaction may be a result of species differences in Se compartmentalization, Hg elimination and possibly also differences in feeding behaviour which would influence uptake of Se.

The Hg : Se molar ratio has been used to compare levels of Se and Hg in tissues of aquatic organisms. It has been suggested that Se protects against Hg in fish when Se is in molar excess of Hg, and even though concentration of available Se has been decreased by the sequestration with Hg, sufficient Se remains to maintain normal functions (Ralston 2008, Peterson et al. 2009). In the present study, the Hg : Se molar ratio indicates Se to be in molar excess of Hg, thus offering a protective interaction, in BT and RT muscle and liver of RT fed Se enriched diets. However, an interaction of Se and Hg on the accumulation of Hg was only observed in the muscle of RT exposed to Hg in

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combination with Se High diet. Additional research is required to further understand the protective ratios in different species and tissues (Burger 2012), and the relationship between the Hg : Se ratio and toxic responses, including oxidative stress.

Selenium is a key component in the glutathione peroxidases (GPx) antioxidant system (Branco et al. 2011), however, SeMet in the presence of the enzyme methioninase forms methylselenol that can cycle with GSH to form a superoxide radical and cause oxidative stress (Palace et al. 2004, Spallholz et al. 2004, Misra et al. 2012a). Mercury is a pro-oxidant and can decrease concentrations of GSH and cause oxidative stress (MonnetTschudi et al. 1996, Gopal 2003, Arabi and Alaeddini 2005, Broniatowski and Dynarowicz-Latka 2009). In the present study, Se exposure did not affect liver GSH levels in BT in the absence of Hg, possibly the concentrations of Se may not have been high enough to induce oxidative stress. However, exposure to Hg alone or in combination with the Se Med diets increased liver GSH while exposure to Hg in combination with Se High diets prevented this increase. Arabi and Alaeddini (2005) reported Hg exposure decreased the concentration of GSH and increased LPO in rainbow trout gill homogenate while co-exposure to Se protected against Hg- induced oxidative stress. However, there is some evidence that GSH concentrations increase in a defensive response to oxidative stress during exposure to metals (Atli and Canli 2008). In the present study, the Hg exposure may not have been damaging enough to overwhelm the defensive response and diminish GSH. Moreover, the Se High diet may have provided sufficient Se to counteract the effect of Hg on GSH, by Hg-Se complex formation (Ralston and Raymond 2010). Selenium may protect against oxidative stress by the action of selenium dependent glutathione peroxidase (Rotruck et al. 1973, Sun et al. 1995, Arabi and Alaeddini 2005,

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Misra et al. 2012b), which eliminates hydrogen peroxide and lipid hydroperoxides (Cohen and Hochstein 1963, Behne et al. 1996).

In RT, Se diets decreased GSH levels in the absence of Hg. The concentration of Se may have elicited the production of methylselenol which can cycle with GSH to form a superoxide radical and lower GSH levels (Palace et al. 2004, Spallholz et al. 2004, Miller et al. 2009a, Miller and Hontela 2011). However, RT GSH levels did not appear to be influenced by Hg or Hg and Se mixtures. This may have been a result of increased elimination of Hg in RT or formation of Hg-Se complexes.

There were differences in GSH levels between BT and RT. Brook trout had lower GSH in the control compared to RT. Similar results have been observed by Miller et al. (2009a), as wild BT had lower GSH reserves than wild RT. There were also differences in LPO levels between BT and RT, with lower LPO in BT. This may be due to RT having higher reserves of GSH that may cycle with excess Se creating reactive oxygen species (ROS) and elevating LPO. Along with differences in LPO between the two trout species, exposure to Hg also elevated LPO in our study. Similar effects of exposure to Hg on LPO were reported in a study with RT gill homogenates (Arabi and Alaeddini 2005). Metal ions, including Hg, catalyze the formations of ROS which can initiate LPO (Chan et al. 1982, Arabi 2004, Arabi and Alaeddini 2005). However, there was no evidence of an interaction between Se and Hg on levels of LPO in our study.

Mercury is a potent neurotoxicant (Aschner and Aschner 1990) and inorganic Hg inhibits AChE activity (Frasco et al. 2007). The present study investigated Hg toxicity on AChE activity and examined whether exposure to Se would alleviate the toxic effects in BT and RT. In BT, AChE activity was inhibited when exposed to Se High diets in the

absence of Hg. In contrast, a study on AChE activity in white suckers reported no effect of Se exposure on AChE activity (Miller et al. 2009b). Also, in the present study, exposure to Hg, and Hg in combination with Se Med diets inhibited AChE. However, exposure to Hg in combination with Se High diets did not have an effect. Therefore, Se may have acted antagonistically against Hg by possibly forming Hg-Se complexes in the brain or by causing Hg redistribution and decreasing the concentration of Hg available to inhibit AChE activity. However, inorganic Hg may have also interfered in Ellman's reaction used to measure AChE activity and caused an overestimation of AChE inhibition (Frasco et al. 2005). In RT, an increase in the activity of AChE was observed in Hg and Hg in combination with Se Med diet exposures. This is in direct contrast with results reported by previous studies (Frasco et al. 2007), including the results observed in BT in the present study.

Species differences were observed between BT and RT in AChE activity. In the absence of Hg, BT had higher AChE activity in the Control and Se Med exposure. Also, BT had lower AChE activity in the Hg in combination with Se Med exposure group. Differences may be attributed to different concentrations of Se and Hg in brain tissue (not analyzed), or Hg induced interference with AChE measurements (Frasco et al. 2005). Our results suggest that AChE activity is a difficult endpoint to use to investigate species differences in the interaction between Se and Hg.

Selenium is a component of the iodothyronine deiodinases which regulate the synthesis and activation of thyroid hormones (Kohrle 1999, Ralston and Raymond 2010). Plasma T3 and T4 concentrations were measured in both species. Exposure to Hg resulted in lower T3 and T4 compared to controls. Exposure to Hg caused

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histopathological alterations to the thyroid gland and interfered with the uptake of iodide in catfish (*Clarias batrachus*) (Kirubagaran and Joy 1989, 1994), anomalies which would lead to low plasma T3 and T4. The decrease in plasma thyroid hormone concentration may also have been a result of Hg's high affinity for Se and the formation of Hg-Se complexes. This process would, in turn, decrease Se concentration and its availability as a component in iodothyronine deiodinases which have a role in regulation of thyroid hormones (Kohrle 1999, Ralston and Raymond 2010). However, even though the molar ratio suggests Se to be in excess of Hg in the muscle of BT and the muscle and liver of RT, there may not have been enough supplemental Se available to maintain normal synthesis of T3 and therefore no alleviation on the effect of Hg was observed. Also, BT had lower concentrations of T4 compared to RT. Brook trout had higher muscle and liver Hg : Se molar ratio and therefore Hg might have been more readily available in BT compared to RT to decrease concentrations of T4. Additionally, trout exposed to Se High diets had higher concentrations of T4 compared to the control. The molar ratio of Hg : Se was less than 1.0 in the muscle of both species and the ratio decreased with increasing concentration of Se in the liver. As a component in iodothyronine deiodinases increased Se may increase the synthesis of thyroid hormones and may have provided enough Se to maintain normal functions after a potential decrease in concentration caused by Hg sequestering Se. These results are consistent with a recent study on brown trout by Mulder et al. (2012) in a lake with low Se concentrations compared to a reference lake. The authors reported a negative relationship between plasma concentrations of thyroid hormones and Hg concentrations in tissues and suggested that the Hg : Se molar ratio in muscle and liver may predict the effect of Hg on thyroid hormones in fish.

Indicators of growth were measured to investigate species differences in the interaction between Se and Hg. Exposure to Hg significantly decreased specific growth rate and condition factor in trout. Similarly, lower fish growth has been observed in walleye and sturgeon fed MeHg contaminated diets (Friedmann et al. 1996, Lee et al. 2011). In the present study, lower specific growth rate and condition factor may have been a response to the toxic effects of Hg, including the decrease in plasma T4. In a previous study, RT growth was promoted by exposure to T4 (Barrington et al. 1961). Also, specific growth rate and condition factor were significantly lower in BT compared to RT. This may have been a result of a significantly higher Hg : Se molar ratio in the muscle and liver, and lower concentrations of plasma T4 in BT compared to RT.

Phase angle, an indicator of overall condition and lipid content (Rasmussen et al. 2012), was lower in BT compared to RT. There was an interaction effect between Se and Hg in phase angle which was higher in trout fed Se diets in the absence of Hg. The current understanding of the effects of Hg or Se on lipid content in fish is limited. Lipid content was not affected by MeHg treatment in sturgeon (Lee et al. 2011) but in largemouth bass fed diets enriched with Se, lipid content of muscle was not affected by Se but liver lipid content increased (Zhu et al. 2012). Metabolism and consequently lipid content may be altered due to increased stress which may mobilize energy reserves in response to a stressor including contaminants such as Hg or Se (Vijayan and Moon 1992, Bleau et al. 1996).

Brook trout also had significantly lower HSI, another indicator of lower growth and condition, compared to RT. Rainbow trout may have had higher specific growth rate, condition factor and HSI as a result of higher feeding activity which may have alleviated

the energetic costs of exposure to metals (D'Cruz et al. 1998, Pyle et al. 2005). A higher feeding activity may also explain higher concentrations of Se in RT compared to BT in Se enriched diet exposure groups. Similarly, Miller et al. (2009a) reported higher HSI in BT and RT exposed to Se in the field. In trout, Hg induced a significant increase in HSI and Se enriched diets did not alleviate this effect. In contrast to the results of the present study, a study on largemouth bass reported HSI was significantly lower when fish were sampled from a site highly contaminated with Hg (Friedmann et al. 2002). Furthermore, Larose et al. (2008) described a negative relationship between HSI and Hg in liver of walleye. Exposure to organic and inorganic Hg caused hyperplasia in gill epithelial cells of RT fingerlings and fry (Wobeser 1975). In the present study, Hg exposure to trout may have caused hyperplasia, detected as higher HSI in the liver but Se enriched diets did not alleviate this effect.

Conclusion

The present study was designed to investigate species differences in the interaction between Se and Hg in BT and RT under controlled laboratory conditions. Exposure to the Se diets and Hg injection increased the muscle and liver burdens of these contaminants in both fish species. Species differences in Se compartmentalization, Hg elimination and feeding behaviour in the laboratory may have caused differences in Se and Hg accumulation. The species differences in the interaction between Se and Hg and in the physiological and toxicological responses may have been caused by different accumulation patterns of Se and Hg. Even though exposure to Se decreased concentrations of MeHg in different studies on fish (Paulsson and Lundbergh 1991, Bjerregaard et al. 1999, Belzile et al. 2006, Bjerregaard et al. 2011), the results of

increased exposure of Se on inorganic Hg accumulation vary (Turner and Swick 1983, Bjerregaard et al. 1999, Bjerregaard et al. 2011). The chemical forms of Se and Hg, the molar ratios, the route and timing of exposure, and different fish species may affect how Se alters Hg accumulation and toxicity (Bjerregaard et al. 2011).

Selenium can act as an antagonist against the toxic effects of MeHg and inorganic Hg (Arabi and Alaeddini 2005, Ralston and Raymond 2010, Cogun et al. 2012). The antagonism of supplemental Se is suggested to be a result of additional Se readily available to ensure normal functions of Se dependent processes, after Se is sequestered by Hg (Ralston and Raymond 2010). This mechanism is compatible with the results of the present study since evidence of antagonistic effects of Se on Hg were reported in Se dependent processes, including preventing oxidative stress and maintaining normal synthesis and activation of thyroid hormones. Additional research is needed on the effects and interactions of Se and Hg in aquatic organisms. Species differences in response to Se and Hg exposure is an important aspect to study as it may impact decisions about water quality management as well as the proposal of using Se to treat Hg contaminated lakes.

Figures and Tables
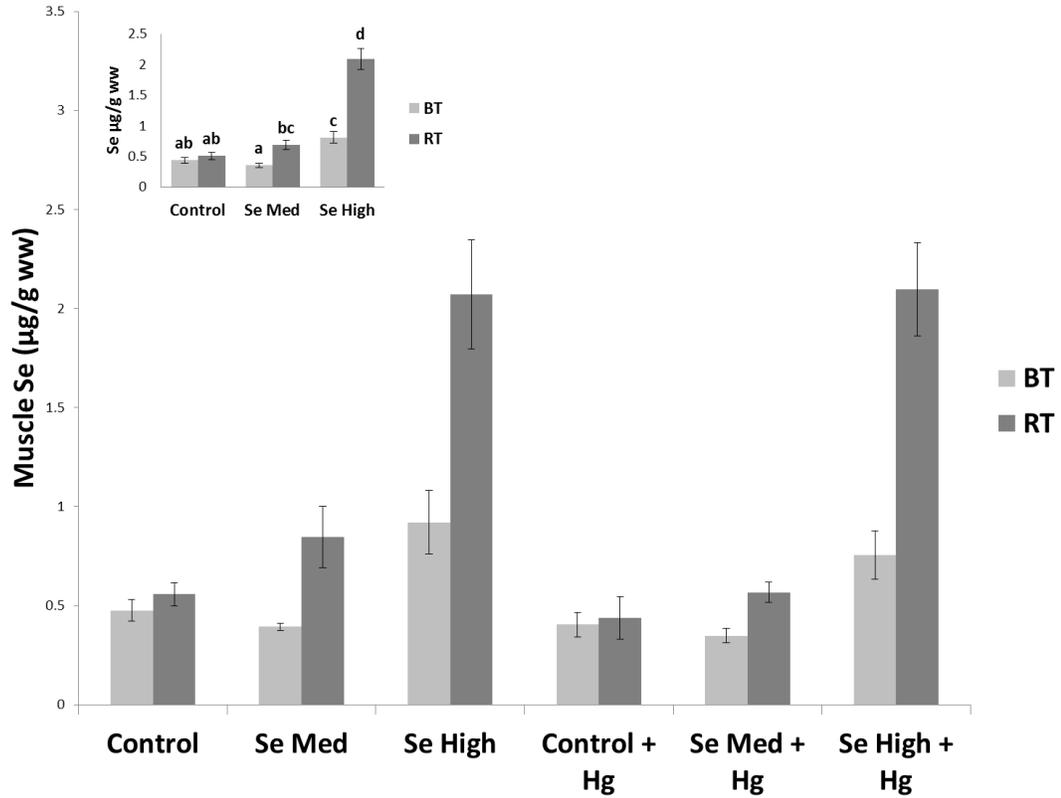
Fig 3.1-

Concentrations of Se (mean $\pm$ SE) on Day 28 in muscle of BT (n= 2-15) and RT (n= 7-13) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant interaction effect between Species and Se exposure (see inset graph, Three-Way ANOVA, $F_{2, 107}=7.0$, $p<0.01$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
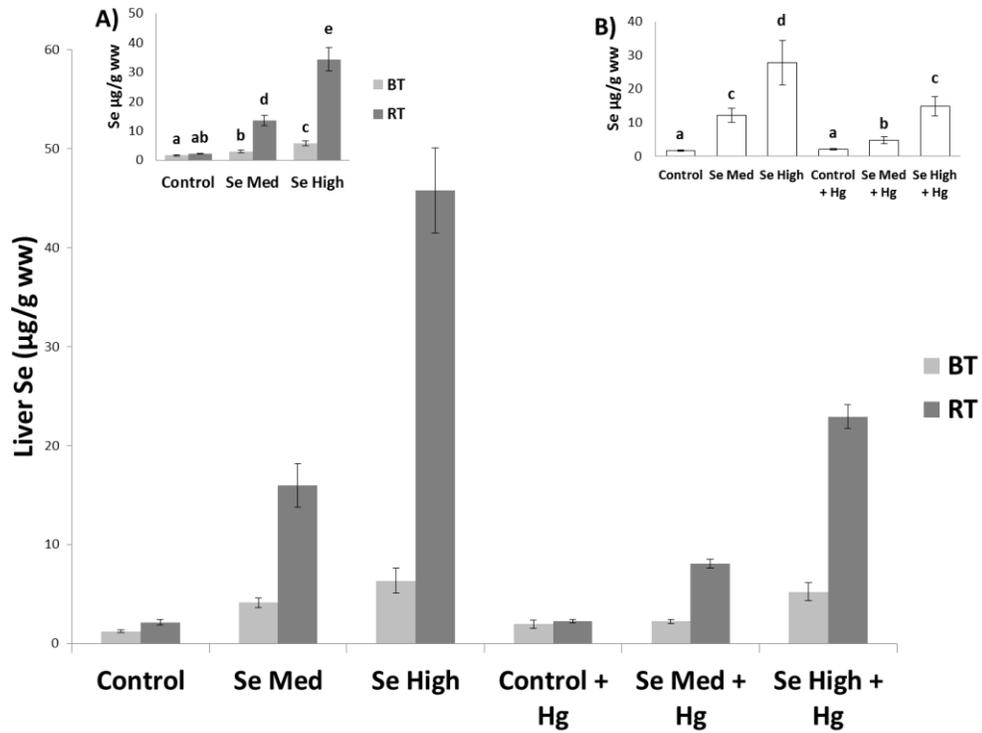
Fig 3.2-

Concentrations of Se (mean $\pm$ SE) on Day 28 in liver of BT (n= 4-6) and RT (n= 5-11) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant interaction effect between Species and Se exposure (see inset graph A, Three-Way ANOVA, $F_{2,61}=31.4$, $p<0.00001$). There was a significant interaction effect between Se exposure and Hg exposure (see inset graph B, Three-Way ANOVA, $F_{2,61}=11.4$, $p<0.0001$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
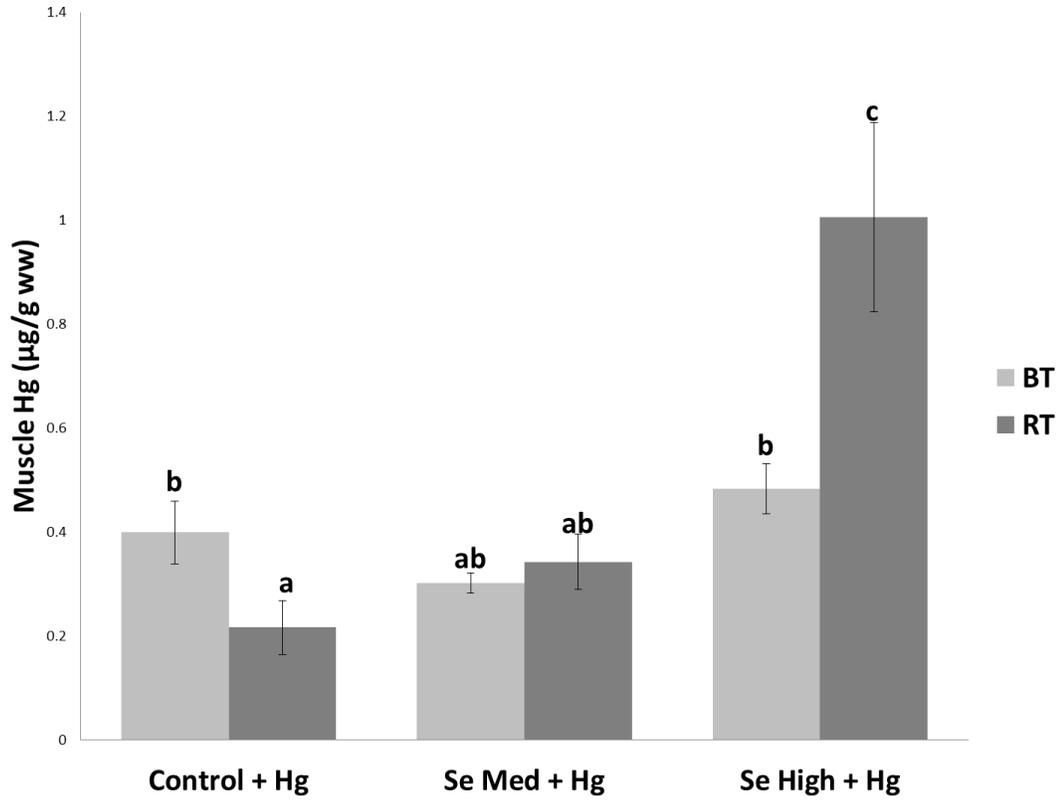
Fig 3.3-

Concentrations of Hg (mean $\pm$ SE) on Day 28 in muscle of BT (n= 11-15) and RT (n= 5-12) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant interaction effect between Species and Se Exposure (Two-Way ANOVA, $F_{2, 60}=10.3$, $p<0.001$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
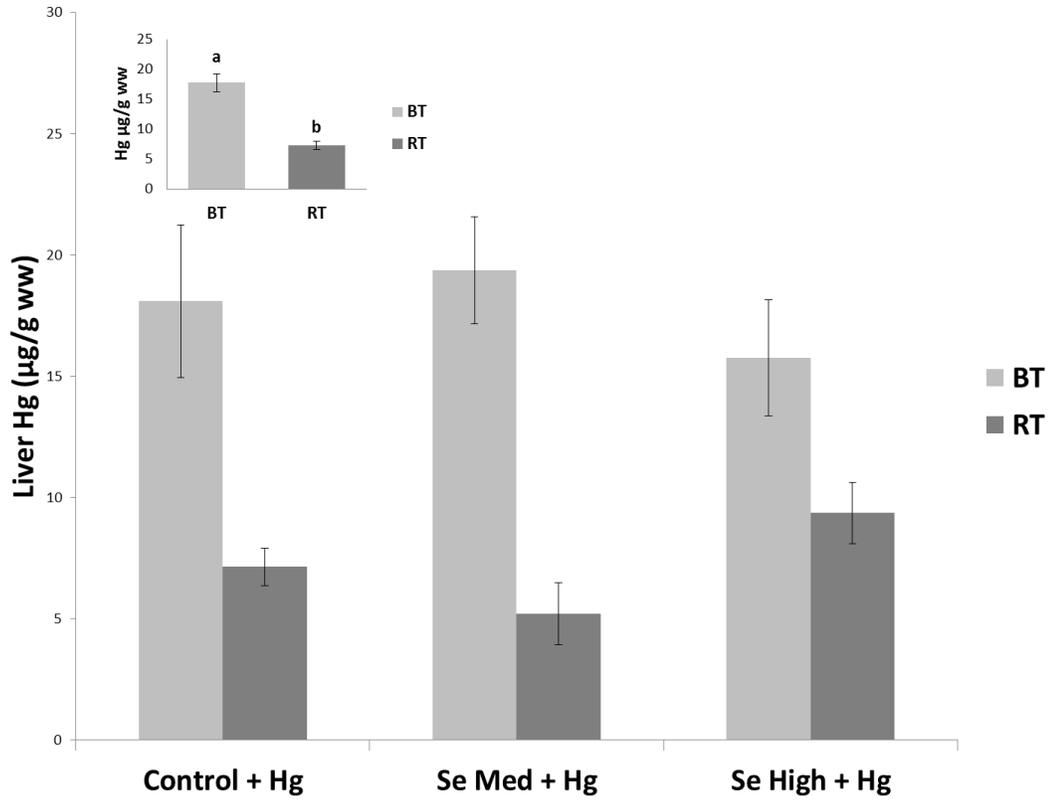
Fig 3.4-

Concentrations of Hg (mean $\pm$ SE) on Day 28 in liver of BT (n= 5-6) and RT (n= 5-8) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Species effect (see inset graph, Two-Way ANOVA, $F_{1, 29}$=43.5, $p<0.00001$). Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.

<u>Table 3.1</u> –
The Hg : Se molar ratios for BT and RT calculated from concentrations in the muscle and liver. There was a significant Species effect in Muscle Hg : Se Molar Ratio (Two-Way ANOVA, $F_{1, 63}=13.0$, $p<0.001$). There was a significant Species effect in Liver Hg : Se Molar Ratio (Two-Way ANOVA, $F_{1, 29}=78.1$, $p<0.0001$). The star (*) represents significant differences between species.   There was a significant Se Exposure effect in Liver Hg : Se Molar Ratio (Two-Way ANOVA, $F_{2, 29}=19.6$, $p<0.00001$). Letters ([A, B, C]) represent significant differences between exposure groups. There were no interactions between Se Exposure and Species ($p>0.05$). Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.

Species	Tissue Molar Ratio	Control + Hg	Se Med + Hg	Se High + Hg
BT *	Muscle Hg : Se	0.53 ± 0.10	0.38 ± 0.03	0.34 ± 0.05
RT	Muscle Hg : Se	0.33 ± 0.07	0.25 ± 0.04	0.19 ± 0.03
Species	Tissue Molar Ratio	Control + Hg [A]	Se Med + Hg [B]	Se High + Hg [C]
BT*	Liver Hg : Se	4.35 ± 0.81	3.59 ± 0.58	1.47 ± 0.48
RT	Liver Hg : Se	1.36 ± 0.14	0.25 ± 0.06	0.16 ± 0.01

Table 3.2 –
Tank water Hg concentrations (ng/L) (mean $\pm$ SE) measured in triplicate from tanks that contained BT and RT exposed to Hg and different concentrations of Se.

Species	Control	Se Med	Se High	Control + Hg	Se Med + Hg	Se High + Hg
BT	0.83 ± 0.11	2.24 ± 0.84	0.83 ± 0.08	16.6 ± 2.27	14.4 ± 2.48	18.2 ± 1.19
RT	0.52 ± 0.04	1.42 ± 0.15	1.20 ± 0.42	57.8 ± 0.59	17.83 ± 0.13	20.9 ± 1.21
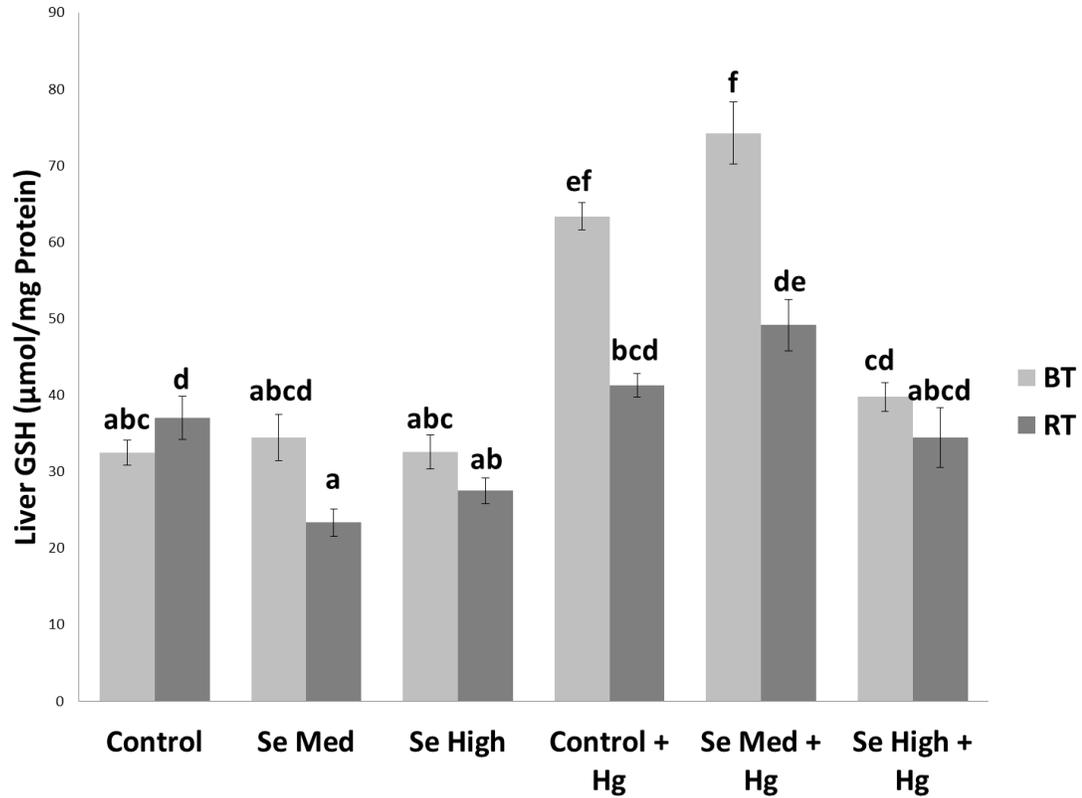
Fig. 3.5 –

Liver GSH (mean $\pm$ SE) concentrations on Day 28 in BT (n= 14-16) and RT (n= 9-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant interaction effect between Species, Se Exposure, and Hg Exposure (Three-Way ANOVA, $F_{2, 163}$=15.7, p<0.00001). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
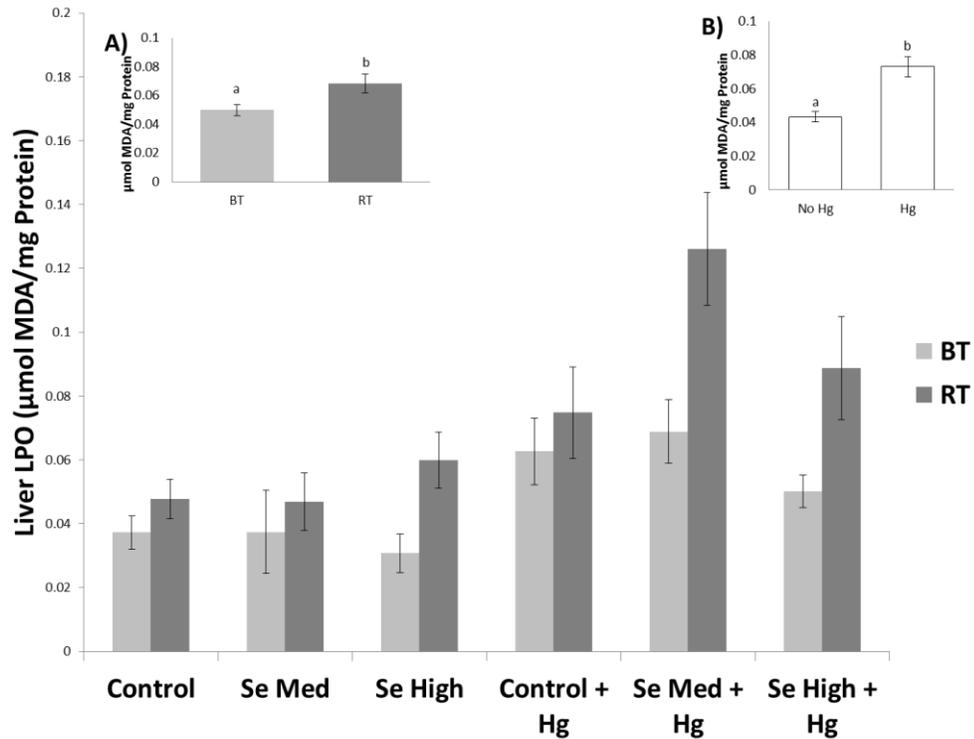
Fig. 3.6 –

Liver LPO (mean $\pm$ SE) concentrations on Day 28 in BT (n= 7-15) and RT (n= 5-16) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Species effect (see inset graph A, Three-Way ANOVA, $F_{2,108}=14.6$, $p<0.0001$) and Hg Exposure effect (see inset graph B, Three-Way ANOVA, $F_{2,108}=21.2$, $p<0.0001$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
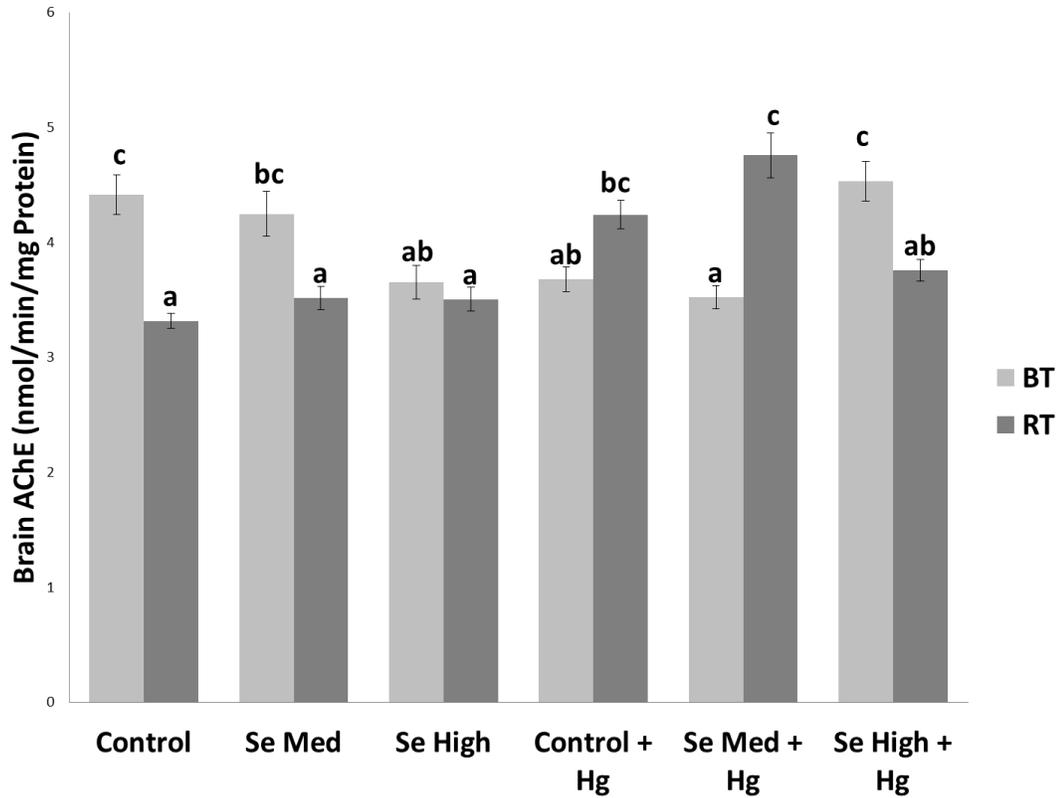
<u>Fig. 3.7</u> –

AChE activity (mean $\pm$ SE) on Day 28 in brain of BT (n= 14-17) and RT (n= 8-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant interaction effect between Species, Se Exposure, and Hg Exposure (Three-Way ANOVA, $F_{2, 162}$=26.4, p<0.00001). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
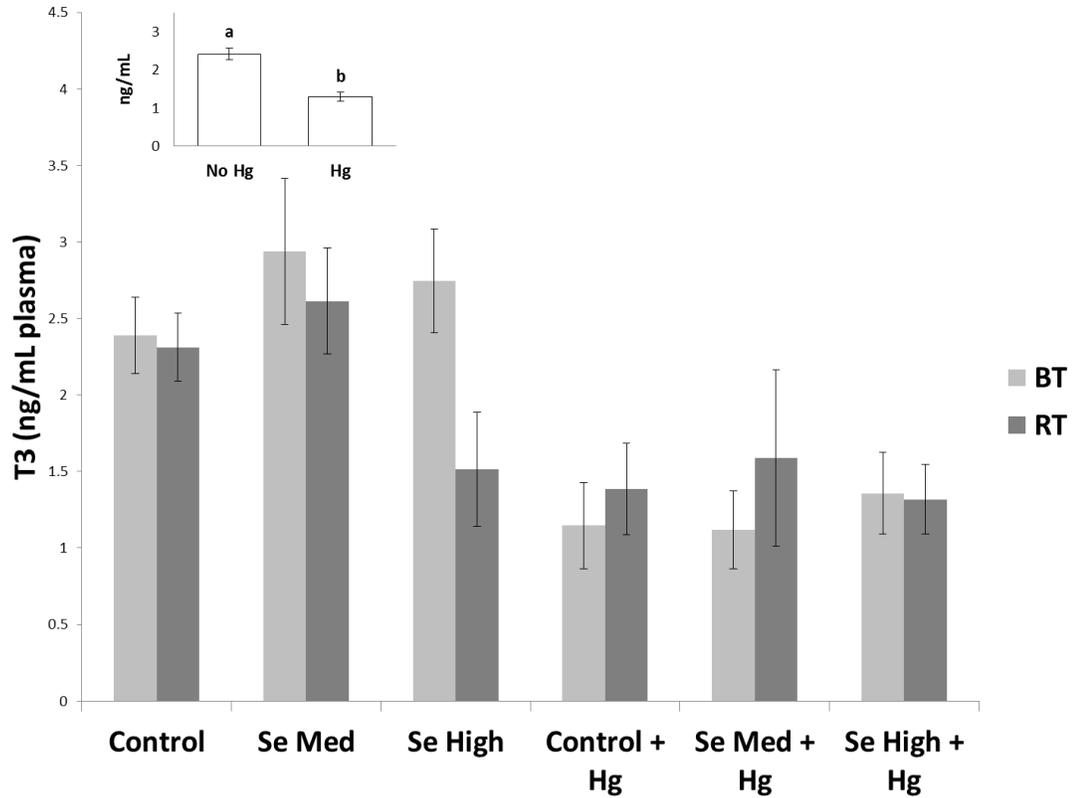
Fig. 3.8 –
Plasma T3 concentrations (mean $\pm$ SE) on Day 28 in BT (n= 9-16) and RT (n= 8-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Hg Exposure effect (see inset graph, Three-Way ANOVA, $F_{1, 154}$=36.3, $p<0.0001$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
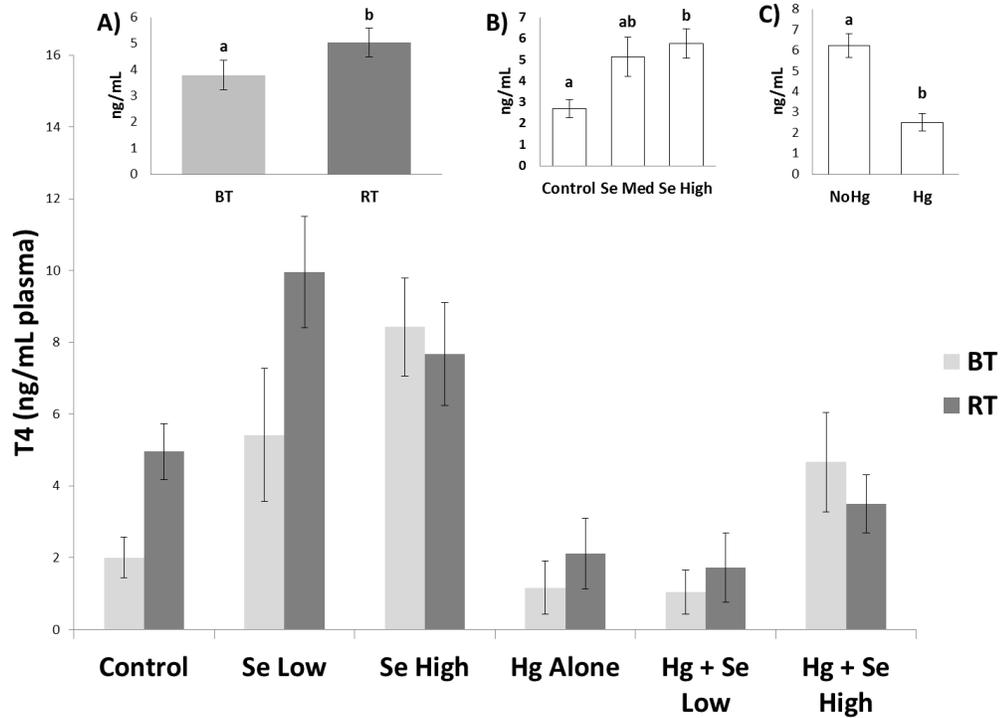
Fig. 3.9 –
Plasma T4 concentrations (mean $\pm$ SE) on Day 28 in BT (n= 9-16) and RT (n= 8-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Species effect (see inset graph A, Three-Way ANOVA, $F_{1, 128}$=10.5, $p<0.01$) and Se Exposure effect (see inset graph B, Three-Way ANOVA, $F_{2, 128}$=7.4, $p<0.01$) and Hg Exposure effect (see inset graph C, Three-Way ANOVA, $F_{1, 128}$=34.4, $p<0.00001$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
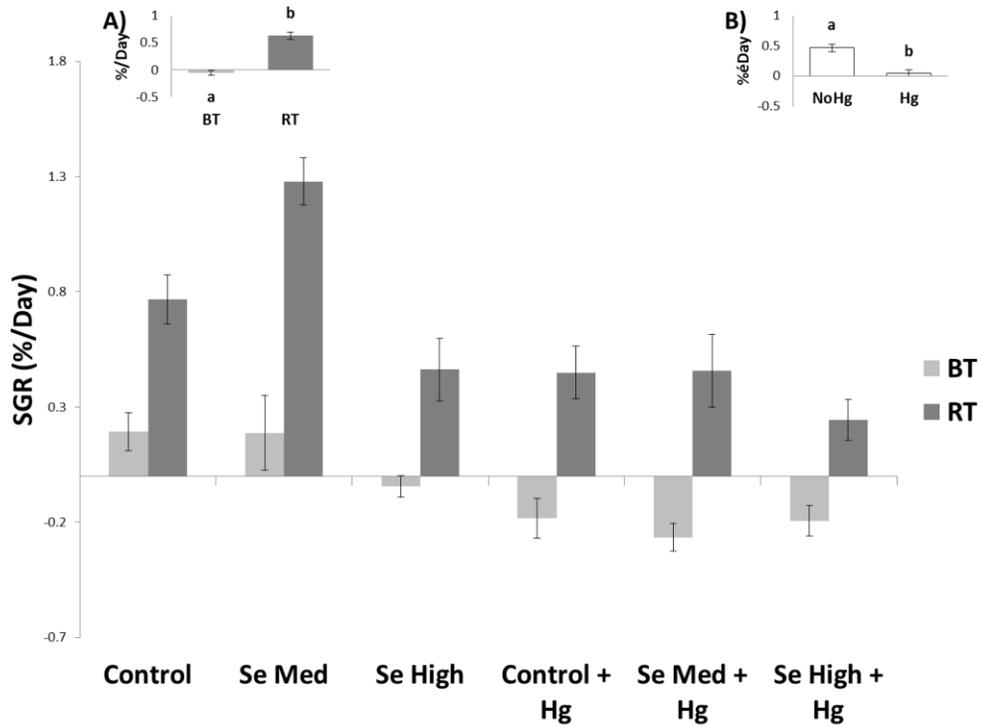
Fig. 3.10 –
Specific growth rate (mean $\pm$ SE) on Day 28 in BT (n= 14-16) and RT (n= 9-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Species effect (see inset graph A, Three-Way ANOVA, $F_{1, 164}$=114.5, p<0.0001) and Hg Exposure effect (see inset graph B, Three-Way ANOVA, $F1_{,164}$=38.5, p<0.00001). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
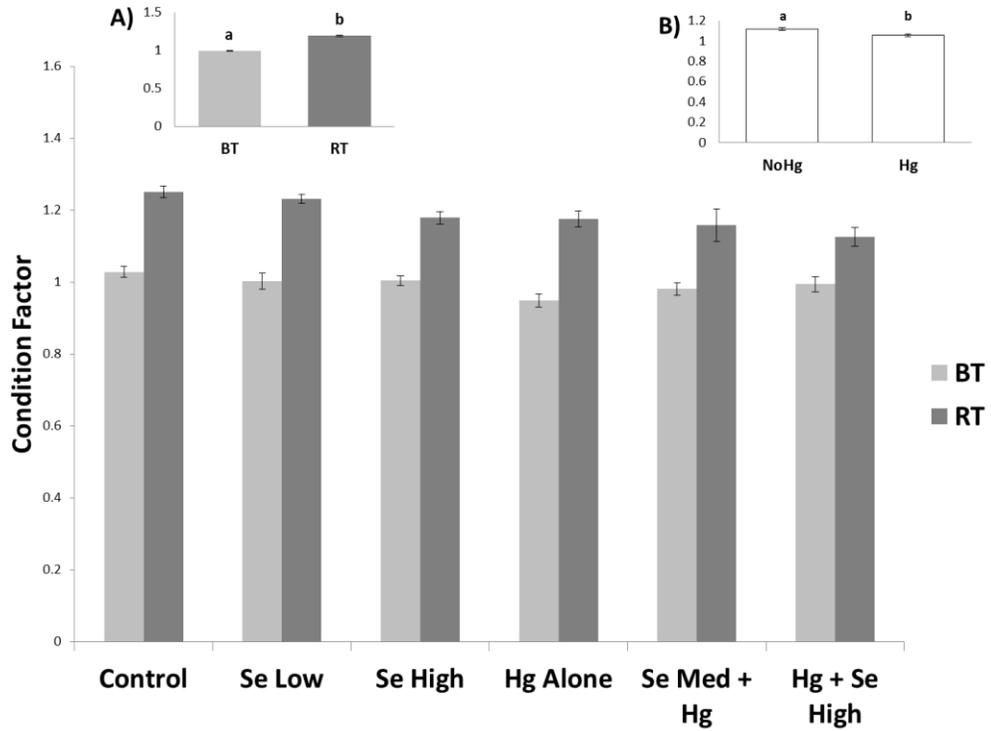
Fig. 3.11 –

Condition factor (mean $\pm$ SE) on Day 28 in BT (n= 14-16) and RT (n= 9-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Species effect (see inset graph A, Three-Way ANOVA, $F_{1, 164}=292.5$, $p<0.00001$) and Hg Exposure effect (see inset graph B, Three-Way ANOVA, $F_{1,164}=20.6$, $p<0.00001$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
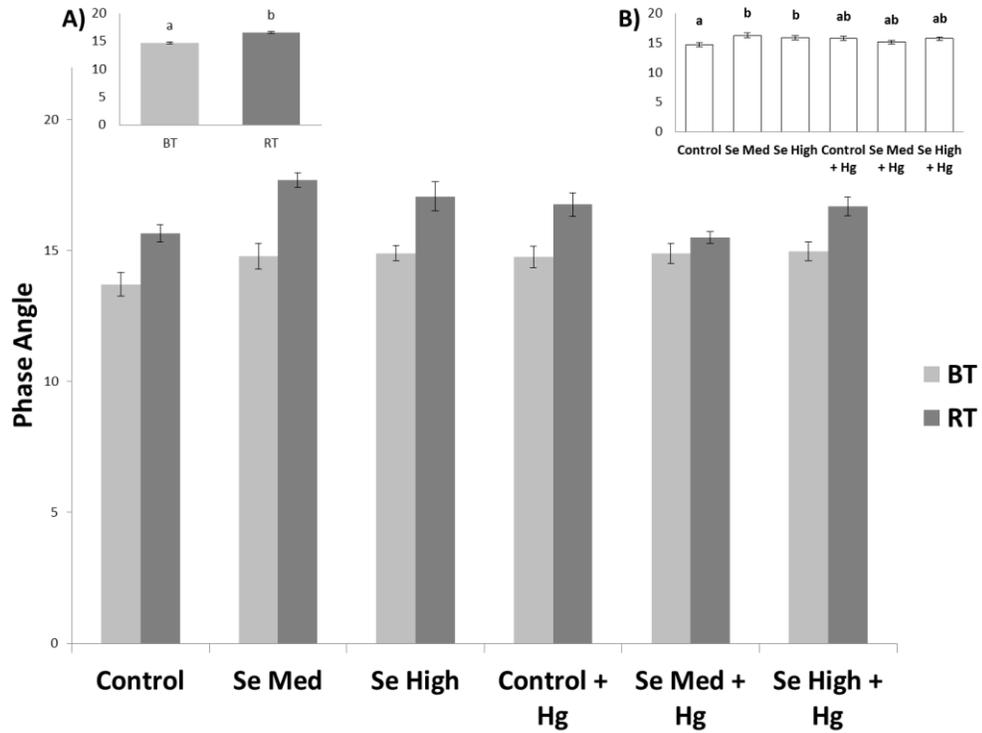
Fig. 3.12 –
Phase angle (mean $\pm$ SE) on Day 28 in BT (n= 14-16) and RT (n= 9-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Species effect (see inset graph A, Three-Way ANOVA, $F_{1, 163}$=69.7, p<0.00001) and Se and Hg Exposure interaction effect (see inset graph B, Three-Way ANOVA, $F_{1, 163}$=6.45, p<0.01). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.
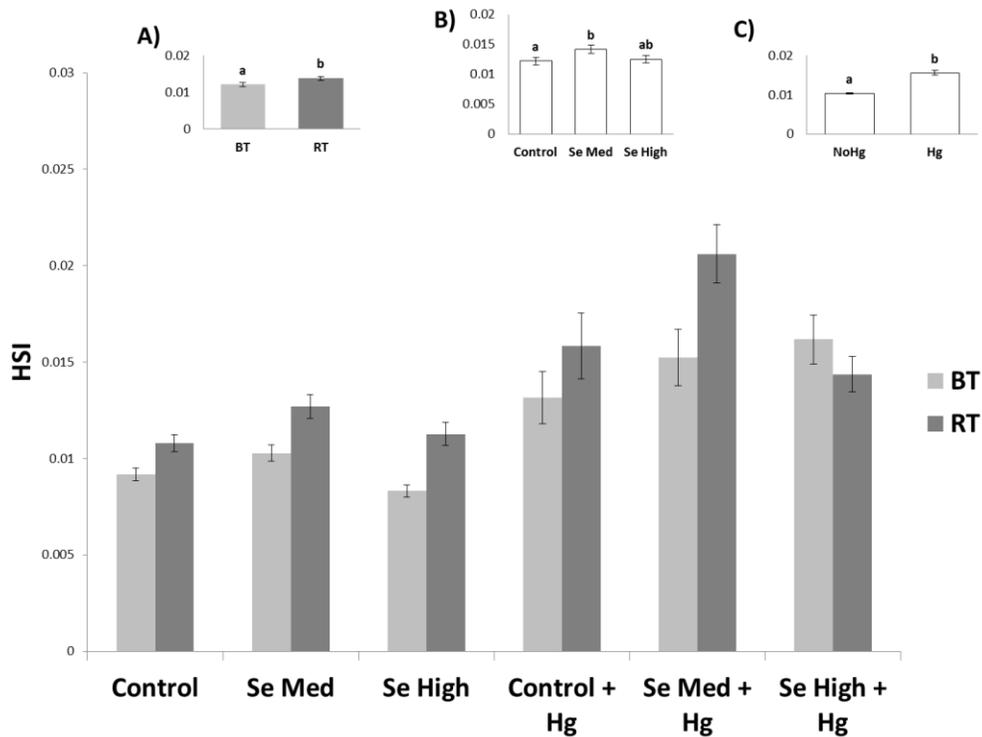
Fig. 3.13 –
Hepatosomatic Index (mean $\pm$ SE) on Day 28 in BT (n= 14-16) and RT (n= 9-17) exposed to a single ip injection of $HgCl_2$ on Day 14 and fed different concentrations of Se through diets on Day 1-28. There was a significant Species effect (see inset graph A, Three-Way ANOVA, $F_{1, 164}=13.2$, $p<0.001$) and Se Exposure effect (see inset graph B, Three-Way ANOVA, $F_{2, 164}=6.32$, $p<0.05$) and Hg Exposure effect (see inset graph C, Three-Way ANOVA, $F_{1, 164}=77.6$, $p<0.00001$). Letters represent significant differences. Analyses have been adjusted using a Bonferroni correction for multiple comparison tests.

CHAPTER 4: Summary
Chapter 1

A literature review of recent studies published on the toxicology of Se, Hg and the Se/Hg interactions is presented in this chapter. Selenium is an essential trace element that can become toxic at concentrations slightly above those required to maintain normal functions. It can enter aquatic systems as a result of anthropogenic processes such as coal mining. Once in the aquatic environment, Se can be taken up by aquatic organisms and accumulate in the food chain and higher trophic level organisms such as fish. Elevated concentrations of Se in fish can result in toxic concentrations. The main mechanism of toxicity was thought to be Se replacing sulphur in proteins and causing improper synthesis. However, recent studies have also provided evidence for oxidative stress as a mechanism of Se toxicity. An indicator of Se toxicity in fish is teratogenicity or the presence of developmental deformities. High concentrations or chronic exposure can also result in negative impacts on adult fish. Elevated concentrations of Se have been reported to cause a range of adverse effects, including metabolic and hormonal alterations in different species of fish, studied in the field and the laboratory. Selenium has also been reported to co-occur with other contaminants including Hg.

Mercury is a nonessential element and a dangerous pollutant. It can be released into the atmosphere through natural and anthropogenic sources. Once in the atmosphere Hg can be transported and deposited into aquatic environments where it can be methylated. Methyl Hg can bioaccumulate and result in high concentrations in predatory fish that are important components in the diets of animals, including humans. Elevated concentrations of Hg may cause cell injury and death, and disrupt essential biological pathways. These mechanisms can elicit a range of toxicological effects in fish. However,

Hg accumulation and toxicity has been reported to be alleviated in the presence of elevated concentrations of Se.

The interaction between Se and Hg has been investigated in a variety of organisms. Interestingly, as a result of the antagonistic interaction between Se and Hg reported in aquatic organisms, Se treatment of aquatic systems has been proposed as a remediation strategy to lower Hg contamination in fish. Several literature reviews have been completed presenting results of earlier studies, with discussions of the mechanisms proposed for the interaction of Se and Hg, and future research directions to be investigated. However, the research on the Se/Hg interaction is vast and ongoing, and therefore a more recent literature review was completed (Chapter 1). As presented in Table 1.1, the antagonism of Se with Hg is reported in recent studies; however, some studies report a lack of interaction or an additive interaction on reproduction. Therefore, additional research is needed on different biological systems and throughout the life cycle of organisms. Also, species may differ in their response to Se and Hg exposure. More research is required on species differences to provide essential information to ensure protective water quality guidelines and management. Species sensitivity data will also provide useful data to the consideration of Se treatment of Hg contaminated aquatic systems.

Chapter 2

To select the dose of injected $HgCl_2$ which would result in measureable muscle and liver Hg burdens near the consumption guidelines of 0.5 mg/kg, a pilot study was completed. Brook trout were injected with either saline or 0.1, 0.3 or 0.5 mg Hg/g body weight, fed control diet and euthanized on Day 7. Dorsal muscle sections and livers were

removed and analyzed for Hg concentration. The injection increased the concentration of Hg in both tissues and at the highest injected dose, 0.5 mg Hg/g body weight, the concentration of Hg was slightly below the consumption guideline in the muscle tissue and was therefore chosen as the ideal injection dose for the main experiment. However, during the main experiment, BT injected with 0.5 mg Hg/g body weight had mortalities and therefore the dose was reduced to 0.3 mg Hg/ g body weight for RT.

In the main experiment, trout were injected with Hg on Day 1 and fed ad libitum, control (0 µg/g), medium (15 µg/g), or high (45 µg/g) SeMet enriched diets until Day 14. On Day 14, fish were euthanized and the concentrations of Se and Hg in muscle and liver, oxidative stress, and hormonal, growth, and energetic status were analyzed. Exposure to Se enriched diets significantly increased the concentration of Se in the muscle and liver of both trout species but was not affected by exposure to Hg. Injection of Hg significantly increased the concentration of Hg in the muscle and liver but the concentration was not affected by exposure to Se. Exposure to Hg also significantly increased liver GSH levels, decreased plasma T3 concentrations, and increased HSI in both species. In rainbow trout, exposure to Hg also significantly decreased plasma T4 concentrations and lowered phase angle, an indicator of body lipid content. However, no effects of Hg were alleviated by supplemental Se. Since the lack of an interaction between Se and Hg, assessed by the concentration of Hg in tissues and protection against the effects of Hg may be a result of insufficient supplemental Se, a follow up experiment with a longer exposure to the Se enriched diets was initiated (Chapter 3).

Chapter 3

To assess species differences in the interaction between Se and Hg, BT and RT were fed control and SeMet enriched diets from Day 1 to Day 28, received a single ip injection of $HgCl_2$ at 0.3 mg Hg/ g body weight on Day 14, and were euthanized and sampled on Day 28. Concentrations of Se and Hg in muscle and liver, oxidative stress indicators, hormonal, growth, and energetic status were evaluated.

Selenium accumulated in the muscle and liver of both trout species, and RT had higher concentrations than BT. Concentrations of Se in the liver was lower in the groups exposed to Hg. In BT, Hg accumulated in the muscle but was not affected by Se diets but in RT, Hg concentrations in the muscle were higher in the Se High diet group. In the liver of both trout species, Hg concentrations were not affected by Se exposure but BT had a higher concentration of Hg in the liver compared to RT. Moreover, higher concentrations of Hg were detected in the water of tanks that held trout exposed to Hg, with a particularly high concentration of Hg in tank water of RT exposed to Hg and fed control diets.

In Hg exposure groups, the average Hg : Se molar ratio in BT was < 1 in the muscle and > 1 in the liver, indicating that Se was in molar excess of Hg in the muscle and Hg was in molar excess of Se in the liver. In RT, the average Hg : Se molar ratio was < 1 in the muscle and in the liver which indicates Se to be in molar excess of Hg in both tissues. Also, BT had a significantly higher molar ratio than RT in the muscle and liver. Species differences in Se compartmentalization, Hg elimination and behaviour in the laboratory may have caused differences in Se and Hg accumulation. The result of the interaction on accumulation may be affected by different chemical forms of Se and Hg,

molar ratios, the route and timing of exposure and different fish species. To further investigate the interaction between Se and Hg, a suite of physiological and biochemical endpoints were measured in both fish species.

Reduced glutathione was measured as an indicator of oxidative stress in the liver. Exposure to Se alone did not alter GSH in BT; however, Hg and Hg in combination with Se Medium diets increased GSH, a response which was not observed with the Se High diet. An increase in GSH may indicate a defensive response to Hg which the supplemental Se in the Se High diet prevented, possibly by forming an inert complex with Hg. In RT, exposure to Se alone lowered liver GSH levels, possibly by the production of reactive radicals. However, exposure to Hg and Se mixtures did not have an effect on GSH. In comparison, BT had significantly higher GSH levels than RT when exposed to Hg and Hg in combination with Se Medium diets which may have been caused by a higher concentration of Hg in BT liver. Also, BT had significantly lower levels in the control compared to RT, which is consistent with earlier literature that reported RT to have higher GSH reserves than BT.

Plasma T3 and T4 concentrations decreased when trout were exposed to Hg and the response was not alleviated by Se. Even though the molar ratio suggests Se to be in excess of Hg in the muscle of BT and the muscle and liver of RT, there may not have been enough Se available to maintain the normal function of iodothyronine deiodinases and synthesis of thyroid hormones, and no alleviation of the Hg effect was observed. Concentrations of T4 differed between Se diet exposures as Se High diet increased the concentrations of plasma T4; however, Se Medium diet did not have an effect. The High concentration of Se in the diet may have provided enough Se to increase T4 production.

Plasma concentrations of T4 increased with diet Se and differed between species, as BT had significantly lower concentrations of T4 than RT. Brook trout had a significantly greater Hg : Se molar ratio in the muscle and liver and therefore Hg might have been more readily available in BT compared to RT to decrease concentrations of T4.

The present study provided evidence of Se antagonism on the adverse effects of Hg on Se dependent processes, including preventing oxidative stress and maintaining normal synthesis and activation of thyroid hormones. Species differences in Se compartmentalization, Hg elimination, and behaviour in the laboratory affected the concentration of the contaminants and therefore affected the physiological and toxicological endpoints. Species differences in the response to Se and Hg exposure require further investigation as they may impact the validity of water quality management and the proposal of using Se as a remediation technique in Hg contaminated aquatic systems.

Future Directions

Trace elements Se and Hg contaminate aquatic systems and are anthropogenic pollutants of concern due to their ability to accumulate in aquatic food chains. Fish are an important component in the diets of humans and therefore Hg tissue guidelines in fish (0.5 mg/kg) have been established to limit the risk of human exposure. Moreover, Se and Hg water quality guidelines are important for overall aquatic habitat health. In order to establish effective water quality guidelines for these contaminants, species sensitivities must be considered and set to ensure the wellbeing of sensitive species. However, the co-occurrence of these contaminants in aquatic systems also needs to be considered, as antagonistic, additive or synergistic interactions may occur.

Selenium interacts with Hg in the biotic and abiotic components of the aquatic environment. Several studies provided evidence for the antagonistic effect of Se on the accumulation and toxicity of Hg in aquatic organisms both in a field and laboratory setting. Subsequently, Se treatment has been proposed as a potential remediation procedure to lower the rate of Hg methylation and uptake, and to decrease tissue burdens in Hg-contaminated aquatic systems (Turner and Rudd 1983, Turner and Swick 1983, Paulsson and Lundbergh 1989, 1991, Mailman et al. 2006). It has even been argued that to protect human health from Hg toxicity, it is the ratio of Se to Hg that should be considered, not the Hg concentrations alone. However, other studies have provided evidence of Se having both antagonistic and synergistic effects on Hg in fish and aquatic birds (Heinz and Hoffman 1998, Heinz et al. 2012, Penglase et al. 2014) and the toxicity of Se remains a major concern. Southworth et al. (2000) discovered that Se decreased the concentration of Hg in largemouth bass but also caused physical deformities and suggested that 5 µg/L of Se or less could lower the uptake of Hg and not cause adverse effects on aquatic life. A Se addition study by Paulsson and Lundbergh (1991) used sodium selenite to raise the lake water concentrations of Se up to 5 µg/L. The Se addition decreased Hg accumulation in fish; however, fish kills also occurred in some of the lakes which may have been a result of the added Se. Yang et al. (2008) proposed that before large application of Se treatment be accepted, additional research be completed to determine an effective and less toxic form of Se to be used, to choose an appropriate technique and to develop a procedure to determine application characteristics such as the dose and timing of Se addition, and consideration of whether the region is naturally low in Se. The effect of the Se/Hg interaction at multiple life stages also need to be

investigated as the interaction may be antagonistic in adults but may result in additive or synergistic effects on reproduction and the young. Finally, more information is required on species specific effects of Se and Hg interactions as species may differ in their response to the co-exposure.

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