

Sublethal effects of selenium on rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*)

By
Jodi Holm

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of

Masters of Science

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AND BROOK TROUT (*SALVELINUS FONTINALIS*)**

BY

JODI HOLM

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University
of Manitoba in partial fulfillment of the requirements of the degree
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Master of Science

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ABSTRACT

While Se is an essential trace element in the diet of all vertebrates, there is a narrow range between dietary requirements and toxic effect threshold concentrations. Se is ordinarily present in uncontaminated water at concentrations of 0.1-0.4 µg/L. Se contamination in Canadian waters is a continuing problem as a result of enrichment downstream from coal, base metal and uranium mining activities. However, the applicability of toxic effect thresholds and guidelines to these cold water habitats is a matter of contention in the literature since almost all reported cases of Se poisoning have been in standing warm water systems.

Recently, a compensation strategy for coal mining in Alberta has raised concerns regarding Se contamination. Specifically, coal mining companies in the northeastern slopes region of Alberta intend to include end pit lakes as part of their reclamation strategy. These lakes are surface pit mines that are allowed to fill with water once mining is completed. Selenium (Se) concentrations in some of these lakes and at other downstream sites exceed water quality guidelines. Although adult rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) from these Se-contaminated sites have accumulated elevated concentrations of Se in their tissues, they show no signs of Se toxicity.

One of the earliest symptoms of Se toxicity in fish populations is the appearance of teratogenic deformities in the offspring of exposed fish. Teratogenesis at the embryo stage of development occurs when the developing fry have assimilated most of the yolks, contaminated with Se that is efficiently transferred from females to their eggs. Teratogenic deformities can result in a significant loss of the early life stages of a fish

population despite an apparently healthy adult population. To examine the possibility of impaired reproduction in rainbow trout and brook trout near coal mining activity, spawn from both species were collected downstream from coal mining activity as well as from reference sites. Eggs and milt were transported to the laboratory, fertilized and reared to the swim-up stage when they were preserved for evaluation of deformities. Three methods were compared to evaluate deformities, including frequency assessment, graduated severity index, and morphometric analysis. Of these methods, frequency assessment was found to be the most time and cost efficient technique. Using this method, higher incidences of deformities were observed in the offspring of fish from the Se-exposed sites compared to the reference sites. The types of deformities were different between the two species. While rainbow trout fry primarily exhibited spinal deformities and yolk sac and pericardial edema, brook trout primarily exhibited craniofacial deformities.

Although Se-induced teratogenesis has most often been attributed to the ability of Se to substitute for sulfur during the assembly of proteins in the developing offspring of exposed females, the role of oxidative stress has recently been identified in aquatic birds. To test for Se-induced oxidative stress in fish, the concentration of non-enzymatic antioxidants were measured in rainbow trout fed Se laden diets for 120 and 302 days. While antioxidant vitamin concentrations were not significantly affected, a significant increase in the ratio of oxidized glutathione to the reduced form was detected in trout fed the highest Se dose. Although the concentration of Se accumulated by adult trout inhabiting Se contaminated streams in Alberta may not be sufficiently elevated to cause

the depletion of non-enzymatic antioxidant defenses in adults, Se concentrations in eggs may sufficiently high to impact the reproductive fitness of these trout.

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ABBREVIATIONS

ANOVA	analysis of variance
bdwt.	body weight
BL	body length
BT	brook trout
CC	cold creek
CF	condition factor
cm	centimeter
CR	craniofacial
°C	degree Celsius
DC	Deerlick Creek
DD	degree day
DDW	double distilled water
DO	dissolved oxygen
DSD	dorso-ventral spinal deviation
dw	dry weight
ED	edema
FF	finfold
FWI	Freshwater Institute
HH	head height
HPLC	high performance liquid chromatography
HW	head width
g	gram
GR	Gregg River
GSH	glutathione, reduced form
GSI	gonado-somatic index
GSSG	glutathione, oxidized form
ICP-MS	inductively coupled plasma-mass spectroscopy
L	liter
LC	Luscar Creek
LED	left eye diameter
LOL	left operculum length
LSD	lateral spinal deviation
LSI	liver somatic index
µg	microgram
ME	measurement error
MFL	mean fry length
MFW	mean fry weight
min	minute
mg	milligram
mm	millimeter
MS222	tricaine methane sulfonate
ND	not done
NT	not taken
pers. comm.	personal communication

ppb	parts per billion
unpubl. observ.	unpublished observation
r	correlation coefficient
r ²	coefficient of determination
RED	right eye diameter
ROL	right operculum length
ROS	reactive oxygen species
RT	rainbow trout
Semet	selenomethionine
SE	standard error
SI	graduated severity index
SL	snout length
SK	skeletal
SSA	sulphosalicylic acid
TBARS	thiobarbituric acid reactive substances
VTG	vitellogenin
WC	Wampus Creek
ww	wet weight
ww calc.	wet weight calculated

GENERAL INTRODUCTION

Selenium is an essential trace element in the diet of aquatic organisms, but elevated levels can lead to reduced growth, reproductive failure and increased mortality. The potential for Se contamination of aquatic ecosystems due to anthropogenic activity has been recognized for nearly two decades (Lemly 1993a) but many of the existing case studies have come from lentic, warm water habitats. Recently, elevated levels of Se have been detected in cold, lotic systems downstream from uranium (TAEM 1996) and coal (McDonald and Stroscher 1998; Casey and Siwik 2000; Kennedy *et al.* 2000) mining activity in Canada. This thesis has been designed specifically to examine the potential for Se toxicity in fish inhabiting these habitats downstream from coal mining activities in Alberta's northeast slopes region. This investigation began after the discovery of elevated Se concentrations in a coal mining "end pit lake" and in waters downstream from the discharge of this lake. Endpit lakes are surface mine pits that have been allowed to fill with water once mining is completed. Several coal-mining companies in the northeastern slopes region of Alberta have proposed to include "end pit lakes" as part of their reclamation strategy, with as many as fifty proposed lakes to be created in Alberta. This thesis examines the relationship between tissue burdens of Se and toxic effects in rainbow trout and brook trout inhabiting Se-rich flowing water habitats downstream from coal mining activity and from nearby reference locations.

The first chapter of this thesis is a review of the literature concerning the effects of Se on fish. It begins with general background information about the chemistry, cycling and toxicity of selenium. The following three sections of the review summarize evidence for the accumulation of Se in the tissues of exposed fish, toxic effects observed in fish that

have accumulated elevated body burdens, and reproductive effects associated with elevated ovary or egg concentrations.

A hallmark of Se toxicity is the appearance of teratogenic deformities in the offspring of exposed fish, resulting from the transfer of Se from females to their eggs. In the second chapter, a comparison is made between three methods for evaluating deformities, including frequency assessment, graduated severity index, and morphometric analysis. These approaches are routinely used to assess the toxicity of environmental contaminants. Of the three methods, frequency assessment was chosen for use in the field study in the following chapter (Chapter 3) as it was found to be the most cost- and time- efficient method of analysis. Frequency assessment also generated the most meaningful data for deriving site and species specific toxic effect thresholds.

Cold water fish inhabiting seleniferous flowing waters have been shown to accumulate elevated body burdens of Se, but still appear healthy (McDonald and Stroscher 1998; Casey and Siwik 2000; Kennedy *et al.* 2000). However, effects on the offspring are the earliest indicators of Se toxicity. The third chapter examines the possibility of impaired reproduction in fish residing in flowing waters with elevated Se. Spawning rainbow trout and brook trout were sampled from sites downstream from coal mining activity in Alberta as well as from reference sites. Fish were artificially spawned and eggs were fertilized and incubated in the laboratory. Once fry reached the swim-up stage, they were preserved and evaluated for deformities using the frequency assessment method identified in Chapter 2. Results from these studies indicate that while adults of both species appear healthy, there are higher incidences of deformities in the offspring of fish sampled from the Se-contaminated sites. The types of deformities were different for each

species. Rainbow trout fry most often displayed spinal curvatures and edema. Brook trout fry exhibited craniofacial and finfold deformities. In rainbow trout, the incidence of deformities was related to the concentration of Se in the eggs but this relationship was not statistically significant in brook trout.

Se-related terata have often been attributed to the simple substitution of Se for sulfur during protein assembly. However, another biochemical mechanism may also explain some of the adverse effects associated with Se toxicity. Oxidative stress has recently been linked with the generation of Se-induced biological lesions in aquatic birds (Hoffman 2002). The final chapter of this thesis (chapter 4) examines the incidence of oxidative stress in fish exposed to environmentally relevant doses of dietary Se. Rainbow trout were exposed to Se as selenomethionine incorporated into the diet (at 2, 10, and 20 $\mu\text{g/g}$) for 120 and 302 days. Rainbow trout accumulated Se in a dose dependant manner in the liver. Oxidative stress was quantified as differences in the concentrations of non-enzymatic antioxidants in the tissues among the 3 dose groups and a control group. No difference was observed among the dose groups and the control group in growth, condition or blood vitamins/ions. However, trout in the highest dose group did show a significant increase in the concentration of oxidized glutathione in the liver as well as a corresponding increase in the ratio of oxidized to reduced glutathione, indicating elevated generation of oxidative radicals. Since oxidative stress was produced in adult rainbow trout and oxidative stress type lesions, including edema near the region of the heart, have been observed in the offspring of trout that have accumulated elevated levels of Se, this mechanism of Se toxicity warrants further investigation.

Chapter 1

Toxic effects of selenium on fish

1) Introduction

During the past decade, increasing concentrations of selenium have degraded many freshwater ecosystems (Lemly 1996; Maier and Knight 1994). While selenium is an essential trace element, it is also an environmental toxin (Draize and Beath 1935; Ellis *et al.* 1937; Rosenfeld and Beath 1946; Hartley and Grant 1961). Toxic effect threshold concentrations in water, food items, and tissues can be as low as 2-5 times normal background concentrations (Maier and Knight 1994). Based on this narrow difference between safe and toxic levels, selenium is thought to be more poisonous than either arsenic or mercury (Sorenson 1991). Selenium is ordinarily present in uncontaminated waters at concentrations of 0.1-0.4 $\mu\text{g/L}$ (Lemly 1985b; Dobbs *et al.* 1996). It is normally made available to aquatic organisms through natural weathering processes (Malchow *et al.* 1995), including erosion and leaching from naturally seleniferous soils (Maier and Knight 1994). Recognition of the potential for widespread contamination of aquatic ecosystems from anthropogenic sources of Se did not occur until the late 1970s (Andren *et al.* 1975; Cherry and Guthrie 1977; Evans *et al.* 1980; National Research Council 1980; Braunstein *et al.* 1981). Skorupa (1998) has reviewed twelve examples where elevated Se levels have adversely affected fish and wildlife. The major anthropogenic sources of selenium include the combustion of fossil fuels, irrigation of seleniferous agricultural soils, mining of various ores, and numerous manufacturing processes (Maier and Knight 1994; Lemly 1996; Eisler 1985).

Although Se does not occur in distinct deposits, it is commonly associated with the sulfide ores and waste rock of mining operations. As a result, it is often released as a waste product from the mining of materials including uranium, bentonite, coal, and

several metals (US Department of Interior 1998). Se is also strongly enriched in coal itself. Concentrations in coal range from 0.2 to 10 $\mu\text{g/g}$ (Moore 1991).

Mining operations can increase the Se load of soil, surface water, and ground water surrounding mining operations. For example, water concentrations of up to 47.1 $\mu\text{g/L}$ were found downstream four active coal mines in west-central Alberta, while water upstream of the mine site ranged from <0.5 to 2.2 $\mu\text{g/L}$ (Casey and Siwik 2000).

Similarly, Se levels of sites immediately downstream from a coal mine in the Elk Basin, British Colombia were 25 $\mu\text{g/L}$, while sites above mining operations were <1 $\mu\text{g/L}$ (McDonald and Stroscher 1998).

Increased levels of Se can affect wildlife and groundwater surrounding mines even after mining has ceased. Large scale mining operations often leave pits to fill with water when mining is complete, resulting in “endpit lakes”, which commonly have poor water quality related to Se enrichment. Unlike natural lakes, which are generally wider than they are deep, end pit lakes are usually extremely deep, with small diameter to depth ratios, which can prevent mixing of the water (Miller *et al.* 1996) and exacerbate the uptake of Se by biota in the limited littoral zone.

1.1) Selenium chemistry

Selenium is a metalloid, having chemical and physical properties of both metals and nonmetals (Haygarth 1994). It also shares many chemical and biochemical properties with sulfur, as both elements are members of Group VIA of the periodic table (Oremland 1994). As a result of these similarities, selenium can serve as an analogue for sulfur and

enter into many of the same abiotic and biotic reactions and pathways (Maier and Knight 1994).

The chemical speciation of selenium in freshwater ecosystems is poorly understood (Lemly 1993a; Maier and Knight 1994). However, it is known that selenium can exist in four oxidation states, each with its own chemical, biological and toxicological properties (Maier and Knight 1994). The most common forms of inorganic selenium in surface waters are the highly soluble selenate (Se^{+6}) and selenite (Se^{+4}). Elemental selenium (Se^0) is stable, insoluble and poorly assimilated by aquatic organisms (National Academy of Sciences 1976). Selenides (Se^{-2}) occur as both organic and inorganic forms. Inorganic selenides are formed naturally in anaerobic environments, often as insoluble metal selenides (National Academy of Sciences 1976). Organic selenides, including Se-amino acids (e.g., selenomethionine, selenocysteine), Se-proteins, and methyl selenides, are formed by the biological reduction of selenium by aquatic organisms (Chau *et al.* 1976; Schrift 1976) that incorporate selenium as a sulfur analogue (Maier and Knight 1994).

1.2) Selenium cycling in aquatic systems

Selenium entering aquatic systems can follow any of four principal pathways: 1) it can be absorbed or ingested by organisms, 2) it can bind or complex with particulate matter, 3) it can remain free in solution or, 4) it can be released to the atmosphere through volatilization (Lemly 1997a). Over time, the majority of aqueous selenium is either taken up by organisms or bound to particulate matter (Lemly and Smith 1987).

Immobilization processes, including chemical and microbial reduction, adsorption, co-precipitation and deposition of plant and animal tissue, are effective at removing

selenium from solution and sequestering it in the sediments (Lemly and Smith 1987). However, the sediments serve only as a temporary repository for selenium (Lemly 1997a). Because aquatic systems are dynamic, selenium can be cycled from the sediments to the food chain by numerous biological, chemical and physical processes, including oxidation by plants and microorganisms, as well as sediment oxidation due to water circulation and biological mixing. Even when surface water concentrations of selenium are low, remobilization of selenium can occur through the direct uptake of selenium from the sediments by rooted plants, bottom-dwelling invertebrates and detritus-eating fish and wildlife (Lemly and Smith 1987). Consequently, selenium levels can remain elevated in the food chain for several years after waterborne inputs of selenium have ceased (Lemly 1997a).

Although immobilization and mobilization processes occur simultaneously in aquatic systems, the relative importance of each varies from one location to another depending on the physical and biological characteristics of the system. Shallow wetlands and marshes tend to accumulate selenium efficiently because of their low flushing rates. Sediments in these systems tend to build up high concentrations of selenium that are remobilized through the benthic-detrital component of the food chain. Conversely, fast flowing systems do not accumulate high concentrations of selenium because their high flushing rates prevent the development of a contaminated surface layer of sediment (Lemly and Smith 1987).

1.3) Bioaccumulation

While there are some dissenting opinions (Deforest *et al.* 2000), it is generally agreed that selenium is bioaccumulated in aquatic organisms (Lemly 1985b; Ogle *et al.* 1988; Maier *et al.* 1988; Ohlendorf 1989; Lemly 1998). This means that organisms can accumulate selenium concentrations greater than those present in either their food or in the surrounding water. The two mechanisms for bioaccumulation are bioconcentration and biomagnification. Bioconcentration involves the direct uptake of dissolved selenium across respiratory and epithelial membranes by either passive or active mechanisms (Maier and Knight 1994). Biomagnification involves the ingestion and assimilation of organic selenium by successive trophic levels of the aquatic food web (Maier and Knight 1994). Bioaccumulation occurs primarily at the levels of primary producer and primary consumer (Lemly 1993a). Field studies report that benthic invertebrates and forage fish have accumulated selenium concentrations of 20-370 $\mu\text{g/g}$, dw (Woock and Summers 1984; Lemly 1985a, 1985b; Saiki 1986; Saiki and Lowe 1987; Barnum and Gilmer 1988; Roth and Horne 1988; Schuler *et al.* 1990). While each of these populations appeared stable and reproducing, tissue selenium concentrations exceed dietary thresholds for fish and wildlife (Lemly 1996).

Laboratory studies have shown that selenomethionine can be bioconcentrated over 16,000 times by algae and over 200,000 times by zooplankton when waterborne concentrations were $<1 \text{ ug/L}$ (Besser *et al.* 1989, 1993). In field studies, biomagnification of selenium has been found to range from 2-6 times between producer and lower consumer (Lemly and Smith 1987). For example, Lemly (1985a) found that benthic aquatic insects ($13.1 \text{ } \mu\text{g Se/g}$, ww) collected from Belews Lake, a site exposed to coal fly

ash effluent, accumulated 3 times more Se than periphyton (attached filamentous algae and diatoms, 4.4 $\mu\text{g/g}$). Similarly, larval aquatic insects (237 $\mu\text{g/g}$, dw) exposed to agricultural run-off in the San Luis Drain were found to have accumulated 4 times more Se than algae (67.0 $\mu\text{g/g}$) from the same sites (Saiki and Lowe 1987). Moreover, McDonald and Stroscher (1998) reported a 6 fold increase in Se accumulation between periphyton (1.3 $\mu\text{g/g}$, dw) and aquatic insects (7.7 $\mu\text{g/g}$) in the Elk River Basin, immediately downstream from an active coal mine. Nevertheless, biomagnification has not been clearly demonstrated in laboratory experiments (Bennet *et al.* 1986; Besser *et al.* 1989, 1993).

1.4) Toxicology

The concentration of selenium determines whether it is an essential nutrient or a toxin (Sorenson 1991). Selenium levels of 0.1-0.5 $\mu\text{g/g}$ (dw) are required in fish diets (Hodson and Hilton 1983; Gatlin and Wilson 1984). Selenium is important in the formation and function of the enzyme glutathione peroxidase, an intracellular antioxidant (Heisinger and Dawson 1983; Bell *et al.* 1986) that protects cell membranes from peroxides formed as by-products of oxidative phosphorylation (Lemly 1998) or from contaminant metabolism (Palace and Klaverkamp 1993; Palace *et al.* 1996a). Symptoms of selenium deficiency include increased mortality, reduced growth, anemia, hepatic injury, as well as muscle lesions (Poston *et al.* 1976; Hilton *et al.* 1980; Bell and Cowey 1985; Bell *et al.* 1985; Gatlin *et al.* 1986). However, at concentrations only 7-30 times higher than dietary requirements, selenium becomes a toxin (Lemly 1998). The effects of excess selenium

include tissue damage, reproductive failure and death (Cumbie and Van Horn 1978; Garrett and Inman 1984; Lemly 1985a; Sorenson 1986)

There are several mechanisms proposed for Se toxicity. The first is inorganic poisoning, in which absorbed selenite or selenate forms transient selenotrisulfide or selenopersulfide bonds with the sulfhydryl groups of amino acids (Frost and Lish 1975). These bonds can then interfere with enzyme functions by altering the three-dimensional shapes of proteins. Laboratory studies have shown that inorganic poisoning typically occurs at concentrations much higher than those observed in contaminated aquatic habitats. Thus, this first toxic mechanism is not likely to be relevant in most aquatic systems (Maier and Knight 1994).

The second manner, organic selenium poisoning, occurs when aquatic organisms utilize selenium accumulated from water, sediments or food as a sulfur analogue, forming endogenous selenoamino acids. Since the normal tertiary structure of protein molecules is dependent on the formation of S-S linkages, the use of selenoamino acids in protein synthesis can result in improperly folded or dysfunctional proteins and enzymes (Diplock and Hoekstra 1976; Reddy and Massaro 1983; Sunde 1984; Maier and Knight 1994). In this manner, selenium has the potential to interfere with the proper functioning of any thiol enzyme or protein (Diplock and Hoekstra 1976).

Se toxicity has also been hypothesized to result from the prooxidant activity of some forms of Se. Those forms of Se that are able to redox cycle are most potentially damaging (Spallholz 1994). While selenomethione is not capable of redox cycling, it has been postulated that conversion to redox cycling forms can occur in embryonic birds (Spallholz and Hoffman 2002) and fish (V. Palace, pers. comm.). Specifically,

selenomethionine can be converted to the selenide anion (RSe^-) that can continuously react with thiols, such as glutathione (GSH), to generate superoxide ($\text{O}_2^{\cdot -}$), hydrogen peroxide and other oxyradicals (reviewed in Spallholz and Hoffman 2002; Spallholz 1994). The production of free radicals can result in damage to cells through the generation of oxidative stress (Saltman 1989). Thus, Se toxicity, as a consequence of oxidative stress, results when the oxidation of thiols by catalytic Se compounds exceeds an organism's endogenous antioxidant defense systems or its ability to methylate excessive Se metabolites (Saltman 1989; Spallholz 1994).

1.5) Acute Toxicity

Acute toxicity tests, such as the lethal concentration for 50% of a sample (LC50), provide a quantitative means to compare the relative acute toxicity of different substances, as well as the relative acute sensitivities among different species. However, it should be noted that their relevance to most aquatic systems is limited (Eaton and Klaassen 1996) since they are short-term tests and often extend to doses that are not environmentally relevant.

The acute toxicity values for selenium vary greatly both within and between species of fishes (Appendix I), with life stage, and with the chemical species used in exposures. However, acute toxicity values do not appear to be substantially affected by feeding status (Hodson *et al.* 1980) or water hardness (Hamilton and Buhl 1990) during exposure. The majority of published data regarding acute Se toxicity is for the more toxic selenite form (Hamilton 1995; Canton 1999; Mauk and Brown 1999).

Within a given genus acute toxicity values for Se can vary greatly. For example, Coho salmon (*Oncorhynchus kisutch*) are significantly more sensitive to both selenite and selenate than are chinook salmon (*Oncorhynchus tshawytscha*) (Hamilton and Buhl 1990). Moreover, within a given species extremely wide ranges of acute toxicity values for selenium exist. It is not unusual for LC50 values to differ 2-5 fold in tests conducted under constant conditions (Fogels and Sprague 1977). Reported selenite LC50s for fathead minnows range from 0.620-11.3 mg/L, an almost 20-fold range in sensitivity (Canton 1999). Within species sensitivities also exist among different life stages. Hamilton and Buhl (1990) found that chinook salmon fry were more sensitive to both selenite and selenate than either embryos or alevins. Buhl and Hamilton (1991) found that juvenile coho salmon and rainbow trout were more sensitive to both selenite and selenate than were alevins of the same species.

Differences in acute toxicity also exist between the different chemical species of Se. Selenite is generally more toxic than selenate, regardless of animal species or life stage tested (Maier and Knight 1994; Canton 1999). In mixtures of various ratios, the combined effect of selenite and selenate is found to be additive (Hamilton and Buhl 1990).

Water is the primary exposure pathway for acute toxicity (Canton 1999). However, most of the Se in fish tissues results from dietary rather than waterborne exposure (Cumbie and Van Horn 1978; Finely 1985; Lemly 1982, 1985a; Hamilton *et al.* 1986, 1990; Woock *et al.* 1987; Besser *et al.* 1993; Coyle *et al.* 1993). Acute toxicity values greatly exceed chronic values, which are based on food-chain bioaccumulation pathways. Concentrations as high as those reported for LC50s are rarely encountered in surface

freshwater, even in Se-contaminated systems (McDonald and Stroscher 1998). Thus, for long term protection of aquatic habitats, chronic toxicity is most meaningful (Canton 1999).

1.6) Chronic Toxicity

Chronic toxicity data describing the accumulation and toxicity of Se in fish tissues is reviewed in the following three sections. Modes of exposure include waterborne and dietary laboratory exposures, as well as field exposures. To reduce the introduction of error, values from the literature are reported as either wet or dry weight (ww or dw, respectively) according to how they were originally reported. The reader is also directed to Appendix II, which summarizes the results of chronic exposure experiments for various species of fish. Note that all values in the table have been standardized to wet tissue weight in order to facilitate comparisons between experiments. Where dry weights were reported, they have been converted to wet weight by assuming 75% moisture content as described by Lemly (1993a). The following values (dw) have been suggested for use as toxic effects thresholds for Se in freshwater and anadromous fish: whole-body, 4 µg/g; skeletal muscle, 8 µg/g; liver, 12 µg/g; ovary and egg, 10 µg/g (Lemly 1993a).

Comparison of the sensitivity between cold- and warm-water species

Based on acute toxicity values, the sensitivity to Se of species found primarily in cold-water does not differ significantly from that of species found primarily in warmer waters. For both warm- and cold- water juvenile and adult fish, acute values for selenite range

from 1-35 mg/L, while those for selenate are generally more variable, ranging from 5 to >300 mg/L (Hamilton and Buhl 1990).

Regarding chronic toxicity values, scientists have taken different views on the applicability of warm-water thresholds for cold-water species. Lemly (1994, 1996) speculated that cold-water species could be more sensitive than their warm-water counterparts. Berdusco *et al.* (2000) also suggested that toxic effect threshold values developed using warm-water species are not applicable to cold-water fish. In an attempt to resolve this problem, Brix *et al.* (2000) fit statistical models to the toxicity data for cold- and warm- water species and found that cold-water anadromous salmonids may be slightly more sensitive than warm-water species. It must be noted that their analysis was based on only one study involving salmonids. The lack of studies involving cold-water species may be a major obstacle in resolving this issue.

2) Evidence for Se accumulation in fish tissues

Several field studies have examined the effects of increased Se on warm-water fishes exposed to either coal fly ash effluent or agricultural irrigation runoff. Baumann and Gillespie (1986) studied bluegills (*Lepomis macrochirus*) and largemouth bass (*Micropterus salmoides*) in the Hyco Reservoir of North Carolina, a power plant cooling reservoir with waterborne Se concentrations of 9-12 mg/L. Fish accumulated higher concentrations of Se than those from a reference site did. In the Hyco Reservoir, tissue concentrations (dw) in largemouth bass were 16 µg/g in the carcass and 30 µg/g in the ovaries, while concentrations in bluegills were 26 µg/g in the carcass and 44 µg/g in the

ovaries. In both species, ovarian tissue was found to accumulate more Se than testicular tissue.

Hopkins *et al.* (2000) examined the effects of coal ash-contaminated sediments on lake chubsucker (*Erimyzon sucetta*), a benthic feeding species common to the floodplain habitats of the Savannah River, NC. Lake chubsucker are not present in a drainage swamp which serves as one of a series of settling basins for ash discharge from a coal fired power plant before ultimately draining into the Savannah River. After four months of exposure to polluted sediments (7.82 $\mu\text{g/g}$, dw) collected from the effluent outflow at the coal ash-polluted site, fish accumulated elevated levels of Se, reaching 5.6 $\mu\text{g/g}$ (dw) compared to the control groups that had Se concentrations of approximately 1 $\mu\text{g/g}$. Although Se levels were particularly elevated, fish also accumulated increased levels of strontium and vanadium. Fish exposed to polluted sediments showed increased mortality, decreased growth and fin erosion.

Se accumulation has also been reported in several species of fish from the San Joaquin Valley in California. Agricultural sumps in this region have been reported to contain Se concentrations as high as 4,200 $\mu\text{g/L}$ (Presser and Barns 1985). Saiki *et al.* (1992) found that fish tissues from contaminated sites (>5 $\mu\text{g Se/L}$) accumulated Se in proportion to waterborne concentrations. Maximum whole-body concentrations (dw) at exposed sites were found to reach 9.4 $\mu\text{g/g}$ in bluegills, 10.0 $\mu\text{g/g}$ in common carp (*Cyprinus carpio*), 11.0 $\mu\text{g/g}$ in mosquitofish (*Gambusia affinis*), and 9.7 $\mu\text{g/g}$ in largemouth bass. Juvenile striped bass (*Morone saxatilis*) from the same area were found to accumulate whole-body concentrations of up to 7.9 $\mu\text{g/g}$ (dw; Saiki and Palawski 1990). All species from the reference sites had whole-body concentrations <2 $\mu\text{g Se/g}$ (dw). As a footnote, Saiki and

Ogle (1995) reported that mosquitofish were the only species to maintain a large population in the San Luis Drain, a region of the same valley, where Se concentrations often exceed 300 µg/L. This species was found to be tolerant of high body burdens of Se, containing more than 100 µg Se/g (dw), which is over 80 times higher than reference fish.

Several researchers have begun documenting the effects of Se on endangered species of fish, for which little to no information previously existed. Muscle biopsies are a non-lethal alternative to whole-body sampling. This procedure involves the use of a 5mm biopsy needle as described by Waddell and May (1995). Using this technique, Hamilton and Waddell (1994) reported that razorback suckers (*Xyrauchen texanus*) collected from Ashley Creek in the Green River, Utah, had muscle levels of Se ranging from 4 to 32 µg/g (dw). Eggs and milt contained Se concentrations of 4-11 µg/g and <1-7 µg/g, respectively. In comparison, eggs from a reference site contained only 2.8 µg/g of Se. A strong correlation was found between the Se concentrations in muscle plugs and those of the eggs and milt ($r = 0.99$ and 0.87 , respectively). Water concentrations in the Ashley Creek are reported to range from 59-78 µg/L (Peltz and Waddell 1991). Wild caught larval razorback suckers from a number of sites in the Green River were found to have correspondingly elevated levels of selenium (Hamilton *et al.* 2000). Mean Se concentrations at all sites ranged from 4.3-5.8 µg/g, dw. The authors speculated that elevated levels of Se could be limiting survival and recruitment of larvae to the juvenile stage.

Using the same technique, Osmundson *et al.* (2000) observed that Se concentrations in muscle plugs of Colorado pikeminnow (*Ptychocheilus lucius*) from the Colorado River

(Colorado) averaged 17 $\mu\text{g/g}$ (dw), whereas measured waterborne Se concentrations in the Colorado River were as high as 8 $\mu\text{g/L}$.

A number of laboratory studies have been conducted that describe the effects of Se on bluegill sunfish and other centrarchid fish species. Lemly (1982) found that juvenile bluegill and largemouth bass exposed to waterborne selenite (10 $\mu\text{g/L}$) for 120 days accumulated whole-body concentrations of 0.9-4.8 $\mu\text{g/g}$ (ww). Despite elevated tissue Se, no mortalities were reported. Tissue distribution was nearly identical for both species, with the relative accumulation in specific tissues as follows: spleen, 2.7-19.1 $\mu\text{g/g}$ > liver, 3.0-11.9 $\mu\text{g/g}$ > kidney, 3.1-11.0 $\mu\text{g/g}$ > gill, 6.0-9.4 $\mu\text{g/g}$ > white muscle, 0.3-3.1 $\mu\text{g/g}$. The largest difference between the species was the level in muscle, which was almost twice as high in bluegills as in bass. The author suggested that this difference might be the result of interspecific differences in the production of intramuscular selenoproteins. In addition, it was noted that Se was not significantly eliminated from the muscle, liver, kidney or spleen after transfer to clean water for 30 days.

In another laboratory study, bluegills were exposed to combined dietary seleno-L-methionine and 10 $\mu\text{g/L}$ waterborne selenate and selenite (6:1) (Coyle *et al.* 1993). Selenium increased in whole-body and reproductive tissue in proportion to exposure concentrations (dw). Whole-body concentrations in control fish remained at around 1 $\mu\text{g/g}$ throughout the 140 days of exposure, while whole-body concentrations in high Se diet fish ranged from approximately 3 $\mu\text{g/g}$ (4.6 $\mu\text{g/g}$ diet) to approximately 19 $\mu\text{g/g}$ (33.3 $\mu\text{g/g}$ diet). Higher concentrations of Se were found in reproductive tissues than in the remaining body tissues. Ovarian concentrations in control fish were 2 $\mu\text{g/g}$, whereas fish fed a diet high in Se had concentrations ranging from 10 $\mu\text{g/g}$ (4.6 $\mu\text{g/g}$ diet) to 34

µg/g (33.3 µg/g diet), and were slightly greater than the testicular Se levels.

Reproductive tissues were found to accumulate greater concentrations of Se than was present in the diet, whereas carcass concentrations remained less than the dietary concentrations. No effects on survival, growth or condition factors of the adult fish were observed in any of the treatments.

In an experiment designed to mimic winter conditions, Lemly (1993b) examined the effects of reduced water temperature and photoperiod on juvenile bluegills exposed to combined dietary seleno-L-methionine (5.1 µg/g, dw) and waterborne selenate and selenite (1:1, 4.8 µg/L). By day 180, fish in warm water (20°C) had accumulated whole-body concentrations of 5-6 µg/g (dw), while fish in cold water (4°C) had accumulated about 30% more whole-body Se (7-8 µg/g). Elevated Se in combination with low water temperatures resulted in significant mortalities at the 60-120 day interval. These mortalities were attributed to "winter stress syndrome", which resulted from the combination of Se-induced increase in energy demand and a reduction in feeding due to cold temperatures and reduced photoperiod. Lemly noted that winter stress syndrome has the potential to significantly reduce the amount of Se necessary to kill fish.

Many laboratory studies have investigated the accumulation of Se in forage fish species. Bennet *et al.* (1986) found that larval fathead minnows (*Pimephales promelas*) grew more slowly after being fed high Se rotifers (46-91 µg/g, dw). After 7 days of feeding, 9-day-old larvae accumulated average whole-body Se concentrations of 61.1 µg/g (dw), while 17-day-old larvae contained 51.7 µg/g, following 9 days of feeding. Ogle and Knight (1989) found that adult fathead minnows showed a comparable decrease in growth after exposure to dietary Se (50% selenite, 25% selenate and 25% seleno-L-

methionine). They observed that whole-body and tissue Se levels were dose-dependent, with female gonads containing significantly higher levels of selenium than other tissues examined. At dietary Se levels of 30 µg/g (dw), female fish had accumulated 10.9 µg/g (dw) in gonads and 7.8 µg/g in muscle, while males had accumulated Se levels of 7.8 µg/g in gonads and 8.8 µg/g in muscle. In contrast, female controls contained Se concentrations of 6 µg/g in gonads and 2.7 µg/g in muscle, while male controls had Se concentrations of 3.9 µg/g in gonads and 1.9 µg/g in muscle. Ovarian concentrations of Se were likely higher than testicular or muscular levels because of the incorporation of Se into egg yolks during vitellogenesis, the process of yolk formation (Ogle and Knight 1989). Se is known to bind to the vitellogenin (VTG) complex (Buhl and Hamilton 2000). Growth inhibition occurred when whole-body concentrations reached 5-8 µg/g, after 56 days of exposure to ≥ 20 µg/g Se. It was suggested that the inhibition of growth was a secondary effect that resulted from reduced palatability of seleniferous food.

Crane *et al.* (1992) found that perch (*Perca fluviatilis*) exposed for one year to 2, 10 or 25 µg Se/L of selenate and selenite (60:40) in experimental ponds accumulated up to 10 times more Se than control fish. Perch in the highest dose group accumulated 5.6 µg Se/g (ww) in muscle and 7.0 µg/g in gonads, compared to 0.5 µg/g and 0.8 µg/g, respectively, in control fish. At the highest dose, Se exposed perch also had approximately 50% higher mortality than fish of any of the other doses.

Several field investigations have documented the effects of high environmental concentrations of Se on different populations of fish species that inhabit northern latitudes. Besser *et al.* (1996) found that spottail shiners (*Notropis hudsonius*), white suckers (*Catostomus commersoni*), and northern pike (*Esox lucius*) exposed to coal fly

ash in Pigeon Lake (Michigan) accumulated higher Se concentrations than fish from a reference site. Mean whole-body Se concentrations (ww) for spottail shiner, white sucker and northern pike were 3.8 µg/g, 1.5 µg/g, and 2.5 µg/g, respectively in Pigeon Lake, which had a mean concentration of 6.7-7.7 µg Se/L. At the reference site whole-body concentrations of all species were <1 µg/g. Low Se concentrations in the white sucker relative to the reference fish may have reflected the movement of this relatively mobile species between the two sites (Besser *et al.* 1996).

Hamilton *et al.* (1986, 1990) and Hamilton and Weidmeyer (1990) examined the accumulation of Se in different life-stages of the chinook salmon (*Oncorhynchus tshawytscha*) in a series of experiments. In each of these studies control fish had whole-body concentrations between 0.2-2.6 µg Se/g (dw). Hamilton and Weidmeyer (1990) exposed eyed eggs and swim-up fry to waterborne selenate and selenite (6:1). Eyed eggs exhibited decreased development when whole-body concentrations reached 4.9 µg/g (dw) and swim-up fry had lower overall survival at 1.9 µg Se/g bdwt after exposure to ≥70 µg Se/g for 90 and 30 days, respectively. After 60 days of exposure to 140 µg/L, swim-up fry showed decreased growth (whole-body 4.9 µg/g). However, juveniles exposed to Se in brackish water did not significantly accumulate Se (1.1-2.0 µg/g) compared with controls. In a similar study, Hamilton *et al.* (1990) exposed chinook salmon as swim-up fry and fingerling (brackish water) to either Se-contaminated mosquitofish (*G. affinis*; SLD diet) or to a diet fortified with seleno-DL-methionine (SeMet diet). After 90 days, survival was reduced in swim-up fry fed ≥9.6 µg/g on either diet (whole-body ≥5.4 µg/g, dw) and growth was reduced in fry fed ≥5.3 µg/g SLD diet (whole-body ≥4.0 µg/g) or at ≥18.2 µg/g SeMet diet (whole-body ≥10.8 µg/g). After 120 days fingerlings grew more

slowly when fed $\geq 18.2 \mu\text{g/g}$ SLD diet (whole-body $\geq 14.4 \mu\text{g/g}$) or $\geq 35.4 \mu\text{g/g}$ Semet diet (whole-body $\geq 23.2 \mu\text{g/g}$). The authors attributed the apparently greater toxicity of the Se-contaminated mosquitofish to possible additional toxins or other forms of organoselenides, such as selenocystine. They also discussed differential uptake, distribution or elimination of protein-incorporated versus free selenoamino acids as possible factors. An earlier study reported that parr fed high Se mosquitofish accumulated mean whole-body concentrations of up to $4.9 \mu\text{g/g}$ (ww) after 33 days exposure to $26 \mu\text{g Se/L}$ (Hamilton *et al.* 1986). Although the authors reported no effects on mortality or growth, exposed fish had impaired parr-smolt transformation, including increased mortality during 24-hour seawater challenges. In all three studies, whole-body concentrations generally increased with increasing Se exposure. There is some evidence of variability, however, as Hamilton and Weidmeyer (1990) reported that concentration factors decreased with increasing exposure, suggesting decreased uptake, increased elimination, or both.

Gissel Nielsen and Gissel-Nielsen (1978) found whole-body Se concentrations of $8.4 \mu\text{g/g}$ (dw) in rainbow trout (*O. mykiss*), following 30-days of exposure to 0.4 mg/L waterborne selenite, resulted in increased mortality. Similarly, Hicks *et al.* (1984) found that juvenile rainbow trout exhibited increased mortality, as well as slower growth following 16 weeks of exposure to $11.4 \mu\text{g/g}$ (dw) dietary selenite. They also found that kidney and liver concentrations of Se (dw) increased in relation to dietary concentrations, reaching levels as high as $51.3 \mu\text{g/g}$ and $11.2 \mu\text{g/g}$ respectively, after 24 weeks of feeding. Control fish, on the other hand, had mean Se concentrations of $0.8 \mu\text{g/g}$ in liver and $0.7 \mu\text{g/g}$ in kidney.

Lorentzen *et al.* (1994) provided data that showed Atlantic salmon (*Salmo salar*) accumulated dietary selenite and selenomethionine (Se-met) differently. Se levels (dw) were highest in the livers of fish fed selenite, whereas whole-body and muscle Se were highest in fish fed Se-met. After 8 weeks of exposure to 2 µg Se/g, fish fed selenite accumulated mean concentrations of 1.1 µg/g whole-body, 4.4 µg/g in liver, and 0.5 µg/g in muscle, whereas fish given selenomethionine accumulated mean concentrations of 2.1 µg/g whole-body, 7.4 µg/g in liver, and 2.5 µg/g in muscle. Tissue concentrations in control fish were significantly lower, averaging 0.6 µg/g whole-body, 2.3 µg/g liver, and 0.5 µg/g muscle.

Se Exposures at Coal and Uranium Mines in Canada

The effects of elevated Se on salmonid populations downstream from coal mines has been examined in only a few prior studies. McDonald and Strosher (1998) and Kennedy *et al.* (2000) examined cutthroat trout (*Oncorhynchus clarki lewisi*) downstream from a surface coal mine in the Elkhorn Basin in British Columbia, where waterborne Se concentrations average 28 µg/L. McDonald and Strosher (1998) found that despite the 100-200 fold increase in water concentration, fish tissues contained only 2-5 times higher Se levels (dw) than fish tissues from reference sites (<1 µg Se/L). Se-exposed trout accumulated between 6.0-12.7 µg/g in muscle, 29.9-50.0 µg/g in liver, and 28.2 µg/g in a composite of non-gravid ovarian tissue; whereas, Se concentrations in reference fish ranged from 3.5-4.4 µg/g in muscle, 13.6-23.5 µg/g in liver, and 22.0 µg/g in the ovarian composite. The authors speculated that bioaccumulation may have been limited by the fast-flowing nature of the system. However, the study of Kennedy *et al.* (2000) did

report an increased level of Se in the tissue of trout inhabiting Se-rich waters compared to those from a reference site. Se-exposed fish accumulated muscle concentrations (dw) ranging from 6.7-41 µg/g and liver concentrations of 18.3-114 µg/g, while fish from the reference site had concentrations of 1.4-3.8 µg/g and 3.4-14.6 µg/g in muscle and liver, respectively.

In a study in the northeastern slope region of Alberta, Casey and Siwik (2000) examined the effects of coal mine discharge on rainbow trout (*O. mykiss*). Fish were collected from Lac Des Roches, a lake created from the end pit of a coal mine, and Luscar Creek, its downstream outlet. Water concentrations of Se in the exposed sites were as high as 47.1 µg/L, while reference sites were generally <2 µg/L. In contrast to trout collected from reference sites, which accumulated Se concentrations averaged 0.8 µg/g in muscle and 4.2 µg/g in eggs, rainbow trout in exposed sites were found to accumulate Se concentrations between 3.0-9.4 µg/g in muscle and 16.2-28.9 µg/g in eggs. Furthermore, the authors reported a strong positive relationship ($r^2 = 0.72$) between the Se concentrations in muscle and eggs.

While many coal mines are known to enriched concentrations of Se in receiving waters, only recently has the potential for Se mobilization from other types of mines become known. For example, TAEM consultants (1996) found increases in Se concentrations in lake chub (*Couesius plumbeus*) exposed to uranium mine effluent in the Fulton Creek drainage in British Columbia. Fish at the exposed site, where waterborne concentrations ranged from 12-14 µg Se/L, accumulated mean tissue concentrations (ww) of 12.3 µg/g in the carcass and 14.0 µg/g in the ovaries. Fish that had accumulated ovary concentrations averaging between 10.3-17.1 µg/g showed cataracts (82%), abdominal

distension (18%), and fin deformities (11.5%). Fin deformities included deformed residual pelvic fins and the absence of pelvic fins. In contrast, chub from the reference sites had mean Se concentrations of 0.3 µg/g in the carcass and 1.3 µg/g in the ovaries.

3) Tissue Se and toxic effects

Fish exposed to elevated concentrations of Se in water and or the diet can exhibit several histopathological and hematological effects. Toxic responses are most often studied in the kidney and liver (Sorenson 1991) because these organs generally accumulate the highest concentrations of Se due to their roles in detoxification and elimination (McConnell 1941; Rosenfeld and Beath 1946; Adams 1976; Hilton *et al.* 1982; Kleinow and Brooks 1986a, b). Histopathological lesions induced by elevated Se are generally permanent (Draize and Beath 1935; Smith *et al.* 1937; Miller and Schoening 1938; Rosenfeld and Beath 1947). It should be noted that the majority of research in this area has involved vertebrates other than fish (Sorenson 1991). Only the existing studies involving fish are reviewed in the following sections.

Sorenson *et al.* (1984) reported a number of pathological changes in the tissues of green sunfish (*Lepomis cyanellus*) collected from a site receiving coal fly ash effluent. For example, fish that accumulated 21.4 µg Se/g (ww) in the liver and 12.9 µg/g in skeletal muscle had lower hematocrit values than reference fish. These fish also had swollen gill lamellae with extensive vacuolation. In addition, livers of the Se-exposed fish were prone to lymphocyte infiltration, extensive vacuolation of parenchymal hepatocytes around the central veins, and proliferation of Kupffer cells, which are phagocytic endothelial cells of the liver (Fawcett 1986) that are normally rare in sunfish

livers. The apparent dilation of the central veins, due to the degeneration of the surrounding parenchymal cells, is an important diagnostic feature because it is considered to be an early sign of cirrhosis (Sorenson *et al.* 1983). The kidneys of sunfish that had accumulated the highest concentrations of Se had focal intracapillary proliferative glomerulonephritis, a condition characterized by excessive numbers of mesangial cells and an abnormally abundant matrix. Finally, the hearts of Se loaded sunfish exhibited pericarditis, a condition characterized by swollen, inflammatory cell-filled pericardial spaces, as well as myocarditis, characterized by numerous inflammatory cells between the ventricular myocardial cells. The authors suggested that all of these cardiac conditions might have been secondary effects from glomerulonephritis-induced uremia.

Sorenson and Bauer (1983) and Sorenson (1988) observed comparable hematological and histopathological changes in redear sunfish (*Lepomis microlophus*). Redears that accumulated liver concentrations of 19.6 µg Se/g (ww) had reduced hematocrit and hemoglobin values, abnormally small and irregularly shaped erythrocytes, and lower mean corpuscular hemoglobin concentrations, a measure of the amount of hemoglobin per volume of packed erythrocytes. Mean corpuscular volumes were also compromised. All of these changes indicate that the Se-exposed redear were either anemic, or becoming so (Sorenson 1986). Moreover, the authors found that redear that had accumulated average liver concentrations of ≥ 7.6 µg/g (ww) had swollen and hypertrophic secondary gill lamellae. As well, liver tissue showed focal necrosis, cytoplasmic vacuolation and increased numbers of Kupffer cells, while kidney tissue showed tubular cell necrosis and blood stasis in some areas.

Lemly (1993b) reported similar hematological changes and gill damage in bluegill sunfish that had accumulated whole-body Se concentrations of 5-8 $\mu\text{g/g}$ (dw). Hematocrit, MCHC (mean corpuscular hemoglobin concentration), MCV (mean corpuscular volumes), and percentage of erythroblasts were all significantly lower in Se treated fish than in the controls, indicating anemia. Se-exposed bluegills also developed significant swelling of blood sinuses in the secondary lamellae of the gills, a condition known as telangiectasia. In many cases, 100% of the lamellae exhibited some degree of swelling. Gill damage may have resulted from the aforementioned hematological changes, which restrict blood flow, increase blood pressure and result in ruptured or swollen lamellar vessels. However, no abnormalities were observed in the liver, kidney, heart, or spleen of the Se-exposed fish, possibly owing to the lower concentrations of accumulated Se.

Coughlan and Velte (1989) observed histopathological changes in the liver and kidney of striped bass (*M. saxatilis*) that had accumulated muscle concentrations averaging 3.8 $\mu\text{g Se/g}$ (ww). Fat vacuoles, swollen or degenerate nuclei, glycogen devacuolization, and granular basophilic cytoplasm were observed in the parenchymal hepatocytes of a number of Se-exposed bass. Renal proximal tubules of Se-exposed fish exhibited sloughed epithelial debris and proteinaceous material, referred to as casts, as well as hydropic vacuolation, degenerated tubules, and lymphocyte infiltration.

There are considerably fewer studies regarding the toxic effects of Se on cold-water species than for their warm-water counterparts (Brix *et al.* 2000). A few studies have examined the effects of dietary Se on rainbow trout (*O. mykiss*). Hilton and Hodson (1983) and Hicks *et al.* (1984) observed elevated renal Ca^{2+} levels and increased

incidence of nephrocalcinosis in rainbow trout that had accumulated tissue concentrations (ww) $>25\mu\text{g/g}$ in the liver and $>9\mu\text{g/g}$ in the kidney. Approximately 25% of Se-exposed trout had chalky deposits in the ureter and posterior sections of the kidney. Renal lesions primarily involved the second proximal convoluted tubule and distal segments of the nephron. Affected tubules were dilated and contained necrotic epithelial cells and casts formed of necrotic debris and mineral precipitates. While the etiology of nephrocalcinosis is not completely understood (Hilton and Hodson 1983), it is thought that excess Se competes with Ca^{2+} for reabsorption in the proximal tubule, resulting in the build up and precipitation of Ca^{2+} within the tubular lumen (Hicks *et al.* 1984).

Hilton *et al.* (1980) found no histopathological lesions or abnormalities in liver, kidney, muscle or spleen of rainbow trout (*O. mykiss*) that had accumulated Se concentrations (dw) up to $100\mu\text{g/g}$ in the liver, and $35\mu\text{g/g}$ in the kidney. However, Sorenson (1991) attributed the lack of effect in this study to a dietary supplement of vitamin E, which, like Se in trace amounts, prevents anemia and serves as a cellular antioxidant (Neal 1977).

In another study, Hamilton *et al.* (1986) fed chinook salmon (*Oncorhynchus tshawytscha*) a diet containing Se contaminated mosquitofish. The experimental design also included a positive control group, which were fed a diet containing uncontaminated mosquitofish, and negative control group, which were fed a diet free of mosquitofish. Subtle pathological changes were reported in the tissues of the Se-treated fish that had accumulated whole-body concentrations $\geq 2.9\mu\text{g/g}$ (ww). The kidneys of Se-exposed fish showed degeneration of the endothelial cells of the glomerular capillary loop, while their livers showed what appeared to be a subtle form of megalocytosis, a condition

characterized by an increase in diameter of the hepatocytes and their nuclei (McCain *et al.* 1982). In addition, the authors reported that the gills of both the Se-treated salmon and the positive controls showed a ragged-looking lamellar epithelium, as well as hyperplasia, hypertrophy, and separation of the epithelium from the pillar cells. Because this condition was not observed in the negative controls, the authors concluded that the mosquitofish incorporated into the diets of the Se-exposed fish and positive controls did not constitute an acceptable feed for chinook salmon.

No field study has examined the histopathological effects of increased Se on fish that exclusively inhabit cold waters. Despite a number of studies that have examined the accumulation of Se in salmonid species downstream active coal mines, none have examined histopathology endpoints in the tissues of exposed fish (McDonald and Strosher 1998; Casey and Siwik 2000; Kennedy *et al.* 2000). TAEM consultants (1996) did report kidney lesions in a number of lake chub (carcass 12.4 µg/g, ww) exposed to uranium mine tailings, but the relationship to Se was not examined.

4) Ovary/egg Se and reproductive effects

Reproductive failure, whether through effects on the adult ovary or on embryonic development and survival, may be the first symptom of Se poisoning (Lemly and Smith 1987). Ovarian Se concentrations are the most relevant measure of reproductive success because Se is efficiently transferred from adults to their offspring through the egg yolk (Lemly 1996). The efficiency of this transfer is due to the fact that Se is carried as part of the yolk precursor proteins, lipovitellin and phosvitin (Kroll and Doroshov 1991). A trademark of Se poisoning is the appearance of teratogenic deformities, or permanent

developmental defects, in the offspring of exposed females. The most common types of teratogenic malformations include lordosis (the concave curvature of the lumbar and caudal regions of the spine), scoliosis (the lateral curvature of the spine), kyphosis (the convex curvature of the thoracic region of the spine, resulting in humpback), missing or abnormal fins, gills, opercula and eyes, as well as abnormally shaped heads and mouths (Lemly 1993c). Deformed individuals often die as a direct result of these malformations, or may be more readily preyed upon (Lemly 1997b). Se-induced edema, exophthalmus and cataracts are not considered true terata because they appear to be reversible and are not formed solely in the embryo-larval stage. Rather, these conditions are considered to be acute or chronic toxic responses to high Se concentrations (Lemly 1993c).

Nevertheless, edema may progress to or be associated with terata (Lemly 1998). For example, an accumulation of fluid surrounding the yolk sac can develop pressure that can lead to spinal curvatures.

Coyle *et al.* (1993) and Hermanutz *et al.* (1992) found a number of reproductive effects in bluegills exposed to Se in outdoor experimental streams that had accumulated ovarian concentrations of 42 $\mu\text{g/g}$ (dw) and 4.5 $\mu\text{g/g}$ (ww), respectively. In both studies, Se exposure did not significantly affect spawning activity or number of eggs per spawn. Both studies also reported decreased survival in the larvae of exposed parents (as low as <10%, versus 70-90% in controls). While Hermanutz *et al.* (1992) found that percent hatch was reduced in exposed fish (60-70 % in exposed fish, versus 90% in controls), Coyle *et al.* (1993) found no effect ($\geq 80\%$). Hermanutz *et al.* (1992) also reported a high incidence of edema, lordosis and internal hemorrhaging in the larvae of exposed fish (6-80%, versus <2% in controls). Edema occurred anterior to the yolk sac, giving the

appearance of a double yolk. Hemorrhaging usually occurred in the head or near the eyes. Larvae suffering from lordosis or hemorrhaging usually failed to survive more than 1 day, while larvae suffering from edema often survived past 5 days post-hatch, but failed to develop. In contrast, Coyle *et al.* (1993) did not report any incidence of deformity in the larvae of exposed fish. The difference in severity of the responses of bluegills in these two studies may have been the result of the form of Se used in the exposure. The study of Hermanutz *et al.* (1992) exposed bluegills in outdoor experimental streams dosed with selenite, while that of Coyle *et al.* (1993) exposed fish to waterborne selenite and selenate in combination with dietary seleno-L-methionine.

In another study, Gillespie and Baumann (1986) performed a series of artificial crosses between the eggs and milt stripped from bluegills collected at either a contaminated reservoir (ovary, 7.0 $\mu\text{g Se/g}$, ww) or a reference site (0.7 $\mu\text{g Se/g}$, ovary). They found that neither percent fertilization nor hatchability differed among any parent combination. However, all crosses involving high Se females resulted in larvae with edema. Edematous larvae were less motile than normal larvae and did not survive to the swim-up stage.

Many studies have focused on the effects of Se on fathead minnows (*P. promelas*). Schultz and Hermanutz (1990) reported a higher incidence of edema (24.6% versus 0.9% in controls) and lordosis (23.4% versus 5.6% in controls) in the larvae of fathead minnows that had accumulated ovary Se concentrations of 5.9 $\mu\text{g/g}$ (ww), compared to 1.4 $\mu\text{g/g}$ in controls. Pyron and Beitinger (1989) and Hermanutz (1992) reported similar morphological abnormalities in the progeny of Se-treated fathead minnows. They did not, however, determine body concentrations of Se. In contrast, Ogle and Knight (1989)

found no significant treatment effects on any of the reproductive parameters measured in their study, including number of spawns per pair, number of eggs per spawn, percent hatch and larval survival. Se-treated females in the highest exposure had average gonad concentrations of 10.9 $\mu\text{g/g}$ (dw), lower than the values from the study of Schultz and Hermanutz (1990). Schultz and Hermanutz (1990) attributed the lower tissue burdens of Se in the Ogle and Knight (1989) study to the forms of Se incorporated into their experimental diets, 75% of which was inorganic Se, a form they theorized was less readily taken up by the fish gut than organoselenium.

Crane *et al.* (1992) examined the effects of increased Se on the reproduction of perch (*Perca fluviatilis*) held in outdoor experimental ponds. They found no effect on fertilization (>90% in all but one case) or time to hatch (10-12 days). The authors also reported no effect on the survival of larvae (>59%) of fish that had accumulated average gonad concentrations of up to 4.5 $\mu\text{g/g}$ (ww). However, 100% of the eggs of perch with mean gonadal concentrations of 7.0 $\mu\text{g/g}$ or higher failed to hatch. Finally, the authors reported limited evidence for an increase in spinal deformities in the larvae of Se-exposed perch (up to 17.1%). Because one of the treatment groups had as low an incidence of deformity as the control groups (<2%), the pattern was not clear.

Over a 21 year period (1975-1996), Lemly (1993c, 1997b) has studied the teratogenic effects of Se in various species of warm-water fish from a lake contaminated with fly ash in North Carolina. The 1982 survey had the highest incidence of malformations in adult and juvenile fish, reaching 70% in green sunfish, 61% in common carp, and 30% in fathead minnows and mosquitofish. Before 1986, Se concentrations in the eggs of Belews Lake fish ranged from 40-159 $\mu\text{g/g}$ (dw). Se inputs into Belews Lake were

curtailed in 1985, and by 1992 Se levels were low enough that some species had begun to recolonize. The incidence of malformations in 1992 fell to less than 11% in all species. In both surveys malformations were found in both juvenile and adult fish, and were predominantly spinal and fin deformities. Lemly believed that the lack of competition and predation in Belews Lake led to the high frequency and persistence of deformities in juvenile and adult fish. In 1996, deformities were reported only in fry, with levels of 3% in carp, 4% in bluegill, 6% in mosquitofish and 3% in largemouth bass, which corresponded to Se concentrations of 3-20 $\mu\text{g/g}$ (dw) in the eggs. Because the earlier studies had shown that the malformed fish could survive to fingerling size, the lack of deformed fingerlings was attributed to increased predation pressure, which was now much higher than in 1992. In all surveys, the prevalence of deformities in reference fish remained below 4%.

In a field study examining western mosquitofish (*G. affinis*) collected from the San Luis Drain, Saiki and Ogle (1995) found that fish that had accumulated whole-body concentrations of $>100 \mu\text{g/g}$ (dw; ovarian concentrations were not reported) experienced lower reproductive success than fish from a reference site. Unlike most fish species, mosquitofish are livebearers, meaning they retain and incubate fertilized eggs within the body of the female. The larvae of Se-exposed fish had a lower survival rate (70-77%) than did the larvae of reference fish (97-99%). In addition, the stillborn larvae of high Se females were shorter (7.5 mm, versus 9.4 mm of controls) and were at an earlier stage of development than the live fry. Stillborn fry often had prominent yolk sacs or occasionally resembled eyed eggs, while most live fry had resorbed their yolk sac. The stillborn fry of the reference fish were not significantly shorter than the live fry, and

nearly all had resorbed their yolk sacs. No terata were reported in the fry of either treatment. In spite of the low survival of the larvae, populations of mosquitofish in the San Luis Drain appeared to be thriving. The authors speculated that low levels of competition and predation in their habitat may explain the success of these populations.

Se-induced reproductive failure can also result from histopathological abnormalities in the ovaries of adult fish. Sorenson (1988) and Sorenson *et al.* (1984) reported pathological changes in the ovaries of redear sunfish (*L. microlophus*) and green sunfish (*L. cyanellus*) that had accumulated mean liver concentrations of 7.6-21.4 µg/g (ww). The ovaries of sunfish from Se-contaminated areas frequently contained degenerating, necrotic or atretic egg follicles; oocytes with irregularly shaped nuclei and enlarged vacuoles; as well as connective tissue hypertrophy. Moreover, Se-exposed females showed asynchronous oocyte development and a reduction in the numbers of developing oocytes. Similarly, TAEM consultants (1996) reported that a number of lake chub (*C. plumbeus*) collected at a site downstream from a uranium mine in Northern Saskatchewan demonstrated edematous ovarian tissue, including degenerate, ruptured and atretic oocytes and follicles, associated inflammation, and resulting fluid accumulation. Ovarian concentrations in these fish were 10.8 µg Se/g (ww, pooled sample), compared to reference fish that had ovarian concentrations of 1.3 µg/g (pooled sample).

Researchers have only begun investigating the effects of Se on salmonid reproduction. Kennedy *et al.* (2000) examined wild cutthroat trout (*O. clarki lewisi*) exposed to high Se concentrations downstream from a coal mine for reproductive effects. Despite elevated concentrations of Se in the eggs of exposed fish (21.0 µg/g, dw), no significant effects were reported for fertilization (>99% in all cases), time to hatch (22-25.5 days versus

25.5-26.6 days in the controls), percent hatch, or larval deformities (<1% in all cases) or mortalities (3.7-100% versus 2.8-55.8% in controls). The authors speculated that the cutthroat trout in this system may have developed a tolerance to high Se levels.

However, these studies have been criticized on the basis of their methodologies and specifically, for the high mortality in reference fish that may have obscured potential differences from the exposed population (Hamilton and Palace 2001)

5) Research Needs

Since Se was recognized as an environmental contaminant for fish and wildlife in the early 1980's, a significant amount of research has focused on the toxicity of Se in aquatic habitats. These efforts have centered primarily on warm-water species from standing water habitats. It is possible that toxic effect thresholds derived using warm-water, lentic species are not applicable to cold-water species inhabiting lotic environments (Lemly 1993a). Only recently, investigations into the potential effects of Se on cold-water species from fast flowing systems have begun. A current area of concern is cold-water salmonids exposed to Se-rich waters downstream from active coal mines in British Columbia and Alberta. The results of a preliminary study are anomalous; cutthroat trout downstream a British Columbia coal mine were found to accumulate high levels of Se, but showed no adverse reproductive effects (Kennedy *et al.* 2000). However, methodological oversights and incomplete reporting of endpoints from this study do not allow conclusions that all salmonids exposed to Se-rich habits near field to coal mining operations suffer no detrimental effects (Hamilton and Palace 2001). Instead, further investigation is required to obtain a thorough understanding of the relationship between

Se exposure resulting from coal mining activities and potential reproductive effects in cold-water salmonid fishes. Future studies should include controlled laboratory feeding experiments to assess toxic effect thresholds for salmonid species, as well as field studies to assess the occurrence and frequency of embryonic mortalities and deformities of salmonids exposed to Se-rich water downstream active coal mines (Selenium Expert Advisory Panel 2000). Another area of concern is the numerous end pit lakes left by deep pit mining operations, which often have high levels of Se (Skorupa 1998). To date, there has been no assessment of the potential adverse effects on fish inhabiting these systems.

Chapter 2

**An assessment of three methods for detecting developmental abnormalities in fry
from brook trout and rainbow trout exposed to elevated Se levels**

2.1) Introduction

Selenium (Se) toxicity resulting from the transfer of Se from females to their eggs during the deposition of yolk lipoproteins. Excess Se can result in the appearance of teratogenic deformities as permanent pathological markers. Teratogenesis at the egg-larval stage of development occurs when the larvae metabolize their yolks contaminated with Se (Lemly 1997c). There are several advantages to identifying teratogenic endpoints as indicators of Se toxicity in fish communities. Teratogenesis is a direct expression of Se toxicosis and represents the sum total of parental exposure, regardless of temporal, spatial or chemical variations in Se exposures. Thus, terata represent a measure of existing rather than potential hazard (Lemly 1997c). Moreover, teratogenic deformities can be subtle, but important, causes of recruitment failure in fish communities. That is, a significant loss of the early life stages of a fish population can occur at the same time that adult fish appear healthy (Lemly 1997c).

The prevalence of teratogenic deformities is regularly used to evaluate the toxicity of various environmental contaminants. A number of field and laboratory studies have already used Se induced teratogenesis to assess the impacts of Se on the reproductive fitness of fish (e.g. Bryson *et al.* 1985; Gillespie and Baumann 1986; Woock *et al.* 1987; Pyron and Beitingner 1989; Schultz and Hermanutz 1990; Hermanutz *et al.* 1992; Hermanutz 1992; Lemly 1993c, 1997b; Kennedy *et al.* 2000). However, in each of these studies no attempt was made to quantify the degree of severity of observed malformations. Consequently, these types of evaluations are dependent on the experience and subjectivity of the investigator in diagnosing the deformities. Characterizing teratogenic deformities by quantitatively measuring morphological

variables in larval fish may eliminate a degree of subjectivity on the part of the investigator, thereby increasing the defensibility of the data. Such a process would also facilitate comparisons between studies by different investigators. The studies described in this chapter focus on the use of morphometrics to quantitatively characterize teratogenic malformations in the offspring of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). These fish were collected from sites with high concentrations of selenium downstream from coal mining activity in the northeastern slopes region of Alberta, Canada as well as from reference areas. The method is compared to two other approaches that have previously been used to assess the effects of Se on fish reproduction. These are 1) the frequency of gross morphological malformations and 2) the use of graduated severity index scores.

2.2) Materials and Methods

2.2.1) Sites and Fish Collection

Spawning rainbow trout were collected in June 2000 from Luscar Creek (high Se site) and Deerlick Creek (reference site) and brook trout were collected from Luscar Creek and Cold Creek (reference site) in October 2000, in the Northeastern slopes region of Alberta, Canada (Figure 2.1). Typical water chemistry parameters for these three sites are given in Table 3.1. Spawning times were monitored by visual identification of redd formation as well as by recording daily water temperatures. For rainbow trout, collection of spawning adults began when daily maximum temperatures reached 6°C, while for brook trout collections began when minimum water temperatures reached 3°C.

Ripe and running males and females were collected using a Smith-Root type VII backpack electroshocker. Fish were netted, separated by sex and held in cages at the site they were captured at for <24 hours before sampling. At sampling, fish were individually anesthetized with 0.4 g/L MS-222 (tricaine methane sulfonate) (SIGMA Chemical Company, St. Louis, MO) until fin movement ceased (~ 3 min.). Each fish was then carefully blotted dry, weighed and measured, and then the area surrounding the urogenital opening was dried thoroughly with paper towels. Eggs or milt were expressed by light pressure on the abdomen. Gametes from each fish were transported on ice in sealed plastic bags filled with oxygen and protected from light.

Tissues were removed from adult fish after spawning and frozen for Se analysis. The liver was separated from the gall bladder, a muscle sample was taken posterior to the opercular bone anterior to the dorsal fin and above the lateral line. Samples of eggs were taken from those remaining in the abdominal cavity after spawning. All tissues were stored at - 20°C until they were analyzed for Se.

2.2.2) Egg and Larval Rearing

After transportation from the Alberta sites to the Freshwater Institute laboratories in Winnipeg (< 24 hours), eggs from each female were fertilized with a consistent volume (10 µL/50 mL) of composite milt derived from 3-5 males captured at the same site. Eggs were combined with the milt in a stainless steel bowl by gentle mixing with the tip of a sterilized goose feather. After standing for 60 seconds, the eggs and milt were just covered with dechlorinated Winnipeg City tap water (approximately 100mL, see water chemistry in Table 3.1) and gently swirled for 3 minutes. An additional 500mL of water

was then added and the eggs were allowed to water harden for a further 5 minutes. The eggs were then placed in vertical Heath tray type incubators. The eggs from each female were assigned randomly to one of 4 trays, each with 12 compartments. Weekly monitoring of water in trays showed no significant differences in temperatures, dissolved oxygen saturation, or Se concentrations. Eggs were incubated at 8 ± 0.8 °C and received 6 L water/min. Dead eggs and fry were removed daily and preserved in Davidson's solution. At swim-up when the fry had resorbed almost all the egg yolk and begun exogenous feeding, fry were sacrificed with an overdose of MS-222 (0.8 g/L) and preserved in Davidson's Solution.

2.2.3) *Tissue Se*

Concentrations of Se were determined in eggs and tissues of adult rainbow trout and brook trout using inductively coupled plasma mass spectroscopy (ICP-MS) as described by Feng *et al.* (1999). Se concentrations in tissues from this study are reported on a wet weight (ww) basis.

2.2.4) *Assessment of Morphological Deformities*

i) Frequency Analysis

All preserved offspring that survived to the swim-up stage were examined using a dissection microscope for gross external malformations. Abnormalities were recorded in four categories: spinal, craniofacial, finfold and edema. Skeletal deformities included kyphosis (convex curvature of the thoracic region of the spine), lordosis (concave curvature of the lumbar region of the spine) and scoliosis (lateral curvature of the spine),

as well as stunting of the trunk or tail. Craniofacial defects included reduction or absence of the jaws and ocular deformities such as noticeably reduced eye diameter (microphthalmia) or pigment irregularities as well as asymmetry. Finfold defects included a noticeable reduction in thickness or absence of a fin. Edema included accumulation of body fluid in the region of the yolk sac, pericardium or head. For the frequency assessment, only the number of deformed fry was determined for each category. In order to account for differences in spawn sizes between individual females, counts for each category were converted into percentages based on the total number of preserved fry obtained from each female. Frequency data were analyzed using a Mann-Whitney U test (SPSS Inc. 1999). Regression analysis was also used to examine relationships between concentrations of Se in eggs and the percentage of deformities (SPSS Inc. 1999). An alpha value of 0.05 was used for all statistical analyses.

ii) Graduated Severity Index

The severity of the four deformity categories were scored using a graduated severity index (SI) as described by Middaugh *et al.* (1988) and Hose *et al.* (1996); where 0 = normal; 1 = slight defect of size or structure; 2 = moderate defect of size or structure; 3 = severe defect of size or structure. A total SI score was calculated for each fry by summing the individual scores in each of the four categories (skeletal, craniofacial, finfold, and edema). Comparisons between reference and high Se sites for the SI data were made using approximate randomization statistical analysis, based on 1000 shuffles (Noreen 1989).

iii) Morphometric Analysis

Morphometric analysis consisted of eight length measurements made on a subsample of 100 fry per female under low power magnification using a SummaSketch III digitizing pad connected to a personal computer with Zoobenthos Biomass Digitizing Program software, Version 1.31 (described in Roff and Hopcroft 1986). Measurements included total body length, head height, head width, snout length, right and left eye diameter, and right and left opercular length as illustrated in Figure 2. All length measurements were corrected for size variance by dividing by total body length. Spinal angles were measured in Corel Draw (Corel Corporation 1995) after larvae were digitized along the lateral line (for dorso-ventral curvatures) and the fin fold (for lateral curvatures). The angles were recorded in 5 regions: cervical; pre-mid dorsal fin; mid-post dorsal fin; anal fin; and caudal, as shown in Figure 3. The total lateral and dorso-ventral spinal curvatures were calculated by adding the deviation from 180° at each of these regions. Measurement errors were calculated using three nonconsecutive repeated measurements of 25 randomly selected larvae (Bailey and Byrnes 1990). When both left and right side measurements were made, only left side measurements were used in measurement error analysis. Morphometric data was analyzed using a mixed model ANOVA (SAS Institute 1999). Before ANOVA, total dorso-ventral and lateral spinal curvature data were log transformed in order to meet the ANOVA assumption of a normal distribution. Quality control measurements were analyzed following the methods of Bailey and Byrnes (1990) by model II nested ANOVA (SAS Institute 1999).

2.3) Results

i) Frequency Analysis

Fish from the Se-exposed site had a significantly higher percentage of fry with abnormalities. Specifically, rainbow trout from Luscar Creek had 38.9% of fry with deformities compared to 0.7% in Deerlick Creek. Brook trout from Luscar Creek had 14.4% of fry with deformities compared to 4.0% of fry from Cold Creek. However, rainbow trout and brook trout did not exhibit comparable types of deformities.

Rainbow trout fry from Luscar Creek had a significantly higher incidence of fin (3.2%) and craniofacial (7.7%) deformities than fry from Deerlick Creek (<0.2%). The prevalence of spinal curvatures was also significantly higher in rainbow trout fry from the exposed site (13.8%) compared to the reference site (0.7%). The most frequent type of spinal abnormality was concave curvature of the spine relative to a normal fry, as shown in Figure 2.4A and B. The most marked difference between rainbow trout fry from Luscar and Deerlick creeks was the incidence of edema. While less than 0.2% of fry from Deerlick Creek had visible signs of edema, 30.8% of fry from Luscar Creek exhibited fluid accumulation. In most cases, edema appeared as fluid surrounding the yolk sac (Figure 2.4C). Yolk sac edema was often associated with spinal curvatures. Edematous fry were most often shorter and had utilized less yolk than had fry that had not developed edema at the time of sampling. In addition, fluid accumulated in the head, often resulting in a spreading of the cranial features (Figure 2.4D) and surrounding the heart.

In contrast to the rainbow trout fry, the incidence of edema (<0.7%), spinal (<2%), or finfold (<2%) deformities in brook trout were not significantly different among sites.

Brook trout from Luscar Creek had significantly higher incidence of craniofacial abnormalities (13.6%) versus those from Cold Creek (3.0%). Approximately 70% of the craniofacial deformities recorded were shortened or misshapen jaws (Figure 2.5E), with another 20% attributable to eye defects (Figure 2.5B and C).

There was a significant relationship between concentrations of Se in rainbow trout eggs from Luscar and Deerlick Creeks and percentages of: deformed fry ($r^2 = 0.988$, $p = 0.002$), craniofacial deformities ($r^2 = 0.898$, $p = 0.01$), spinal deformities ($r^2 = 0.884$, $p = 0.013$), finfold deformities ($r^2 = 0.980$, $p = 0.005$), and edema ($r^2 = 0.999$, $p = 0.000$) (Figure 2.6). The point of rapid rise in deformity in each of the Se regressions occurs near 6 $\mu\text{g/g}$. No such relationships were identified between concentrations of Se in the eggs of brook trout and any of the parameters examined (Figure 2.7).

ii) Grduated severity index (SI) scores

Total SI scores were significantly higher in rainbow trout fry at the Se-exposed site than in those from the reference sites (Figure 2.8A). Individual index scores paralleled the total SI scores. Edema contributed the most to the total index score, accounting for nearly 50% of its value. For brook trout, the difference in mean total SI scores between Luscar and the reference site was not as great as that observed for rainbow trout (Figure 2.8B). Of the individual indices, only craniofacial scores were significantly higher in brook trout fry from Luscar compared to those from the reference site, contributing approximately 60% to the total SI score. Conversely, finfold SI scores were significantly higher in Cold Creek brook trout compared to those from the Se-exposed site. Neither edema nor spinal index scores differed significantly between sites.

iii) Morphometric Analysis

Morphometric analysis revealed substantial variation in measured parameters among the offspring of different females within a single site. Even with this variability, rainbow trout fry were found to have significantly greater lateral and dorso-ventral spinal curvatures at the Se-exposed compared to the reference sites (Table 2.1). Similar results were obtained for brook trout (Table 2.1). Brook trout from Luscar Creek also had significantly shorter left and right opercular lengths compared to those from Cold Creek. Among all eight parameters measured, percent measurement error (%ME) of the morphometric variables ranged over two orders of magnitude (Table 2.2), from 0.2% for brook trout body length to 95.0% for brook trout snout length.

2.4) Discussion

The most sensitive indicators of Se toxicity are reproductive based endpoints (Lemly 1996). As such, the assessment of the incidence of deformities in larvae is an important component of evaluating the impact of Se on fish populations. The primary goal of this study was to compare three methods of evaluating deformities in larval fish: frequency assessment, SI method, and morphometrics and to provide information on the effects of Se on deformity rates using these methods.

Rainbow trout and brook trout were found to exhibit different types of deformities, but the results of the frequency and SI analyses were generally consistent for each of the two species. Frequency assessment showed that Luscar rainbow trout fry had significantly more skeletal deformities and edema compared to the reference site, whereas SI analysis indicated more severe skeletal deformities and edema. At first it may appear that the the

finfold and craniofacial results from the two methods appear contradictory. However, the differences indicate that although there were not significantly more craniofacial or finfold deformities in Luscar Creek rainbow trout, the overall severity of their deformities was greater than those from the reference site.

Brook trout had significantly more craniofacial deformities in Luscar Creek compared to the reference site. SI scores also indicated that the severity of craniofacial deformities was higher in these fish. Moreover, SI analysis indicated that finfold deformities were more severe at the reference site, despite there being no statistical difference between sites in the number of individuals with finfold malformations.

Morphometric analysis generally did not return results that were similar to the other two methods. Significant differences between sites were seen only for dorso-ventral and lateral spinal curvatures in both species. Therefore, for rainbow trout, morphometric analysis only confirmed that spinal curvatures were the most consistent teratogenic deformity observed at the seleniferous site. Results from the morphometric analysis were similar for brook trout, even though spinal deformities were not identified by the two other methods. None of the measured craniofacial parameters (HW, HH, SL, LED, RED) were found to differ between the Se-exposed site and the reference site using morphometric analysis even though craniofacial defects were identified in brook trout by the prior two analyses. Finally, morphometrics did reveal shorter opercula in brook trout fry from Luscar Creek compared to those from Cold Creek. It should be noted that opercular anomalies are routinely observed (~ 30%) in adult brook trout collected from Luscar Creek (V. Palace, unpubl. observ.).

Several advantages and disadvantages are associated with each of the methods used to evaluate deformities. Frequency assessment is a cost-effective method of evaluation, requiring few specialized instruments and lending itself well to rapid analysis. Up to 5,000 fry can be assessed in approximately 40 hours. Statistical analysis of data using a nonparametric t-test equivalent is appropriate as the deformity data are expressed as percentages. Finally, regression analysis to evaluate the relationships between Se content of the eggs and the incidence of the endpoints examined are useful to derive site and species specific toxic effect thresholds and can readily be performed with the type of data returned from the analysis.

The SI method has comparable equipment and analysis time requirements to frequency assessments. However, the data generated is non-continuous, requiring analysis using computer-intensive statistics, for which software application packages are not readily available. Furthermore, because SI scores are scaled values, they are not particularly suitable for regression analysis, which is the most appropriate tool to report toxicological effects in populations.

Morphometric analysis requires specialized equipment to magnify as well as digitize the images of individual fry. Analysis requires considerably more time, with an analysis of 1,500 fry taking approximately 700 hours. Also, statistical analysis of the morphometric data required complex, nested designs to account for variation between the offspring of different females in addition to variation among sites. Morphometric evaluation also lacked sensitivity to detect some deformities while being oversensitive to others. The lower power of this method to detect craniofacial deformities in brook trout likely resulted from the craniofacial categories used in the other two tests being, in effect

subdivided into several different parameters (HH, HW, SL, RED, LED) for this analysis. Some deformities identified by frequency analysis including edema, fin alterations, differences in jaw length, and ocular pigment, could not be detected with morphometric analysis. The possibility of adding more morphometric parameters to a particular study in order to ensure more thorough inspections is time prohibitive. Morphometric analysis may be more suited to detecting minute differences in the sizes of structures than SI or frequency evaluations, as evidenced by the detection of differences in opercular lengths.

Table 2.1) Morphological measurements (mm) of rainbow trout and brook trout fry (mean $\pm$ SE) taken from a Se-rich site (Luscar Creek) and reference sites (Deerlick Creek and Cold Creek) in northeastern Alberta.

	Rainbow trout		Brook trout	
	Deerlick Cr.	Luscar Cr.	Cold Cr.	Luscar Cr.
n	261	297	1560	4904
BL ^a	$19.8 \pm 8.0 \times 10^{-2}$	$20.2 \pm 6.0 \times 10^{-2}$	$17.2 \pm 1.0 \times 10^{-4}$	$16.6 \pm 6.0 \times 10^{-4}$
HH	$2.8 \pm 1.0 \times 10^{-2}$	$2.8 \pm 1.0 \times 10^{-3}$	$2.1 \pm 1.0 \times 10^{-4}$	$2.0 \pm 6.0 \times 10^{-5}$
HW	$3.1 \pm 1.0 \times 10^{-2}$	$3.1 \pm 1.0 \times 10^{-3}$	$2.5 \pm 2.0 \times 10^{-4}$	$2.4 \pm 7.0 \times 10^{-5}$
SL	$0.6 \pm 3.0 \times 10^{-3}$	$0.6 \pm 6.0 \times 10^{-4}$	$0.38 \pm 8.0 \times 10^{-5}$	$0.4 \pm 4.0 \times 10^{-4}$
RED	$1.7 \pm 7.0 \times 10^{-3}$	$1.8 \pm 6.0 \times 10^{-4}$	$1.5 \pm 1.0 \times 10^{-4}$	$1.4 \pm 4.0 \times 10^{-5}$
LED	$1.7 \pm 1.0 \times 10^{-2}$	$1.7 \pm 6.0 \times 10^{-4}$	$1.5 \pm 1.0 \times 10^{-4}$	$1.4 \pm 4.0 \times 10^{-5}$
ROL	$1.9 \pm 8.0 \times 10^{-3}$	$1.9 \pm 1.0 \times 10^{-3}$	$1.4 \pm 1.0 \times 10^{-4}$	$1.3^b \pm 6.0 \times 10^{-5}$
LOL	$1.9 \pm 6.0 \times 10^{-3}$	$1.9 \pm 1.0 \times 10^{-3}$	$1.4 \pm 1.0 \times 10^{-4}$	$1.3^b \pm 6.0 \times 10^{-5}$
LSD	$6.7 \pm 6.0 \times 10^{-2}$	$13.1^b \pm 1.0 \times 10^{-1}$	$9.6 \pm 6.0 \times 10^{-3}$	$10.6^b \pm 5.0 \times 10^{-3}$
DSD	$4.4 \pm 2.0 \times 10^{-2}$	$6.1^b \pm 4.0 \times 10^{-2}$	$8.7 \pm 4.0 \times 10^{-3}$	$10.5^b \pm 6.0 \times 10^{-3}$

^a BL = total body length, HH = head height, HW = head width, SL = snout length, RED = right eye diameter, LED = left eye diameter, ROL = right opercular length, LOL = left opercular length, LSD = lateral spinal deviation, DSD = dorso-ventral spinal deviation.

^b Significantly different from the reference site at $\alpha = 0.05$.

Table 2.2) Percent measurement error associated with the different morphological variables examined in rainbow trout and brook trout fry from a Se-rich site (Luscar Creek) and reference sites (Deerlick Creek and Cold Creek) in northeastern Alberta.

	Rainbow trout		Brook trout	
	Deerlick Cr.	Luscar Cr.	Cold Cr.	Luscar Cr.
BL ^a	6.1	0.4	0.2	0.2
HH	20.1	3.9	11.7	14.4
HW	5.4	1.5	1.1	2.3
SL	22.4	1.0	95.0	9.9
LED	1.5	0.7	4.6	8.1
LOL	8.7	3.5	2.2	3.6
LSD	34.4	7.1	15.9	22.2
DSD	14.3	10.1	23.1	52.0

^a BL = total body length, HH = head height, HW = head width, SL = snout length, LED = left eye diameter, LOL = left opercular length, LSD = lateral spinal deviation, DSD = dorso-ventral spinal deviation.

Figure 2.1) Collection sites for rainbow and brook trout in McLeod River drainage of Alberta, Canada in 2000. Luscar Creek is a selenium exposure site downstream from coal mining activity and Deerlick and Cold Creeks are reference sites for rainbow and brook trout, respectively. Rainbow trout were collected in June, whereas brook trout were collected in October.

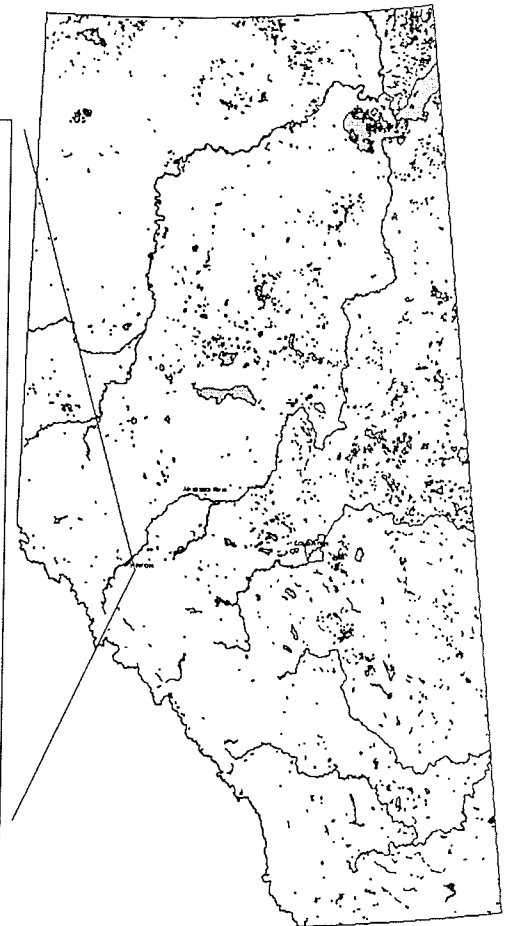
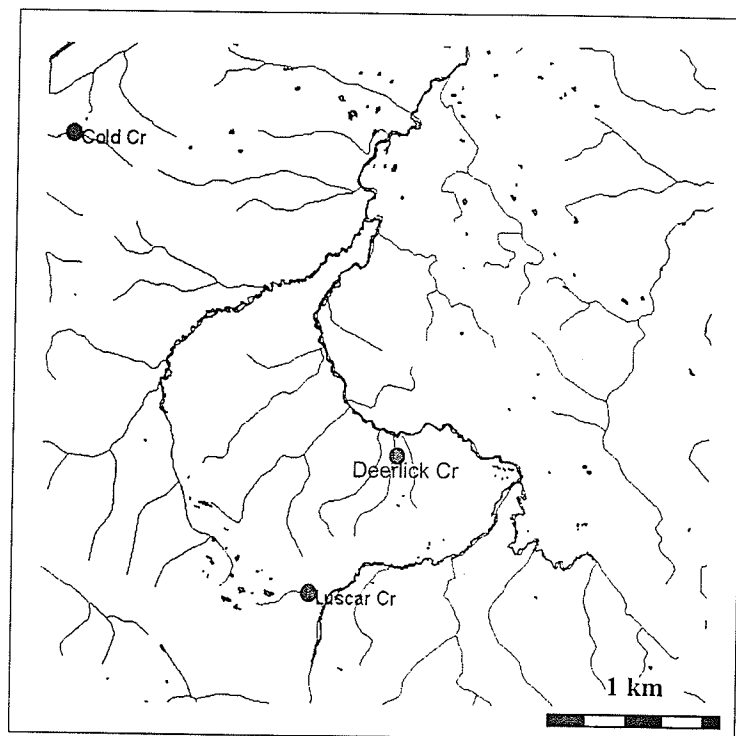


Figure 2.2) Diagram of larval trout showing the different morphometric parameters measured (A) in side view and (B) in top view. BL = body length, HH = head height, HW = head width, SL = snout length, LED = left eye diameter, LOL = left opercular length.

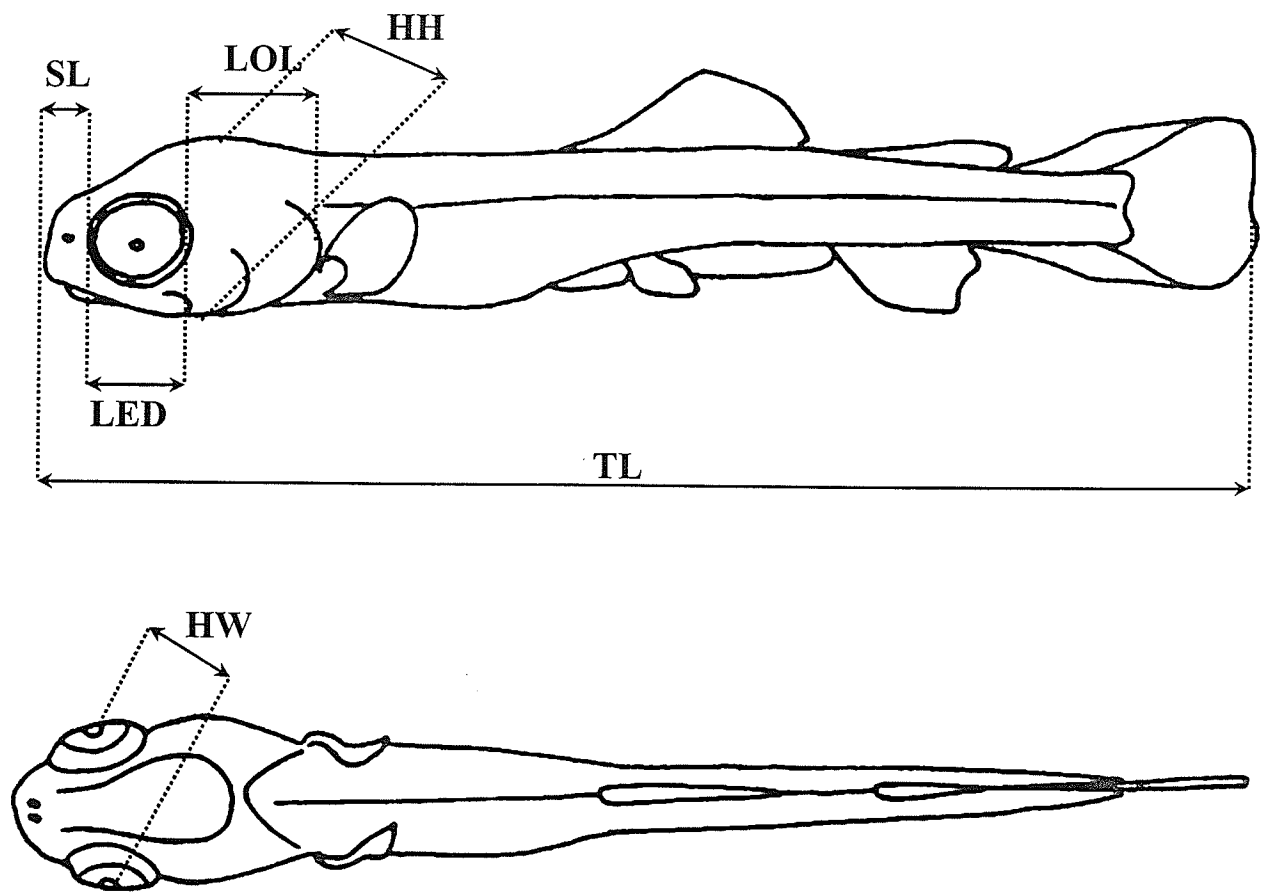


Figure 2.3) Diagram of larval trout showing the different regions for spinal angle measurements for morphometric analysis.

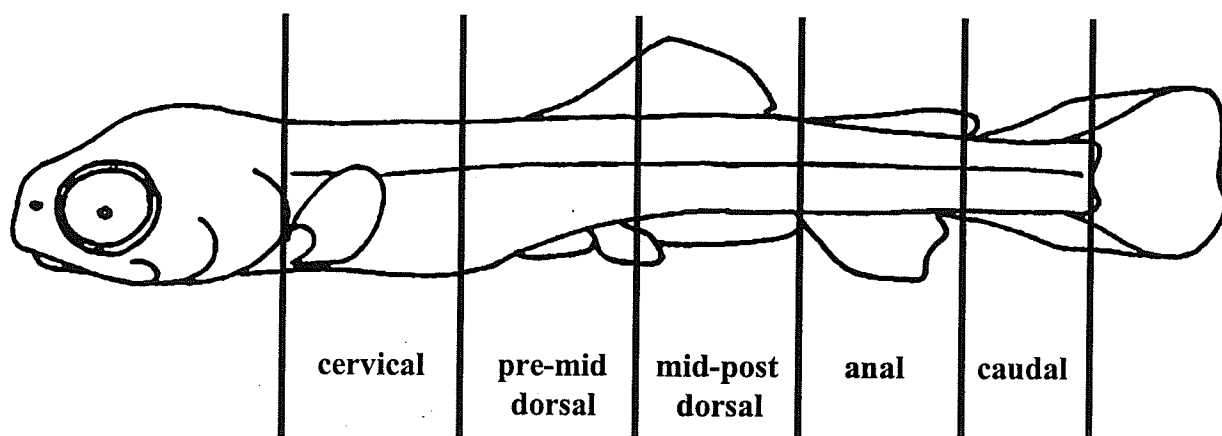
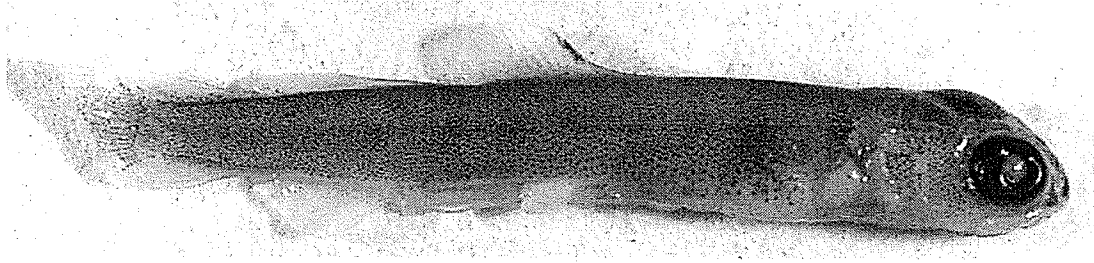
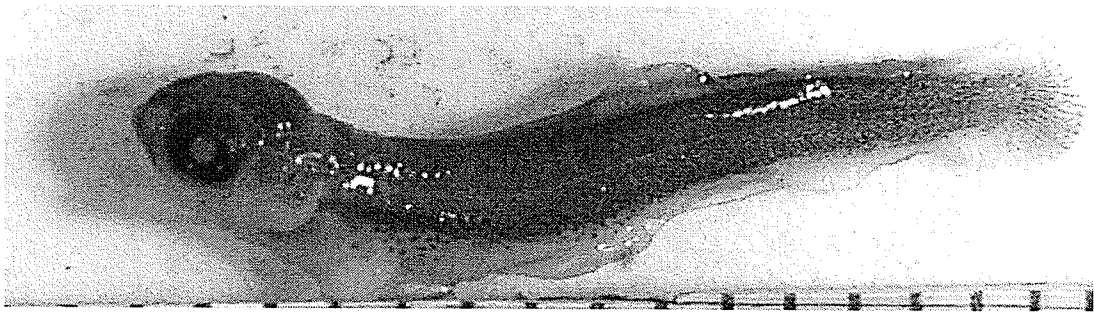


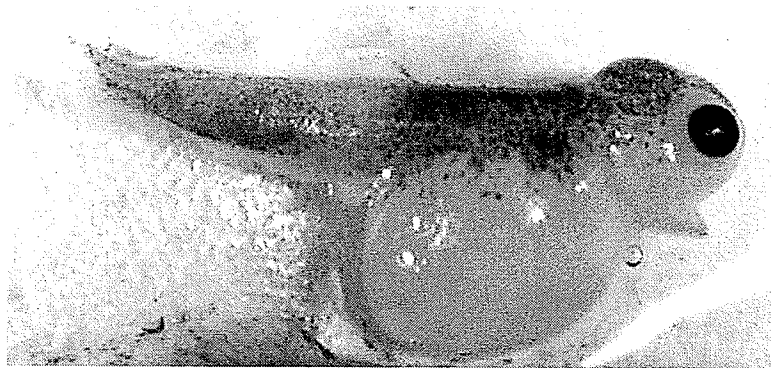
Figure 2.4) Rainbow trout fry from the Se-exposed site, Luscar Creek, showing the most commonly observed deformities and scored using a graduated severity index in each of four categories: craniofacial (CF), skeletal (SK), finfold (FF), and edema (ED). (A) normal larva, scored as CR 0, SK 0, FF 0, ED 0, (B) larva with spinal curvature, scored as CR 0, SK 1, FF 0, ED 0, (C) edematous larva, scored as CR 3, SK 2, FF 0, ED 2, (D) fry on right shows a spreading of the cranial features, scored as CF 0, SK 0, FF 0, ED 1 compared to normal fry on the left, scored as CR 0, SK 0, FF 0, ED 0.



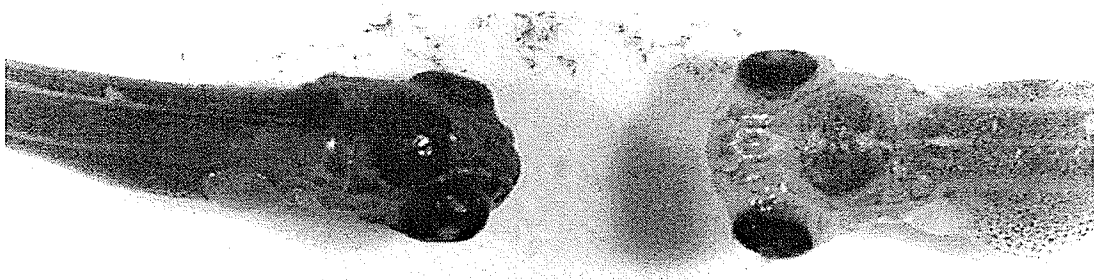
A



B

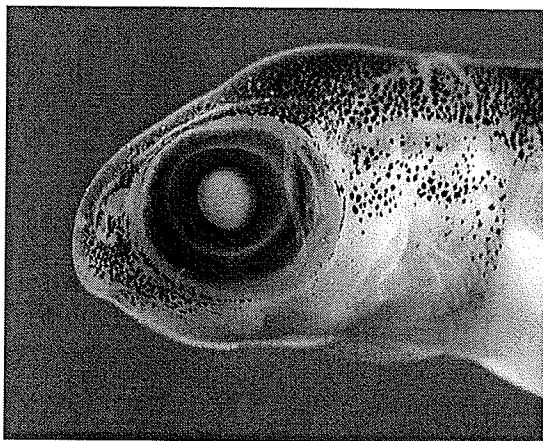


C



D

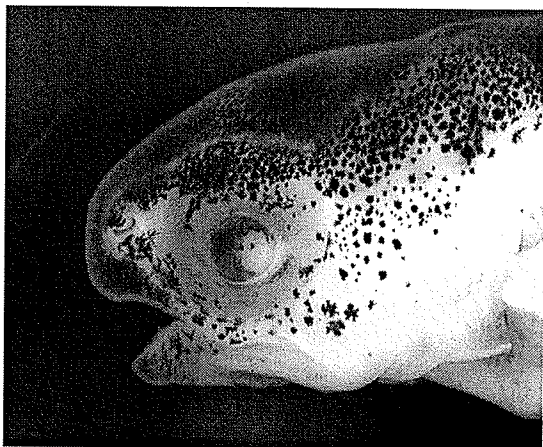
Figure 2.5) Brook trout fry from the Se-exposed site, Luscar Creek, showing the most commonly observed deformities and scored using a graduated severity index for craniofacial (CF) defects. (A and D) normal fry, scored as CR 0, (B) fry with microphthalmia, scored as CR 3, (C) ocular malformation, scored as CR 1, (E) shortened, malformed lower jaw, scored as CR 1.



A



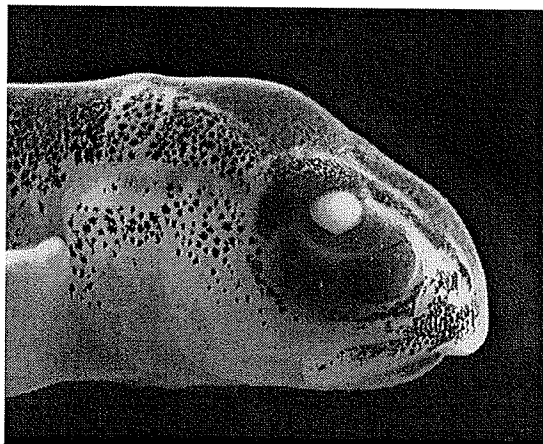
D



B

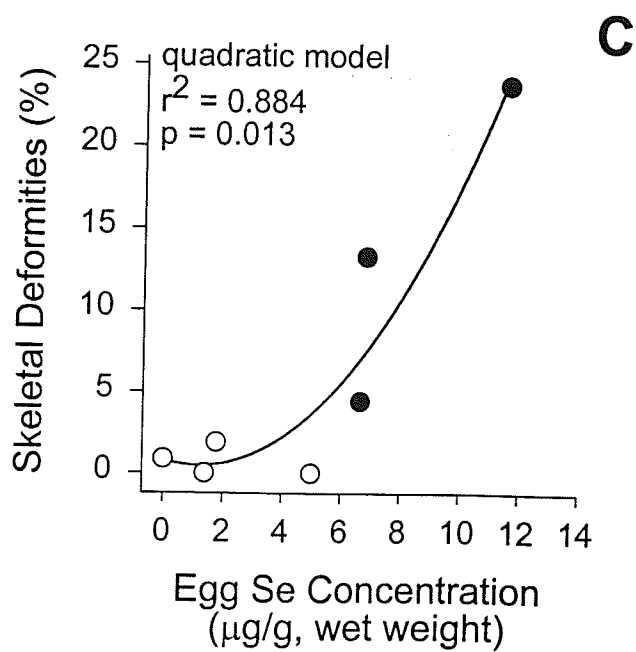
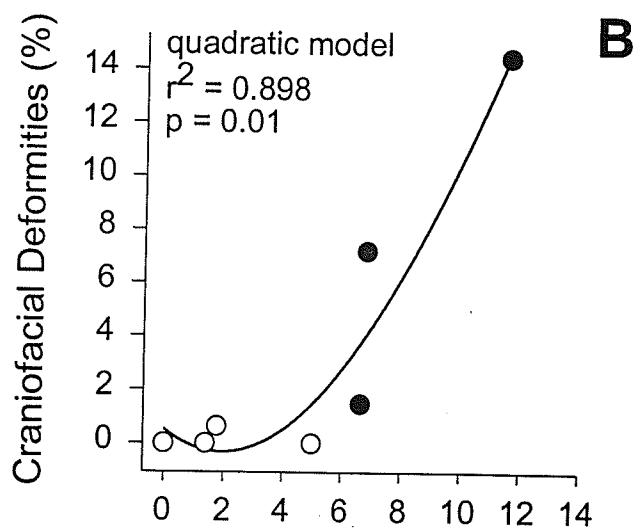
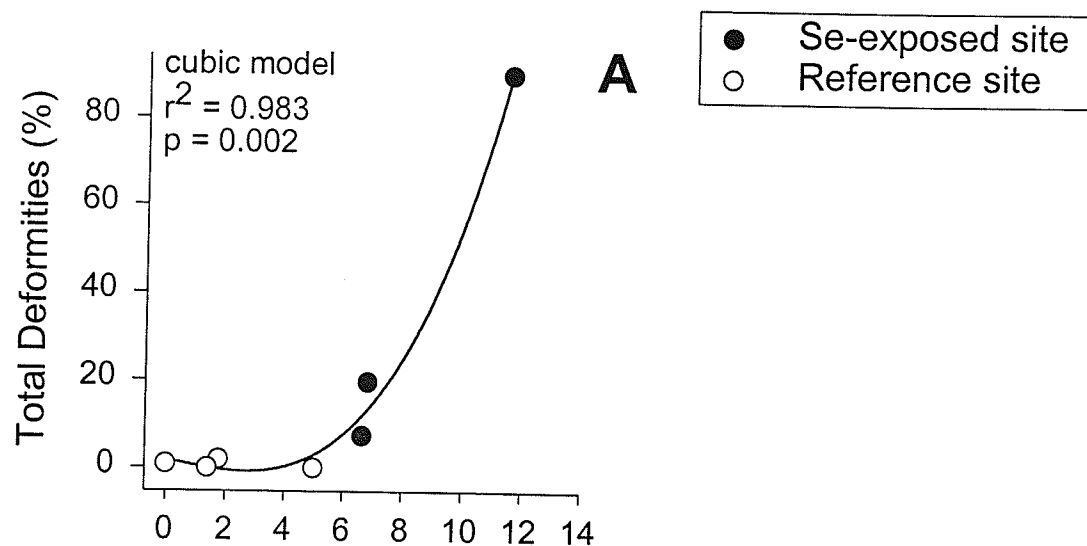


E



C

Figure 2.6) Relationship between Se concentrations in subsamples of eggs obtained from adult female rainbow trout collected at the selenium exposed site, Luscar Creek, and the reference site, Deerlick Creek and deformities in the offspring from each female in each of five categories: (A) total deformities, (B) craniofacial deformities, (C) skeletal deformities, (D) finfold deformities and (E) edema. Each symbol represents data from one female's offspring.



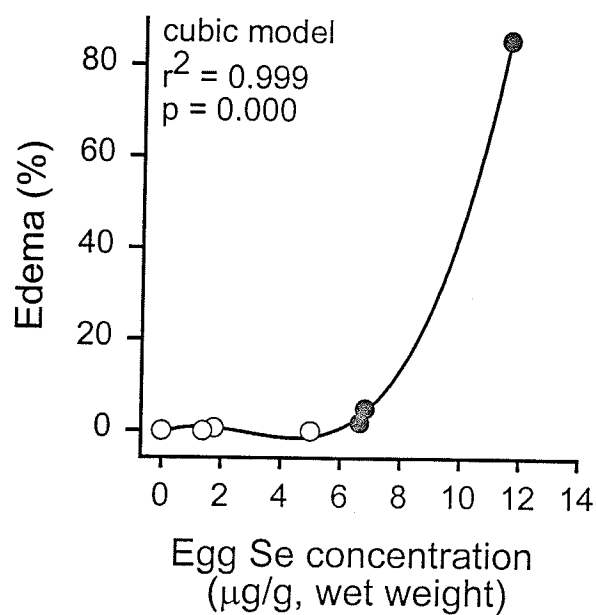
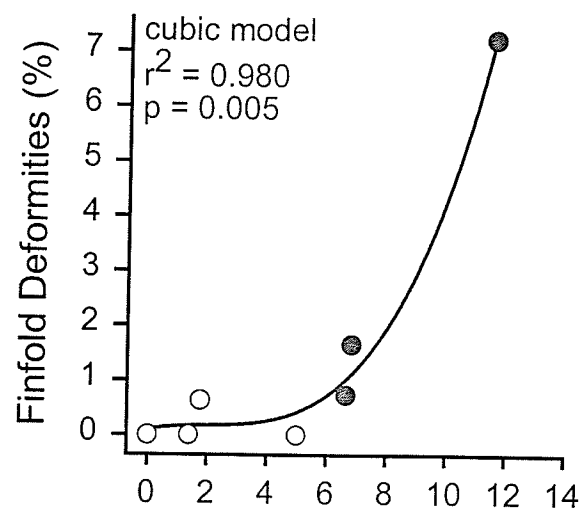
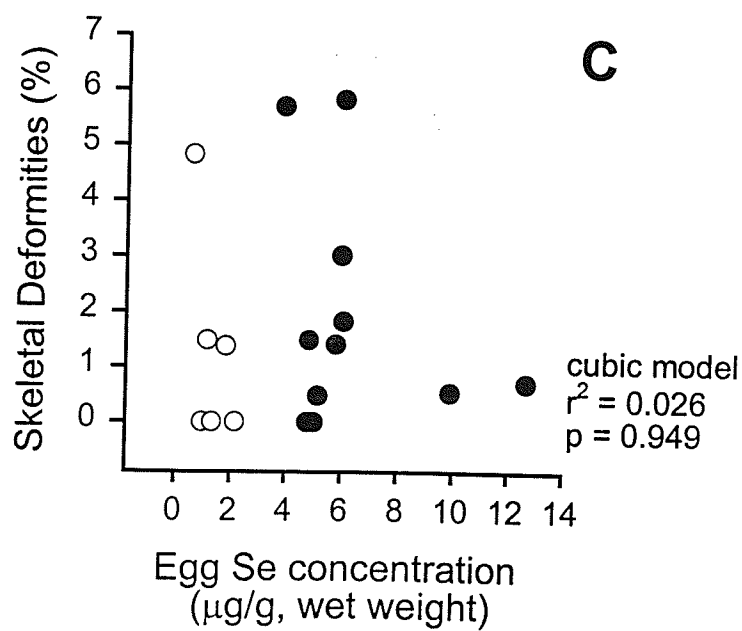
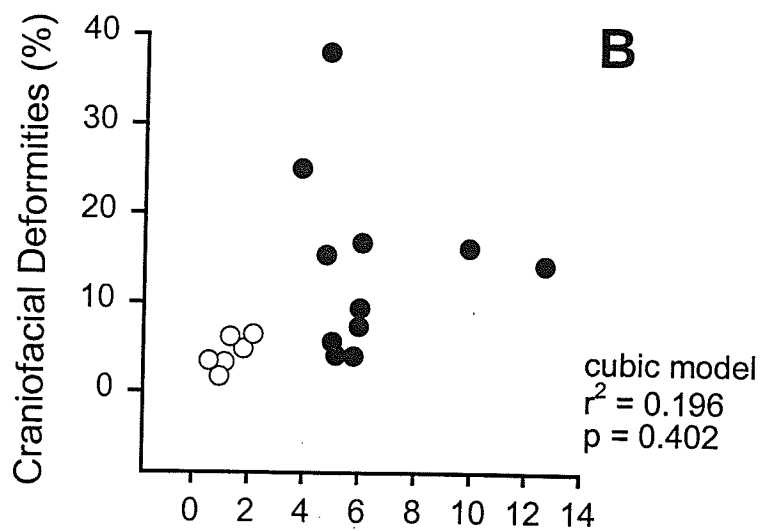
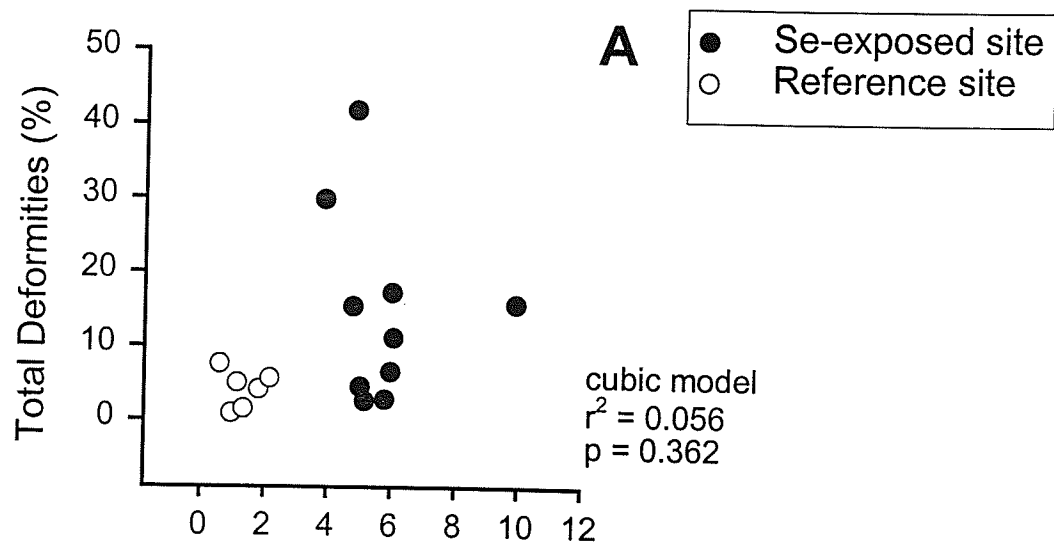


Figure 2.7) Relationship between Se concentrations in subsamples of eggs obtained from adult female brook trout collected at the selenium exposed site, Luscar Creek, and the reference site, Cold Creek and deformities in the offspring from each female in each of five categories: (A) total deformities, (B) craniofacial deformities, (C) skeletal deformities, (D) finfold deformities and (E) edema. Each symbol represents data from one female's offspring.



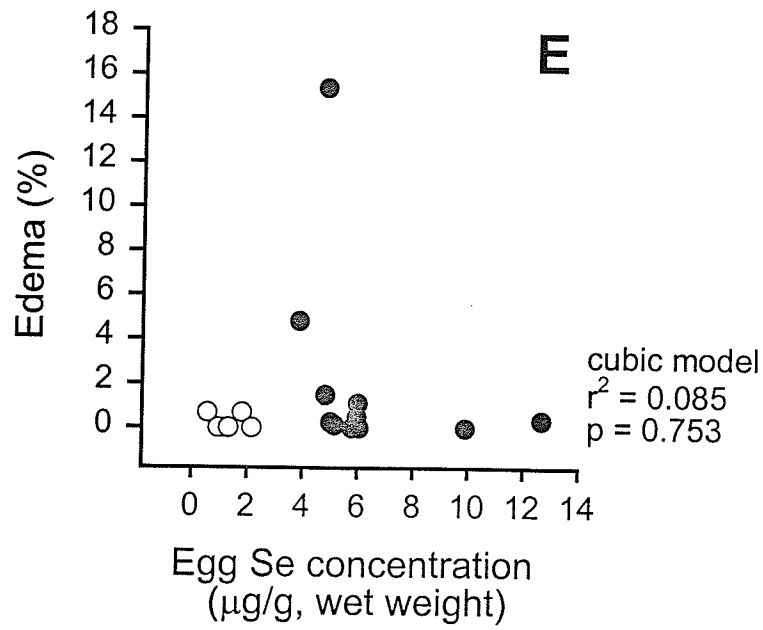
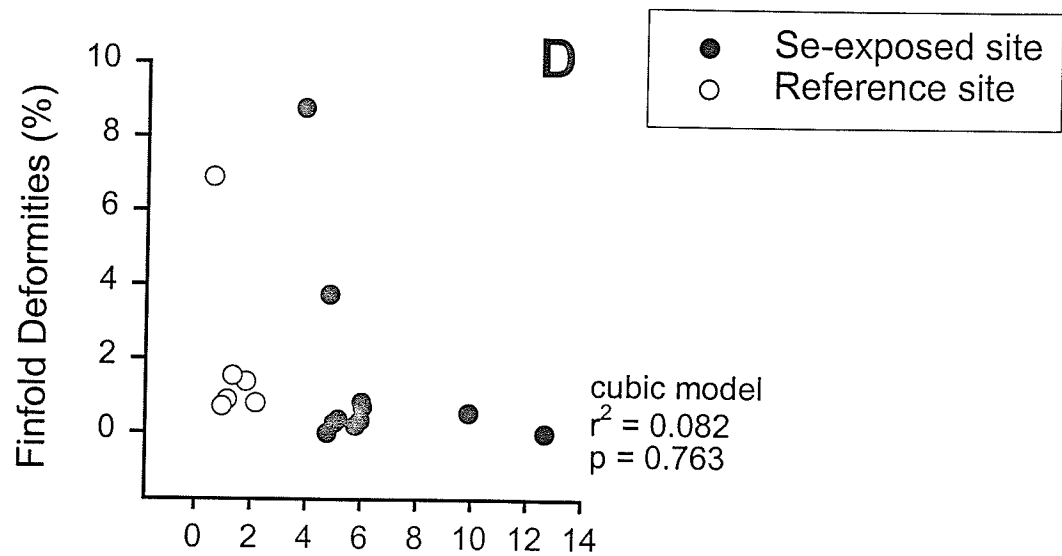
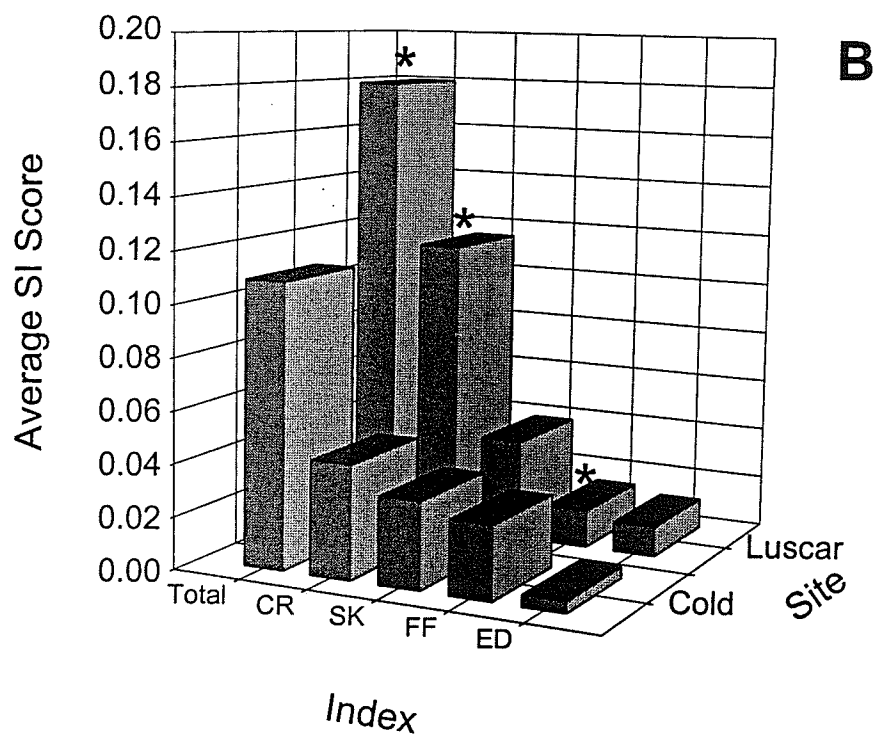
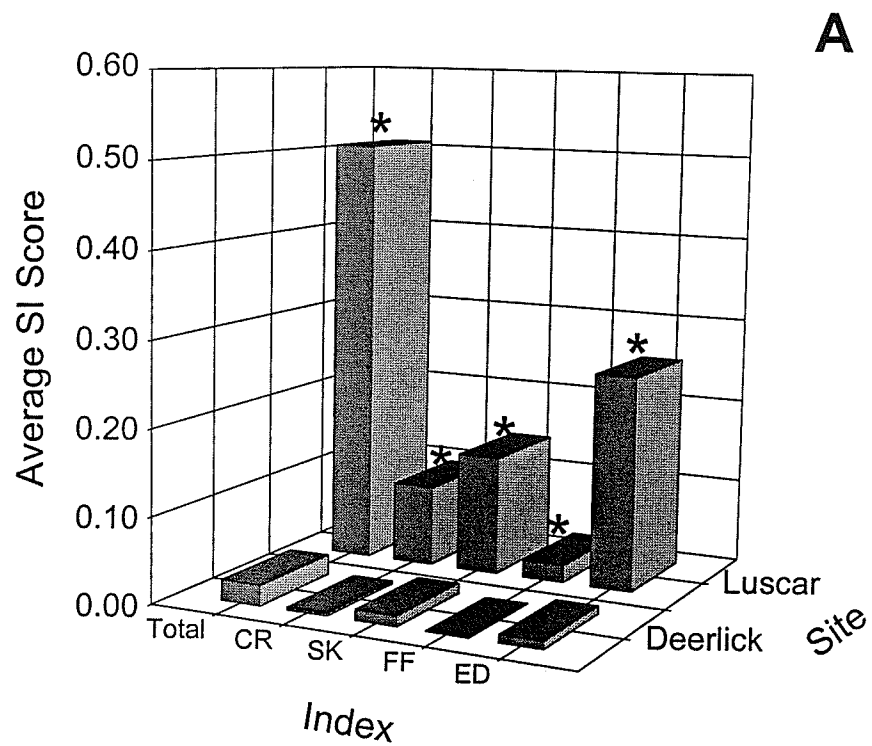


Figure 2.8) Mean graduated severity index (SI) scores for total, craniofacial (CR), skeletal (SK), finfold (FF), edema (ED) defects in (A) rainbow trout and (B) brook trout collected from a Se-exposed site (Luscar Creek) and reference sites (Deerlick Creek for rainbow trout and Cold Creek for brook trout) in northeastern Alberta. Scores from the Se exposed site that were significantly different from the reference site are marked with an asterisk.



Chapter 3

Development and survival of fry from wild rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) exposed to elevated selenium downstream of coal mining activities.

3.1) Introduction

The potential for Se contamination of aquatic ecosystems due to anthropogenic activities has been recognized for nearly two decades (Lemly 1993a). However, to date almost all reported cases of Se poisoning in natural fish populations have been obtained from standing, warm water habitats. Consequently, the applicability of toxic effect thresholds and guidelines developed from these systems to cold flowing water habitats is a matter of contention in the literature (Lemly 1997c; Brix *et al.* 2000; Kennedy *et al.* 2000).

Recently, elevated Se levels have been detected in cold, flowing water habitats downstream from uranium (TAEM 1996) and coal (McDonald and Strosher 1998; Casey and Siwik 2000; Kennedy *et al.* 2000) mining activity in Canada. Fish from these systems have concentrations of Se in their tissues that exceed toxic effect thresholds that have been established for warm water fishes (Lemly 1993a), but to date, no effects have been reported. A study by Kennedy *et al.* (2000) examined deformities in the larvae of cutthroat trout from a cold water system with elevated Se. They did not observe any deformities in the progeny of the cutthroat trout from their study and concluded that the absence of toxic responses may be the result of an evolved Se-tolerance in this population. Extrapolating the results from this one study for assessing risk to other species is difficult. Moreover, the results of this paper have been criticized on the basis of the methodologies employed (Hamilton and Palace 2001).

The conclusion that salmonid fishes are more tolerant to the effects of Se than their warm water counterparts is of concern because of a current and proposed reclamation strategy employed and proposed for further use by coal mining companies in Alberta's

northeast slopes region. Specifically, coal mining companies in the region have proposed to develop end pit lakes following mine pit closure. These lakes are created when surface pits are allowed to fill with water once mining is completed. A problem arises because the physical and geochemical characteristics of pit lakes may potentiate the mobility of Se and its entry into aquatic food webs (Miller *et al.* 1996). Although Se concentrations in some of these endpit lakes, and at other downstream sites, have been found to exceed water quality guidelines, there has been no assessment of the potential adverse effects in fish populations in these systems (Skorupa 1998). The objective of this study was to determine if exposure to elevated levels of Se results in teratogenesis in the progeny of two salmonid species.

3.2) Materials and Methods

3.2.1) Sites and Fish Collection

Spawning rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) were collected during two consecutive years (2000 and 2001) using the same protocol described in Chapter 2. In addition to the collections already described for 2000, the 2001 survey involved an increased number of sample sites (Figure 3.1). Specifically, rainbow trout were collected in June 2001 from Luscar Creek (high Se site), Gregg River (intermediate Se site), Wampus Creek and Deerlick Creek (reference sites). Brook trout were collected in October 2001 from Luscar Creek, Gregg River and Cold Creek (reference site). Typical water chemistry data for the 2000-2001 study sites appear in Table 3.1. After being anesthetized (as described in Chapter 2), adult trout were first weighed and measured. Condition factor (CF) was calculated using the formula: [body

weight (g)] / [fork length³ (cm)] *100. To limit potential impacts to already declining populations, fish were not sacrificed after gametes were stripped in 2001.

3.2.2) *Tissue Se Determination*

Concentrations of Se were determined in eggs and tissues of adult rainbow trout and brook trout using an ICP-MS method described by Feng *et al.* (1999). Because several other elements are known to affect the availability and toxicity of Se (Hamilton and Palace 2001, and references therein) concentrations of Al, Sb, Ba, Be, Bi, B, Cd, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Hg, Mo, Ni, Ag, Sr, Tl, Th, Sn, Ti, U, V, Zn were also determined. Correlation analysis was used to evaluate relationships between muscle Se concentrations and egg Se concentrations (SPSS Inc. 1999). Se concentrations in tissues are reported on a wet weight basis. Wet weight Se concentrations can be approximated from the literature where dry weight concentrations are reported by assuming that most tissues contain 75% moisture (Lemly 1997). Where this calculation is used in the discussion of this chapter, concentrations are reported as calculated wet weight (ww calc.).

2.3) *Egg and Larval Rearing*

The method used to rear fry of both species to the swim-up stage in 2001 was the same as that for the 2000 studies described in Chapter 2. The exceptions were that rainbow trout eggs from the 2001 study were incubated at $5 \pm 0.6^{\circ}\text{C}$, instead of at 8°C , the temperature of incubation used for all other eggs. This change was incorporated to more closely approximate the ambient temperature of the streams from which the

rainbow trout were captured. Percent fertilization was calculated based on the number of dead eggs when visual examination did not reveal a developing embryo, compared to the total number of eggs fertilized. Mortality was calculated based on the number of dead eggs or fry removed until the swim-up stage compared to the total number of fertilized eggs. Time, in degree-days ($DD = \Sigma$ daily temperature for the duration of incubation), to the eyed stage and hatch was also recorded.

3.2.4) Assessment of Morphological Deformities in Larvae

Frequency assessments, as described in Chapter 2, were used to quantify deformities in rainbow and brook trout fry in both years of the study. Of the three methods assessed in Chapter 2, this method was most efficient and the data generated were easiest to analyze. Mean fry length (MFL) in 2001 was quantified by taking the mean lengths of 10 randomly selected fry per female using the same equipment described in the previous chapter. Mean fry weight (MFW) was calculated in both 2000 and 2001 by finding the average weight of 50 randomly selected fry from each female.

3.2.5) Statistical Analysis

All statistical analyses were conducted with SPSS version 10.0 (SPSS Inc. 1999). Endpoints for adult females, eggs and larvae were compared between sites in 2000 using Mann-Whitney U nonparametric tests and in 2001 using Kruskal-Wallis nonparametric tests. If results from the Kruskal-Wallis test were significant ($P \leq 0.05$), data were ranked and analyzed using a one-way ANOVA. If results from the one-way ANOVA were significant, Bonferroni's test was used to identify significant differences between sites.

In addition, data from Luscar Creek and Deerlick/Cold Creek were combined over both years of the study for each species (rainbow trout or brook trout). To test for year effects (2000 vs. 2001), the pooled data set was split into subsets for each site and analyzed using Mann-Whitney U test. To test for site effects (Luscar Creek vs. Deerlick Creek or Cold Creek), the pooled data were split into subsets for each year and analyzed by Mann-Whitney U test. Because the relationship between Se accumulation in eggs and teratogenesis was of primary interest, pooled data from both years were also analyzed using regressions. An alpha value of 0.05 was used for all statistical analyses.

3.3) Results

3.3.1) Adult characteristics

Weights, lengths and condition factors of rainbow trout (Table 3.2) and brook trout (Table 3.3) adult females were not significantly different between Luscar Creek and the reference sites in 2000. However, in 2001, there were significant differences in the size of both species among sites. Rainbow trout from Luscar were heavier and longer than fish from Gregg or Deerlick. Even with these differences, condition factors were not found to differ significantly between sites. Brook trout from Gregg River were lighter than fish from Luscar Creek and shorter than those from Cold or Luscar Creeks. Condition factors of Luscar brook trout were significantly higher than at Cold Creek in 2001.

One-way Mann-Whitney U analysis of the combined data sets from 2000 and 2001 indicated that weight, length, and condition factor were not significantly different within years for either species (Table 3.4). However, significant differences were found

between sites when the data was pooled (Table 3.5). Specifically, rainbow trout were significantly heavier and longer in Luscar Creek compared to the reference site.

Condition factors did not vary between the sites. Condition factors of brook trout from Luscar Creek were significantly higher than the reference site.

With the exception of Se, there were no significant differences in the concentrations of metals or metalloids in rainbow trout (Table 3.6) and brook trout (Table 3.7) eggs collected from Luscar Creek and the reference sites. In both 2000 and 2001, significantly higher concentrations of Se (mean $\pm$ SE) were found in the tissues of rainbow trout and brook trout from Luscar Creek versus the reference sites. In the 2000 survey (Table 3.2), Luscar rainbow trout had significantly higher concentrations of Se in axial muscle and eggs compared with those from the reference site. In 2001 (Table 3.2), rainbow trout eggs from Luscar and Gregg River were found to have significantly higher concentrations than those from both Deerlick and Wampus creeks.

In 2000, brook trout from Luscar Creek had significantly higher concentrations of Se in axial muscle, liver, and eggs versus muscle, liver, and eggs in fish from Cold Creek (Table 3.3). In 2001, brook trout captured from Luscar and the Gregg River were found to have significantly higher egg Se concentrations than fish from the Cold Creek (Table 3.3).

Selenium in eggs was positively correlated to selenium in muscle tissue for both rainbow trout ($r = 0.864$; Figure 3.2A) and brook trout ($r = 0.954$; Figure 3.2B) in 2000. Correlation analysis for Se in muscle tissue could not be performed on 2001 data or 2000/2001 combined data since Se muscle concentrations were not analyzed for the 2001 study. These samples were not taken on the basis that Se concentrations in the eggs is the

most meaningful for predicting teratogenesis and that additional lethal sampling of potentially declining fish populations was not required to confirm these relationships.

When the data from the two years were pooled, Se concentrations in the eggs of both species were found to be significantly higher at Luscar Creek compared to the reference site (Table 3.5). There were no significant differences in Se concentrations between the two years of the study for either brook trout or rainbow trout (Table 3.4).

3.3.2) Embryo-larval characteristics

In 2000 and 2001, there were no significant differences in fertilization, mortality, or degree-days to hatch between the Se-exposed site and the reference sites in either rainbow trout (Table 3.8) or brook trout (Table 3.9). There was a significant difference in 2001 in degree-days to the eye stage for brook trout, with Gregg River eggs developing faster than those from Luscar and Cold Creeks. When results from the two years were pooled, none of the aforementioned parameters were found to differ significantly between the Se-exposed site and the reference sites for either species (Table 3.10).

However, there were significant differences in percent fertilization and mortality for a given species between the two study years (Table 3.11). Specifically, significantly higher fertilization, mortality rates and increased degree-days to eye and hatch were observed for rainbow trout in the 2001 study. Higher fertilization and mortality rates in 2000 and increased degree-days to the eye stage in 2001 were found for brook trout.

Percentages of larval deformities were different between species and between years. The frequency assessment for 2000 discussed in Chapter 2 found significantly higher incidence of craniofacial (CR), skeletal (SK), finfold (FF) deformities and edema (ED) in

rainbow trout fry from the exposed site. However, in 2001 there were no significant differences in the occurrence of any of these deformities among brook trout fry from any of the sites (Table 3.8). Analysis of the pooled data from 2000 and 2001 showed that there were significant differences in the incidence of all types of deformities between years, with each category of deformity having a higher mean percentage in 2001 compared to 2000 (Table 3.9). However, no significant differences were found between deformity levels at Deerlick and Luscar for the pooled data (Table 3.10). Rainbow trout fry were significantly larger (MFL and MFW) in 2001 than in the previous year. The weights of Luscar fry at swimup were significantly greater than fry from the reference site.

The types of deformities observed in brook trout fry were also different between the two years of the study. In 2000, brook trout had a significantly higher incidence of CR deformities at the Se-exposed site compared to the reference site. But in 2001 brook trout fry from Gregg had a significantly higher incidence of FF deformities than at the reference site (Table 3.9). Analysis of the pooled data from 2000 and 2001 show that CR deformities were significantly higher in 2000 than in 2001 (Table 3.11). CR deformities were also higher in Luscar fry than in fry from the reference site (Table 3.10). Brook trout from the second year of the study had greater MFL values than fry from 2000.

The relationships between the incidence of deformities in rainbow trout fry and the concentrations of Se in eggs were similar for the combined 2000/2001 data (Figure 3.3) and the data already discussed in Chapter 2 for 2000 (Figure 2.6). There were significant relationships between Se and edema (cubic model, $p < 0.001$), craniofacial defects (cubic model, $p = 0.002$), and skeletal defects (cubic model, $p < 0.001$). The edema data set

showed the strongest relationship to the mathematical model ($r^2 = 0.838$) compared with either the craniofacial ($r^2 = 0.381$) or skeletal ($r^2 = 0.486$) data sets. The inflection point in the regression curves for craniofacial, finfold deformities and edema occurred at approximately 6 $\mu\text{g/g}$. In contrast, an association between the occurrence of deformities in brook trout and egg Se concentrations was not evident (Figure 3.4).

3.4) Discussion

Elevated levels of Se were found in the tissues of two salmonid species captured downstream from coal mining activity in the northeastern slopes region of Alberta. The levels of Se were found to exceed the toxic effect thresholds of 8 $\mu\text{g/g}$ (dw) in muscle, 10 $\mu\text{g/g}$ (dw) in eggs and in the limited number of liver samples analyzed from brook trout (Lemly 1993a). In spite of the high concentrations of Se in their tissues, adult rainbow and brook trout did not show histopathological lesions typically associated with Se in either the gill, liver or kidney (R.E. Evans, DFO – Winnipeg, unpubl. observ.).

Histopathological effects of Se can include hematological changes, as well as gill, liver, kidney and ovary damage as described in redear sunfish with liver concentrations of 7.5 $\mu\text{g Se/g}$ (ww calc.; Sorenson 1988) and green sunfish with muscle concentrations of 13 $\mu\text{g Se/g}$ (ww calc.; Sorenson *et al.* 1984). In light of these findings, examinations of the differences in species susceptibility of adults to Se-induced tissue damage in adult fish warrant further consideration.

In 2000, rainbow trout and brook trout consistently had higher concentrations of Se in their eggs than in muscle tissue, which is similar to the pattern of accumulation observed for cutthroat trout (Kennedy *et al.* 2000), largemouth bass and bluegill sunfish (Baumann

and Gillespie 1986). Se concentrations in the eggs of both species also showed a strong relationship with Se concentrations in muscle, suggesting that maternal transfer of Se is efficient in both brook trout and rainbow trout. A similar relationship between Se concentrations in muscle and eggs have been reported for razorback suckers (Hamilton and Waddell 1994) and cutthroat trout (Kennedy *et al.* 2000).

Se levels in the tissues of female rainbow and brook trout in the present study were found to be highly variable. This was true even for females collected from the same site. This variability is likely a consequence of fish mobility within the study area. For example, fish are free to move into or out of Luscar Creek via the McLeod River. This means that exposure to Se laden waters or prey items may be intermittent. In fact, fish captured in Luscar Creek may feed at sites other than Luscar Creek that differ widely in their Se concentrations. Similarly, trout captured at the reference sites may have spent a significant portion of their feeding times at sites with elevated Se concentrations. This is one of the most important reasons that actual tissue Se concentrations must be considered in examining potential teratogenesis rather than comparisons based only on capture site.

Fertilization, mortality and size at swim-up were not different between fry collected from the Se-exposure sites and the reference sites for either species. This is consistent with results from other studies in bluegills (Coyle *et al.* 1993; Gillespie and Baumann 1986), fathead minnows (Ogle and Knight 1989), perch (Crane *et al.* 1992) and cutthroat trout (Kennedy *et al.* 2000). The apparent lack of response in endpoints associated with the early egg stage of development to elevated concentrations of Se could be related to the rate of consumption of the yolk sac (reviewed in Heming and Buddington 1988). Se is incorporated into the platelet components, lipovitellin and phosvitin, of the egg yolk

(Kroll and Doroshov 1991) and exerts toxic effects only after it has been assimilated by the developing larval fish. Prior to hatch, the yolk is absorbed at a slow but steadily increasing rate. The rate of absorption increases rapidly right before hatch and continues at a relatively high and constant rate. During this second phase the yolk platelets are preferentially consumed over the oil globule component of the yolk. The rate of yolk consumption then slows as the platelets are depleted. Thus, even though Se is present in the egg yolk throughout development, it may affect larval, rather than egg development because it is, in effect, not available to affect development until after hatch.

The increase in degree-days to the eye stage and to hatch for rainbow trout eggs from 2000 compared to those from 2001 is likely a consequence of the different incubation temperatures for those two years. In 2000, eggs were incubated at a higher temperature, 8°C, than in 2001, 5°C. This change was incorporated to the study design for 2001 to more closely approximate actual incubation temperatures for rainbow trout eggs. It is well known that developmental rates for fish are dependent on the physical conditions of incubation, including water temperature (Vernier 1960).

Teratogenic effects observed in fish from this study were consistent with those reported in other fish species that had accumulated similar tissue burdens of Se. Gillespie and Baumann (1986) reported that eggs from female bluegills (ovary Se, 6.96 µg/g) captured from a Se-contaminated reservoir produced up to 100% edematous larvae. Bluegill sunfish (Hermanutz *et al.* 1992) and fathead minnows (Schultz and Hermanutz 1990) exposed to elevated levels of Se in outdoor experimental streams had a higher incidence of edema, lordosis and internal hemorrhaging. These effects were identified at Se concentrations of 5.96 µg/g in ovary.

Although Se concentrations in the eggs of rainbow trout were consistent in both years of the study, the types and incidence of larval deformities differed between years. A potential source of variability between the two years of the study is incubation temperature. In addition to affecting developmental rate, temperature could influence the appearance of teratogenic endpoints. Lemly (1993b) has shown how environmental conditions can affect the toxicity of Se by influencing an organism's physiology. He found that cold weather increased the sensitivity of bluegill sunfish to Se by leading to a depletion of stored body lipids, a condition he termed "Winter Stress Syndrome", that ultimately resulted in increased mortality among exposed fish. However, replication of these studies at both temperatures is required to unequivocally determine whether teratogenesis arising from Se exposure is temperature dependent in rainbow trout or brook trout.

Recent evidence indicates that Se toxicity may be related to the ability of Se to generate oxidative stress (Spalholz 1994). Spalholz has shown that several forms of Se can redox cycle to produce reactive oxygen species. However, selenomethionine, the predominant form of Se found in fish eggs, is not capable of redox cycling. It may be that generation of oxygen radicals from organic forms of Se requires transformation or cleavage to metabolites that are redox active. Specifically, Spalholz and Hoffman (2002) speculated that selenomethionine might liberate the redox active compound methylselenol in the presence of an unidentified enzyme found in the embryos of birds or fish. In vitro evidence from our laboratory indicates that this enzyme is indeed present in rainbow trout eggs (Palace *et al.* 2002). Furthermore, temperature appears to affect the rate of this enzyme activity with a tendency for higher activity to be detected at higher

incubation temperatures. This would suggest that embryos incubated at 8°C during 2000 would be more likely to exhibit oxidative effects than those incubated at 5°C during 2001. Finally, the generation of edema as a result of oxidative stress has been identified in situations where fish are exposed to specific contaminants (Fitzsimons 1995; Bengtsson *et al.* 1994). A more thorough examination of the potential for Se to generate oxidative stress in fish is clearly required.

Associations were identified in this study between the concentration of Se in the eggs of rainbow trout and the frequency of larval deformities. The inflection point (or rapid rise in deformities) is consistent for all regression curves and occurs between 4 and 6 µg/g. Lemly (1993c) reported a similar relationship between whole-body Se concentrations and frequency of deformed centrarchid fish species inhabiting a Se-laden reservoir. Similar to the results from the rainbow trout in these studies, the relationship for centrarchids also fit an exponential function (cubic model), with the point of rapid rise in deformities occurring between 4 and 12.5 µg/g (ww calc.).

The toxic effect threshold of 4-6µg/g in eggs identified in this study for rainbow trout inhabiting seleniferous flowing water habitats are similar to those reported in other studies. Most importantly, there is no evidence that these thresholds are significantly different between cold water and warm water species. Adverse effects in both habitat types are linked with whole-body Se concentrations of 1 to 3 µg/g and concentrations of Se in eggs of 4 to 12 µg/g (ww; Appendix II). However, within habitat types there may be differences in the sensitivity of different species to the effects of Se. At similar burdens of Se in eggs, brook trout and rainbow trout did not exhibit comparable types or frequency of deformities. Brook trout primarily showed craniofacial deformities,

specifically malformed jaws and eyes, which could interfere with feeding. These deformities can become lethal at the larval transition stage between endogenous and exogenous feeding when an inability to feed results in starvation. While not being directly lethal, spinal distortions and edema, most often observed in rainbow trout alevins, can lead to increased susceptibility to predation by decreasing swimming ability. What significance these deformities will ultimately have on the natural populations of rainbow trout and brook trout requires further examination.

Table 3.1) Typical study site water characteristics of the sites sampled in 2000 and 2001 of this study at the sampling times.

Site	Water temperature (°C) ^a	% dissolved oxygen ^a	pH ^a	Conductivity (mS/cm) ^a	Se concentration (µg/L)
Field Sites					
Luscar Creek	2-6	93	8.3	0.538	6-32 ^c
Gregg River	2-6	76.8	8.0	0.506	1.4-5.4 ^c
Deerlick Creek	2-7	91	7.5	0.116	0.71 ^d
Cold Creek	2-8	97	8.0	0.393	0.66 ^d
Wampus Creek	2-5	89.6	7.3	0.257	< 0.5 ^c
Laboratory					
FWI	8.0 ± 0.8 ^b	> 98	7.8	0.018	1.6 ± 0.1 ^e

^a data obtained using a Hydrolab multiprobe (Houston, TX)

^b except for rainbow trout raised in 2001 where water temperature was 5.0 ± 0.6

^c Casey and Siwik 2000

^d data from 2002 (V. Palace, unpubl. observ.) using inductively coupled mass spectroscopy (ICP-MS) as described by Feng *et al.* (1999)

^e data obtained using Hydride generation with atomic fluorescence detection (PSA Analytical, Kent, UK)

Table 3.2) Mean morphological measurements and tissue Se concentrations of adult female rainbow trout collected from a high Se site (Luscar Creek), an intermediate Se site (Gregg River), and reference sites (Deerlick Creek and Wampus Creek) in northeastern Alberta over two consecutive years (mean $\pm$ SE). Values that are significantly different at $\alpha = 0.05$ are marked with dissimilar letters.

Site	n	Weight (g)	Length (cm)	CF ^a	[Se] muscle ^b	[Se] eggs ^b
2000						
Luscar	3	177.0 $\pm$ 112.0	22.7 $\pm$ 5.2	1.12 $\pm$ 0.02	1.50 $\pm$ 0.28 ^c	8.37 $\pm$ 1.62 ^c
Deerlick	4	70.0 $\pm$ 7.5	19.2 $\pm$ 0.6	0.98 $\pm$ 0.04	0.48 $\pm$ 0.15 ^d	2.05 $\pm$ 1.06 ^d
2001						
Luscar	10	159.0 $\pm$ 23.7 ^c	23.6 $\pm$ 1.1 ^c	1.14 $\pm$ 0.04	NT	6.49 $\pm$ 0.89 ^c
Gregg	6	72.0 $\pm$ 7.95 ^d	18.3 $\pm$ 0.7 ^d	1.16 $\pm$ 0.04	NT	6.65 $\pm$ 1.83 ^c
Deerlick	10	79.3 $\pm$ 12.4 ^d	18.9 $\pm$ 1.0 ^d	1.11 $\pm$ 0.03	NT	2.77 $\pm$ 0.20 ^d
Wampus	2	65.1 $\pm$ 1.6	18.1 $\pm$ 0.5 ^d	1.11 $\pm$ 0.06	NT	2.35 $\pm$ 0.31 ^d

^a CF = condition factor = weight (g) / length (cm)³ $\times$ 100

^b μ g/g, wet weight

NT = not taken for Se analysis

Table 3.3) Mean somatic measures and concentrations of Se in adult female brook trout collected from a high Se site (Luscar Creek) an intermediate Se site (Gregg River), and a reference site (Cold Creek) in northeastern Alberta over two consecutive years (mean $\pm$ SE). Values that are significantly different at $\alpha = 0.05$ are marked with different letters.

Site	n	Weight (g)	Length (cm)	CF ^a	[Se] muscle ^b	[Se] eggs ^b	[Se] liver ^b
2000							
Luscar	11	167.8 $\pm$ 45.3	23.4 $\pm$ 1.8	1.10 $\pm$ 0.04	3.79 $\pm$ 0.51 ^c	6.37 $\pm$ 0.78 ^c	13.14 $\pm$ 1.41 ^c
Cold	7	117.4 $\pm$ 22.8	22.0 $\pm$ 1.6	1.04 $\pm$ 0.04	0.55 $\pm$ 0.10 ^d	1.35 $\pm$ 0.24 ^d	2.13 $\pm$ 0.33 ^d
2001							
Luscar	11	112.5 $\pm$ 11.5 ^d	21.0 $\pm$ 0.8 ^d	1.18 $\pm$ 0.04 ^c	NT	8.02 $\pm$ 0.77 ^d	NT
Gregg	8	56.1 $\pm$ 8.3 ^c	17.0 $\pm$ 0.9 ^c	1.08 $\pm$ 0.03	NT	6.88 $\pm$ 0.51 ^d	NT
Cold	5	99.0 $\pm$ 16.6	21.0 $\pm$ 1.4 ^d	1.02 $\pm$ 0.02 ^d	NT	1.25 $\pm$ 0.15 ^c	NT

^a CF = condition factor = weight (g) / length (cm)³ $\times$ 100

^b μ g/g, wet weight

NT = not taken for Se analysis

Table 3.4) Mean somatic measures and concentrations of Se in adult female rainbow trout and brook trout combined over both years of the study by year (mean $\pm$ SE). Values that are significantly different at $\alpha = 0.05$ are marked with different letters.

Year	n	Weight (g)	Length (cm)	CF ^a	[Se] eggs ^b
Rainbow trout					
2000	7	116.0 $\pm$ 47.6	20.7 $\pm$ 2.1	1.04 $\pm$ 0.04	4.75 $\pm$ 1.52
2001	28	105.1 $\pm$ 12.1	20.4 $\pm$ 0.7	1.13 $\pm$ 0.02	4.89 $\pm$ 0.60
Brook trout					
2000	18	148.2 $\pm$ 29.1	22.8 $\pm$ 5.2	1.08 $\pm$ 0.03	4.6 $\pm$ 0.78
2001	24	90.9 $\pm$ 41.2	19.7 $\pm$ 3.3	1.11 $\pm$ 0.02	6.23 $\pm$ 0.66

^a CF = condition factor = weight (g) / length (cm)³ $\times$ 100

^b μ g/g, wet weight

Table 3.5) Mean somatic measures and concentrations of Se in adult female rainbow trout and brook trout combined over both years of the study by site (mean $\pm$ SE). Luscar Creek is the Se-exposed site, while Deerlick Creek and Cold Creek are reference sites. Values that are significantly different at $\alpha = 0.05$ are marked with different letters.

Site	n	Weight (g)	Length (cm)	CF ^a	[Se] eggs ^b
Rainbow trout					
Luscar	13	162.9 $\pm$ 28.4 ^c	23.4 $\pm$ 1.3 ^c	1.14 $\pm$ 0.03	6.92 $\pm$ 0.78 ^c
Deerlick	14	76.7 $\pm$ 33.6 ^d	19.0 $\pm$ 0.7 ^d	1.07 $\pm$ 0.03	2.56 $\pm$ 0.32 ^d
Brook trout					
Luscar	22	140.1 $\pm$ 23.6	22.2 $\pm$ 1.0	1.14 $\pm$ 0.03 ^c	7.20 $\pm$ 0.56 ^c
Cold	12	109.8 $\pm$ 14.7	21.5 $\pm$ 1.1	1.03 $\pm$ 0.02 ^d	1.30 $\pm$ 0.14 ^d

^a CF = condition factor = weight (g) / length (cm)³ $\times$ 100

^b μ g/g, wet weight

Table 3.6) Mean trace element concentrations ($\mu\text{g/g}$, wet weight) in rainbow trout eggs collected over two consecutive years (2000 and 2001) from a high Se site (Luscar Creek), an intermediate Se site (Gregg River), and reference sites (Deerlick and Wampus creeks) in northeastern Alberta (mean $\pm$ SE).

	Luscar Creek	Gregg River ^a	Deerlick Creek	Wampus Creek ^a
# fish	13 ^b	6	14 ^b	2
Ag	< 0.005	0.009 $\pm$ 0.001	< 0.005	0.008 $\pm$ 0.002
Al	1.5 $\pm$ 0.8	0.45 $\pm$ 0.05	1.5 $\pm$ 0.8	1.11 $\pm$ 0.75
As	0.20 $\pm$ 0.02	< 0.2	0.20 $\pm$ 0.02	< 0.1
B	< 0.8	0.811 $\pm$ 0.007	< 0.8	< 0.8
Ba	0.34 $\pm$ 0.04	0.24 $\pm$ 0.03	0.34 $\pm$ 0.04	0.38 $\pm$ 0.05
Be	< 0.1	< 0.1	< 0.1	< 0.1
Bi	< 0.003	0.0031 $\pm$ 0.0004	0.008 $\pm$ 0.005	< 0.002
Ca	423 $\pm$ 14	486 $\pm$ 9	482 $\pm$ 13	400 $\pm$ 24
Cd	0.023 $\pm$ 0.004	< 0.01	< 0.2	< 0.02
Co	0.028 $\pm$ 0.005	0.038 $\pm$ 0.005	0.018 $\pm$ 0.003	< 0.01
Cr	< 0.1	< 0.2	< 0.1	< 0.02
Cu	2.4 $\pm$ 0.2	2.7 $\pm$ 0.2	2.7 $\pm$ 0.2	2.12 $\pm$ 0.08
Fe	17.5 $\pm$ 1.5	14.0 $\pm$ 2.0	18.6 $\pm$ 1.9	17.0 $\pm$ 1.5
Hg	0.015 $\pm$ 0.003	< 0.01	< 0.03	< 0.01
Li	< 0.1	< 0.1	< 0.1	< 0.1
Mg	591 $\pm$ 14	652 $\pm$ 20	587 $\pm$ 20	529 $\pm$ 18
Mn	0.53 $\pm$ 0.03	0.45 $\pm$ 0.03	0.79 $\pm$ 0.07	0.90 $\pm$ 0.07
Mo	0.013 $\pm$ 0.002	0.017 $\pm$ 0.004	0.016 $\pm$ 0.002	0.017 $\pm$ 0.007
Ni	< 0.03	< 0.06	< 0.09	0.34 $\pm$ 0.31
Pb	0.012 $\pm$ 0.005	0.007 $\pm$ 0.001	0.03 $\pm$ 0.02	0.025 $\pm$ 0.010
Sb	< 0.004	< 0.004	< 0.004	< 0.006
Se	6.9 $\pm$ 0.8	6.65 $\pm$ 1.83	3.0 $\pm$ 0.3	2.35 $\pm$ 0.31
Sn	0.016 $\pm$ 0.005	0.010 $\pm$ 0.004	0.014 $\pm$ 0.005	0.023 $\pm$ 0.012
Sr	2.7 $\pm$ 0.5	2.22 $\pm$ 0.23	0.81 $\pm$ 0.05	0.73 $\pm$ 0.03
Th	0.011 $\pm$ 0.003	0.009 $\pm$ 0.002	0.017 $\pm$ 0.005	0.014 $\pm$ 0.005
Ti	7.09 $\pm$ 0.11	7.23 $\pm$ 0.24	6.7 $\pm$ 0.1	6.11 $\pm$ 0.14
Tl	0.0010 $\pm$ 0.0002	0.0020 $\pm$ 0.0005	0.0020 $\pm$ 0.0005	0.003 $\pm$ 0.001
U	< 0.001	< 0.001	< 0.001	< 0.002
V	0.15 $\pm$ 0.01	0.14 $\pm$ 0.01	0.15 $\pm$ 0.01	0.16 $\pm$ 0.02
Zn	26.2 $\pm$ 0.8	26.1 $\pm$ 1.1	28.0 $\pm$ 1.2	26.1 $\pm$ 1.2

^a sites sampled only in the second year (2001) of the study

^b sample size for As, Ca, Cd, Hg, Pb, Se, Zn, elements which were analyzed in both years (2000 and 2001) of the study, for all other elements analyzed only in 2001, the sample size is 10

Table 3.7) Mean trace element concentrations ($\mu\text{g/g}$, ww) in brook trout eggs collected over two consecutive years (2000 and 2001) from a high Se site (Luscar Creek), an intermediate Se site (Gregg River), and reference sites (Deerlick and Wampus creeks) in northeastern Alberta (mean $\pm$ SE).

	Luscar Creek	Gregg River ^a	Cold Creek
# fish	22	8	12
Ag	0.005 $\pm$ 0.001	0.006 $\pm$ 0.001	0.010 $\pm$ 0.002
Al	4.4 $\pm$ 3.0	2.7 $\pm$ 1.8	1.5 $\pm$ 0.4
As	0.07 $\pm$ 0.01	< 0.03	< 0.3
B	9 $\pm$ 5	0.58 $\pm$ 0.12	4 $\pm$ 3
Ba	0.21 $\pm$ 0.03	0.23 $\pm$ 0.04	0.12 $\pm$ 0.02
Be	0.10 $\pm$ 0.05	< 0.04	0.09 $\pm$ 0.04
Bi	0.006 $\pm$ 0.003	0.007 $\pm$ 0.001	0.0049 $\pm$ 0.0003
Ca	412 $\pm$ 23	414 $\pm$ 34	399 $\pm$ 45
Cd	0.020 $\pm$ 0.007	0.0083 $\pm$ 0.0003	0.010 $\pm$ 0.001
Co	0.06 $\pm$ 0.01	0.034 $\pm$ 0.020	0.05 $\pm$ 0.01
Cr	0.17 $\pm$ 0.09	0.056 $\pm$ 0.011	0.09 $\pm$ 0.02
Cu	2.9 $\pm$ 0.1	3.0 $\pm$ 0.1	3.3 $\pm$ 0.4
Fe	10.7 $\pm$ 1.2	12.5 $\pm$ 1.9	16.3 $\pm$ 2.7
Hg	0.017 $\pm$ 0.006	0.013 $\pm$ 0.002	0.018 $\pm$ 0.003
Li	< 0.3	< 0.1	< 0.1
Mg	425 $\pm$ 31	511 $\pm$ 14	374 $\pm$ 52
Mn	0.99 $\pm$ 0.14	0.67 $\pm$ 0.14	1.7 $\pm$ 0.3
Mo	0.019 $\pm$ 0.003	0.021 $\pm$ 0.003	0.021 $\pm$ 0.002
Ni	0.15 $\pm$ 0.05	0.034 $\pm$ 0.003	0.07 $\pm$ 0.02
Pb	0.16 $\pm$ 0.09	0.007 $\pm$ 0.002	0.08 $\pm$ 0.04
Sb	< 0.01	< 0.003	< 0.005
Se	7.2 $\pm$ 0.6	6.9 $\pm$ 0.5	1.3 $\pm$ 0.1
Sn	0.07 $\pm$ 0.05	0.042 $\pm$ 0.011	0.041 $\pm$ 0.007
Sr	3.9 $\pm$ 0.3	0.90 $\pm$ 0.14	0.64 $\pm$ 0.10
Th	< 0.1	0.0043 $\pm$ 0.0007	0.0046 $\pm$ 0.0008
Ti	3.98 $\pm$ 0.13	3.8 $\pm$ 0.2	4.2 $\pm$ 0.7
Tl	0.014 $\pm$ 0.002	0.0036 $\pm$ 0.0008	0.006 $\pm$ 0.001
U	< 0.002	< 0.001	< 0.001
V	0.031 $\pm$ 0.016	0.130 $\pm$ 0.003	0.016 $\pm$ 0.003
Zn	23.6 $\pm$ 1.4	23.5 $\pm$ 1.4	23.4 $\pm$ 1.5

^a site sampled only in the second year (2001) of the study

Table 3.8) Mean embryo-larval parameters for rainbow trout collected from a high Se site (Luscar Creek), an intermediate Se site (Gregg River), and reference sites (Deerlick Creek and Wampus Creek) in northeastern Alberta over two consecutive years (mean $\pm$ SE). Values that are significantly different at $\alpha = 0.05$ are marked with different letters.

Site	2000		2001			
	Luscar	Deerlick	Luscar	Gregg	Deerlick	Wampus
n ^a	297	261	2021	720	1342	209
% fertilization	79.8 $\pm$ 4.3	51.5 $\pm$ 10.9	81.5 $\pm$ 5.0	79.4 $\pm$ 5.2	88.0 $\pm$ 2.1	94.0 $\pm$ 4.8
% mortality	3.3 $\pm$ 1.0	0.7 $\pm$ 0.4	27.8 $\pm$ 7.3	38.3 $\pm$ 13.7	26.5 $\pm$ 4.7	4.2 $\pm$ 0.8
DD eye ^b	296.0 $\pm$ 5.2	299.0 $\pm$ 0.0	181.8 $\pm$ 2.6	178.2 $\pm$ 2.1	179.3 $\pm$ 3.1	185.3 $\pm$ 0
DD hatch	432.7 $\pm$ 4.6	434.0 $\pm$ 2.3	326.3 $\pm$ 4.5	325.4 $\pm$ 2.2	317.7 $\pm$ 2.0	327.5 $\pm$ 2.9
% CR	7.7 $\pm$ 3.7 ^b	0.2 $\pm$ 0.2 ^c	14.7 $\pm$ 3.4	11.7 $\pm$ 2.7	10.6 $\pm$ 1.9	12.0 $\pm$ 4.1
% SK	13.8 $\pm$ 5.6 ^b	0.7 $\pm$ 0.5 ^c	19.4 $\pm$ 8.2	11.1 $\pm$ 2.3	15.6 $\pm$ 4.7	4.9 $\pm$ 4.9
% FF	3.2 $\pm$ 2.0 ^b	0.2 $\pm$ 0.2 ^c	6.8 $\pm$ 3.0	15.5 $\pm$ 6.6	4.0 $\pm$ 0.9	1.5 $\pm$ 0.2
% ED	30.8 $\pm$ 27.4 ^b	0.2 $\pm$ 0.2 ^c	19.9 $\pm$ 8.5	13.9 $\pm$ 5.3	10.8 $\pm$ 2.5	7.5 $\pm$ 0.4
% TD	38.9 $\pm$ 25.6 ^b	0.7 $\pm$ 0.5 ^c	ND	ND	ND	ND
MFL (mm)	20.2 $\pm$ 1.2	19.7 $\pm$ 0.1	18.7 $\pm$ 0.6	19.0 $\pm$ 0.5	18.1 $\pm$ 0.5	19.4 $\pm$ 1.1
MFW (mg)	61.2 $\pm$ 8.2	46.0 $\pm$ 3.0	52.0 $\pm$ 6.1	42.5 $\pm$ 4.2	37.5 $\pm$ 2.7	46.7 $\pm$ 7.2

^a number of fry to reach the swim-up stage

^b DD= degree-days, CR = craniofacial defects, SK = skeletal defects, FF = finfold defects, ED = edema, TD = total defects, MFL = mean fry length, MFW = mean fry weight

ND = not done

Table 3.9) Mean embryo-larval parameters for brook trout collected from a high Se site (Luscar Creek), an intermediate Se site (Gregg River), and a reference site (Cold Creek) in northeastern Alberta over two consecutive years (mean $\pm$ SE). Values that are significantly different at $\alpha = 0.05$ are marked with different letters.

Site	2000		2001		
	Luscar	Cold	Luscar	Gregg	Cold
n ^a	4904	1560	3440	1892	1440
% fertilization	97.4 $\pm$ 0.8	96.1 $\pm$ 1.2	87.2 $\pm$ 2.6	85.2 $\pm$ 5.4	77.8 $\pm$ 14.2
% mortality	12.6 $\pm$ 3.8	9.3 $\pm$ 2.4	2.9 $\pm$ 0.8	2.9 $\pm$ 0.9	3.7 $\pm$ 1.6
DD eye ^b	233.2 $\pm$ 0.7	234.2 $\pm$ 1.2	246.0 $\pm$ 1.3 ^c	237.4 $\pm$ 2.6 ^b	248.2 $\pm$ 1.7 ^c
DD hatch	449.5 $\pm$ 26.8	504.0 $\pm$ 20.2	512.4 $\pm$ 2.5	506.0 $\pm$ 2.7	512.0 $\pm$ 10.0
% CR	13.6 $\pm$ 3.5 ^b	3.0 $\pm$ 0.5 ^c	5.6 $\pm$ 3.2	2.12 $\pm$ 1.0	0.7 $\pm$ 0.3
% SK	1.9 $\pm$ 0.8	1.3 $\pm$ 0.8	2.1 $\pm$ 1.1	0.81 $\pm$ 0.3	0.6 $\pm$ 0.4
% FF	1.1 $\pm$ 0.6	1.2 $\pm$ 0.8	3.7 $\pm$ 1.8	4.1 $\pm$ 2.4 ^c	0.1 $\pm$ 0.1 ^b
% ED	0.6 $\pm$ 0.4	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.2	1.7 $\pm$ 1.2
% TD	14.4 $\pm$ 3.6 ^b	4.0 $\pm$ 2.3 ^c	ND	ND	ND
MFL (mm)	16.6 $\pm$ 0.3	17.3 $\pm$ 1.0	17.9 $\pm$ 0.3	18.1 $\pm$ 0.3	18.4 $\pm$ 0.3
MFW (mg)	32.2 $\pm$ 3.9	33.4 $\pm$ 7.3	31.5 $\pm$ 1.6	32.9 $\pm$ 1.7	36.8 $\pm$ 1.8

^a number of fry to reach the swim-up stage

^b DD = degree-days, CR = craniofacial defects, SK = skeletal defects, FF = finfold defects, ED = edema, TD = total defects, MFL = mean fry length, MFW = mean fry weight

ND = not done

Table 3.10) Mean embryo-larval parameters for rainbow trout and brook trout collected from a high Se site (Luscar Creek) and reference sites (Deerlick Creek and Cold Creek) in northeastern Alberta over two consecutive years, combined over both years of the study by site (mean $\pm$ SE). Values that are significantly different at $\alpha = 0.05$ are marked with different letters.

Site	Rainbow trout		Brook trout	
	Luscar	Deerlick	Luscar	Cold
n ^a	2318	1603	8344	3000
% fertilization	81.1 $\pm$ 3.9	77.6 $\pm$ 5.6	92.3 $\pm$ 17.7	88.5 $\pm$ 6.2
% mortality	22.2 $\pm$ 6.3	19.1 $\pm$ 4.6	7.7 $\pm$ 2.1	6.9 $\pm$ 1.7
DD eye ^b	208.1 $\pm$ 14.0	213.5 $\pm$ 15.2	239.6 $\pm$ 1.6	240.1 $\pm$ 2.3
DD hatch	350.8 $\pm$ 13.4	350.9 $\pm$ 54.8	481.0 $\pm$ 14.8 ^c	507.6 $\pm$ 6.2 ^d
% CR	13.1 $\pm$ 3.2	7.6 $\pm$ 7.1	9.9 $\pm$ 2.4	2.7 $\pm$ 0.6
% SK	18.1 $\pm$ 6.3	11.4 $\pm$ 3.8	2.0 $\pm$ 0.6	1.0 $\pm$ 0.4
% FF	6.0 $\pm$ 2.4	2.9 $\pm$ 0.8	2.6 $\pm$ 1.0	1.2 $\pm$ 0.5
% ED	22.4 $\pm$ 8.5	7.8 $\pm$ 2.2	1.3 $\pm$ 0.7	0.9 $\pm$ 0.5
MFL	19.1 $\pm$ 0.5	18.5 $\pm$ 0.4	17.3 $\pm$ 0.2	17.8 $\pm$ 0.3
MFW	54.2 $\pm$ 5.0 ^c	39.9 $\pm$ 2.2 ^d	31.9 $\pm$ 2.1	34.8 $\pm$ 1.8

^a number of fry to reach the swim-up stage

^b DD = degree-days, CR = craniofacial defects, SK = skeletal defects, FF = finfold defects, ED = edema, MFL = mean fry length, MFW = mean fry weight

Table 3.11) Mean embryo-larval parameters for rainbow trout and brook trout collected from a high Se site (Luscar Creek) and reference sites (Deerlick Creek and Cold Creek) in northeastern Alberta over two consecutive years, combined over both years of the study by year (mean $\pm$ SE). Values that are significantly different at $\alpha = 0.05$ are marked with different letters.

Site	Rainbow trout		Brook trout	
	2000	2001	2000	2001
n ^a	557	3363	6464	4880
% fertilization	63.6 $\pm$ 8.3 ^c	84.2 $\pm$ 12.3 ^d	96.9 $\pm$ 0.7 ^c	84.6 $\pm$ 3.5 ^d
% mortality	1.8 $\pm$ 0.7 ^c	27.9 $\pm$ 4.3 ^d	11.3 $\pm$ 2.4 ^c	3.1 $\pm$ 0.6 ^d
DD eye ^b	297.7 $\pm$ 1.3 ^c	180.4 $\pm$ 1.5 ^d	233.6 $\pm$ 0.6 ^c	243.6 $\pm$ 1.4 ^d
DD hatch	433.4 $\pm$ 1.6 ^c	323.1 $\pm$ 1.9 ^d	468.8 $\pm$ 18.4	510.2 $\pm$ 2.4
% CR	3.39 $\pm$ 2.1 ^c	12.4 $\pm$ 1.7 ^d	10.3 $\pm$ 2.2 ^c	3.4 $\pm$ 1.5 ^d
% SK	6.3 $\pm$ 3.4 ^c	15.4 $\pm$ 3.5 ^d	1.7 $\pm$ 0.5	1.4 $\pm$ 0.5
% FF	1.5 $\pm$ 1.0 ^c	7.0 $\pm$ 1.9 ^d	1.6 $\pm$ 0.6	3.1 $\pm$ 1.2
% ED	13.3 $\pm$ 12.1 ^c	14.5 $\pm$ 3.4 ^d	1.5 $\pm$ 0.9	0.6 $\pm$ 0.1
MFL	19.9 $\pm$ 0.5 ^c	18.6 $\pm$ 0.3 ^d	16.9 $\pm$ 0.2 ^c	18.1 $\pm$ 0.2 ^d
MFW	52.6 $\pm$ 4.4 ^c	44.5 $\pm$ 2.8 ^d	32.7 $\pm$ 2.5	33.1 $\pm$ 1.0

^a number of fry to reach the swim-up stage

^b DD = degree-days, CR = craniofacial defects, SK = skeletal defects, FF = finfold defects, ED = edema, MFL = mean fry length, MFW = mean fry weight

ND = not done in both years of the study

Figure 3.1) Collection sites for rainbow and brook trout in McLeod River drainage of Alberta, Canada in 2001. Luscar Creek is the high Se site, Gregg River is an intermediate Se site and Deerlick and Wampus creeks are reference sites for rainbow trout and Cold Creek is a reference site for brook trout.

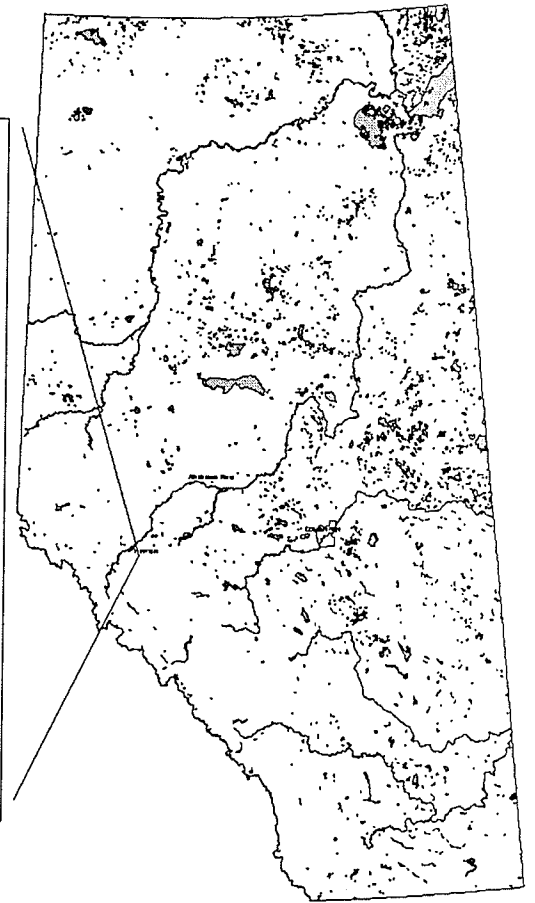
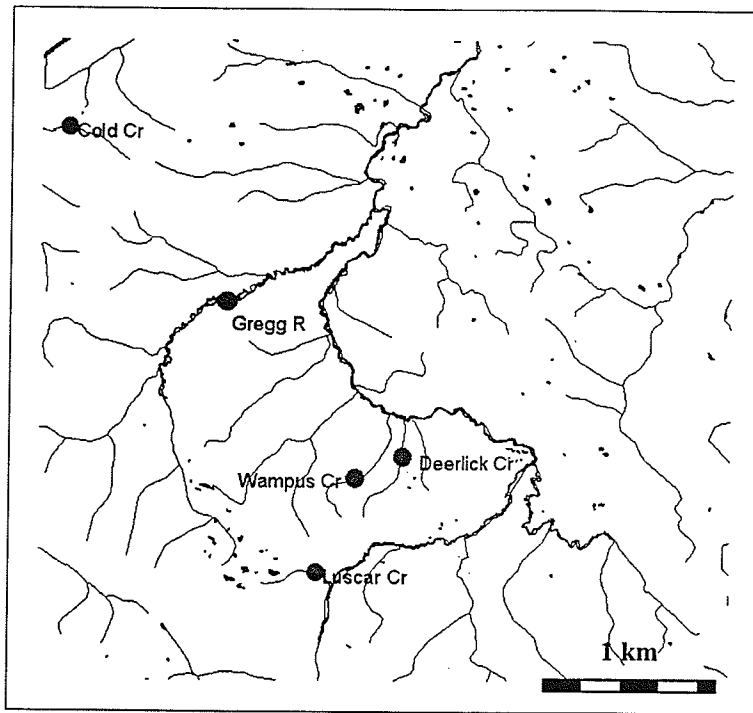


Figure 3.2) Correlation between muscle and egg Se concentrations in (A) rainbow trout and (B) brook trout collected from Se-exposed and reference sites in northeastern Alberta. Each symbol represents data from tissues obtained from one female.

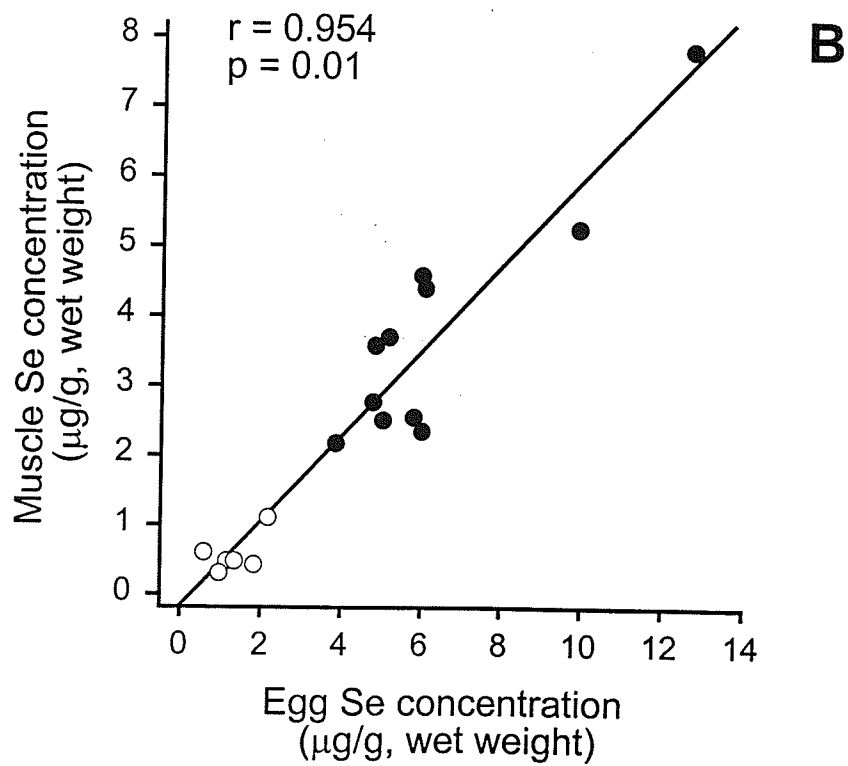
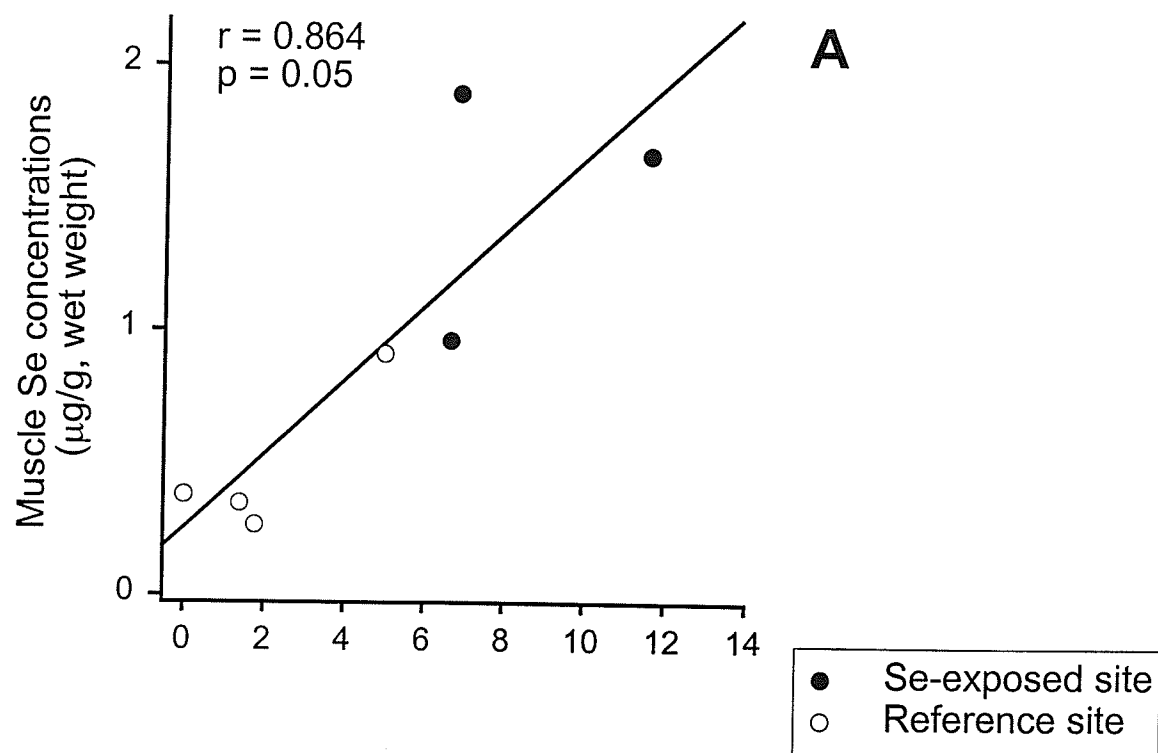
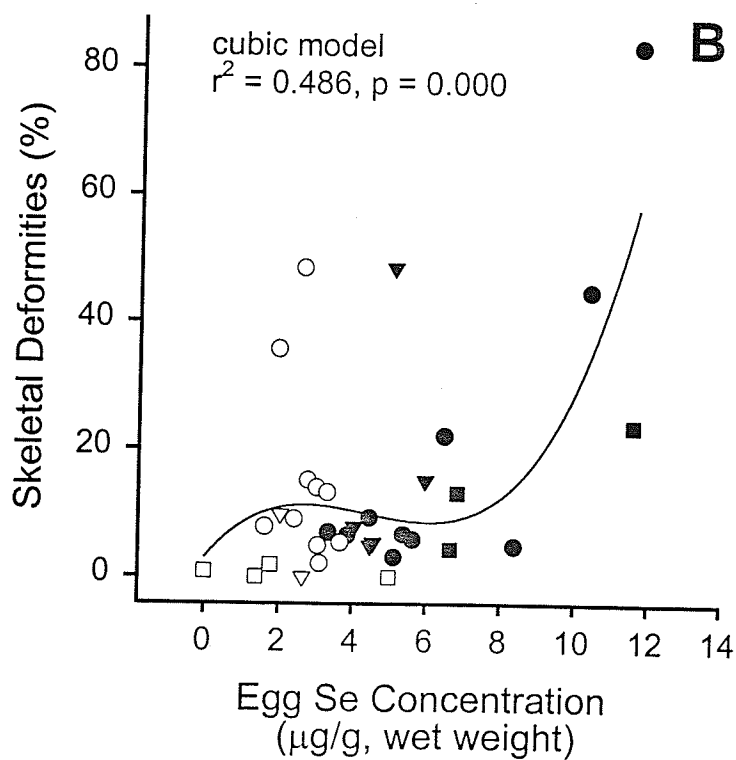
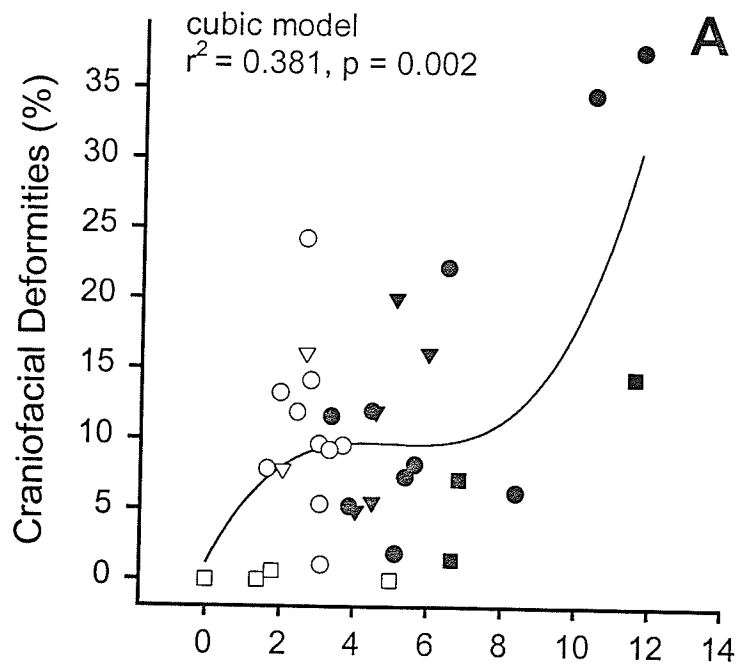


Figure 3.3) Relationship between Se concentrations in subsamples of eggs obtained from adult female rainbow trout collected over two consecutive years (2000 and 2001) from a high Se site (Luscar Creek), an intermediate Se site (Gregg River) and reference sites (Deerlick and Wampus creeks) and deformities in the offspring from each female in each of four categories: (A) craniofacial deformities, (B) skeletal deformities, (C) finfold deformities and (D) edema. Each symbol represents data from one female's offspring.



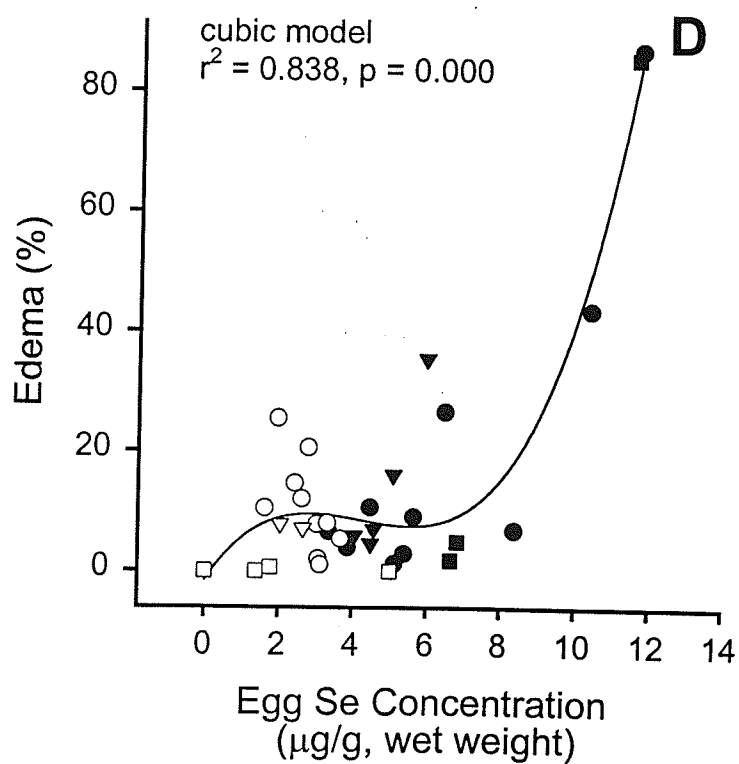
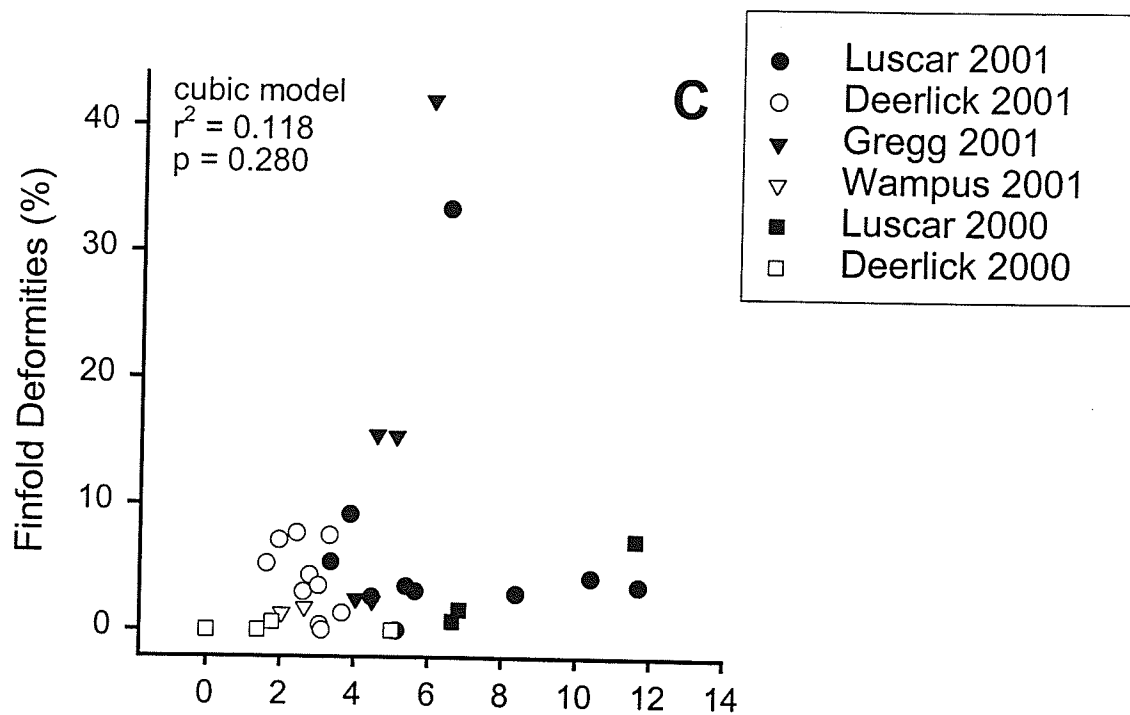
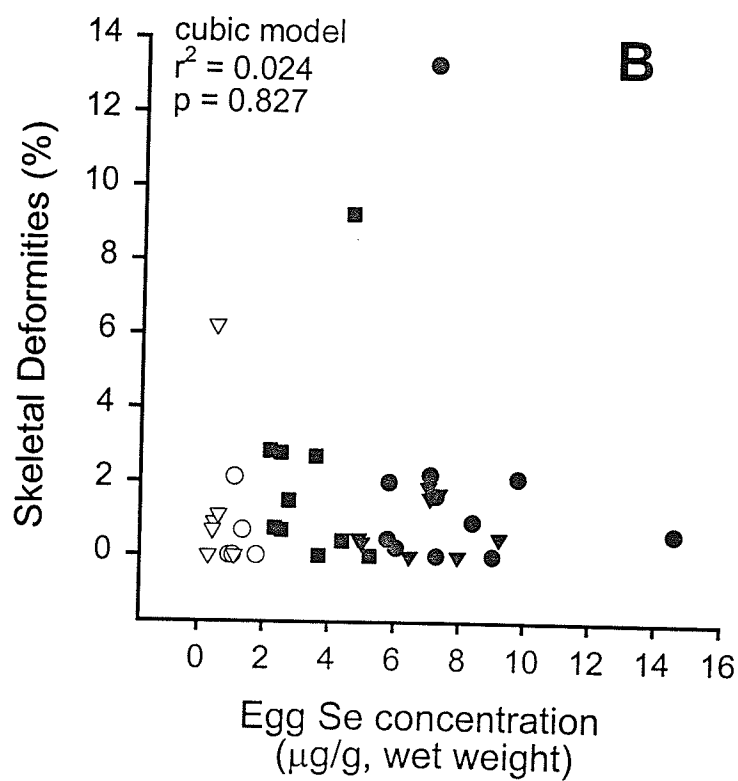
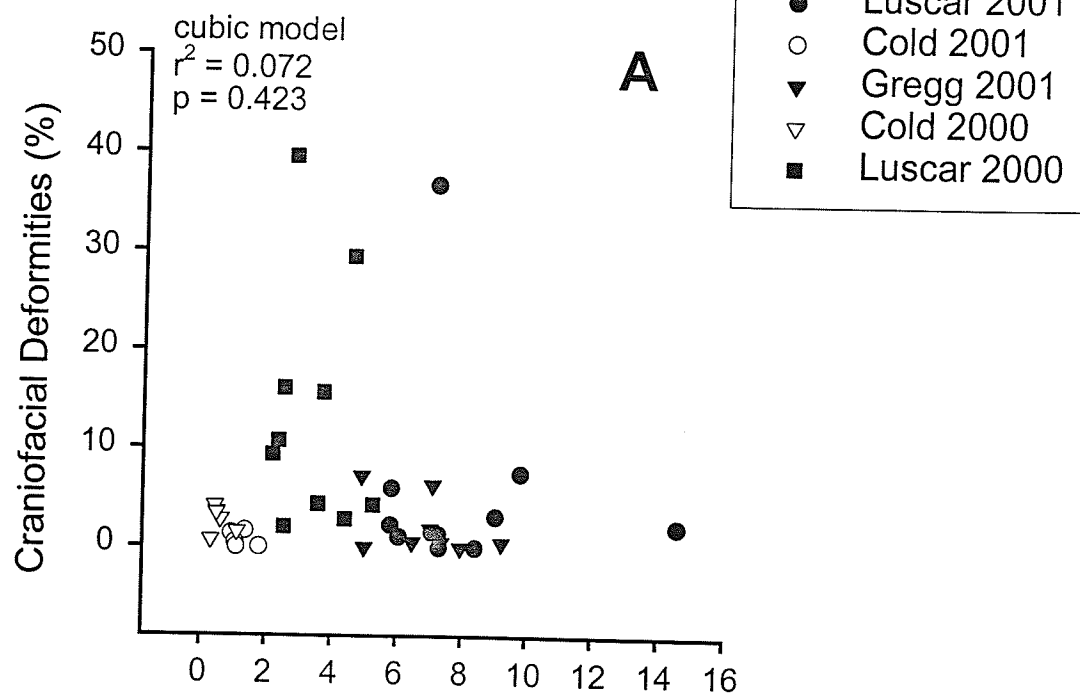
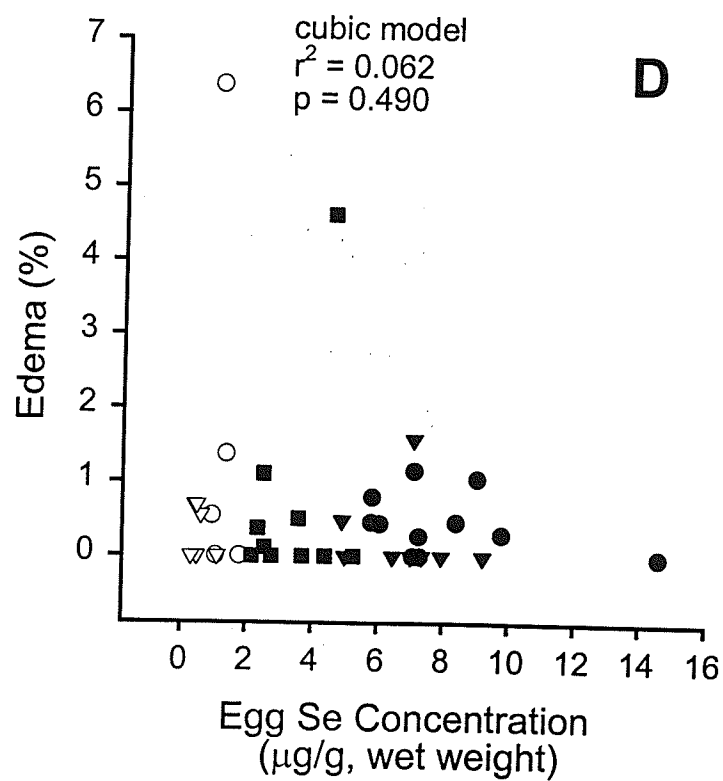
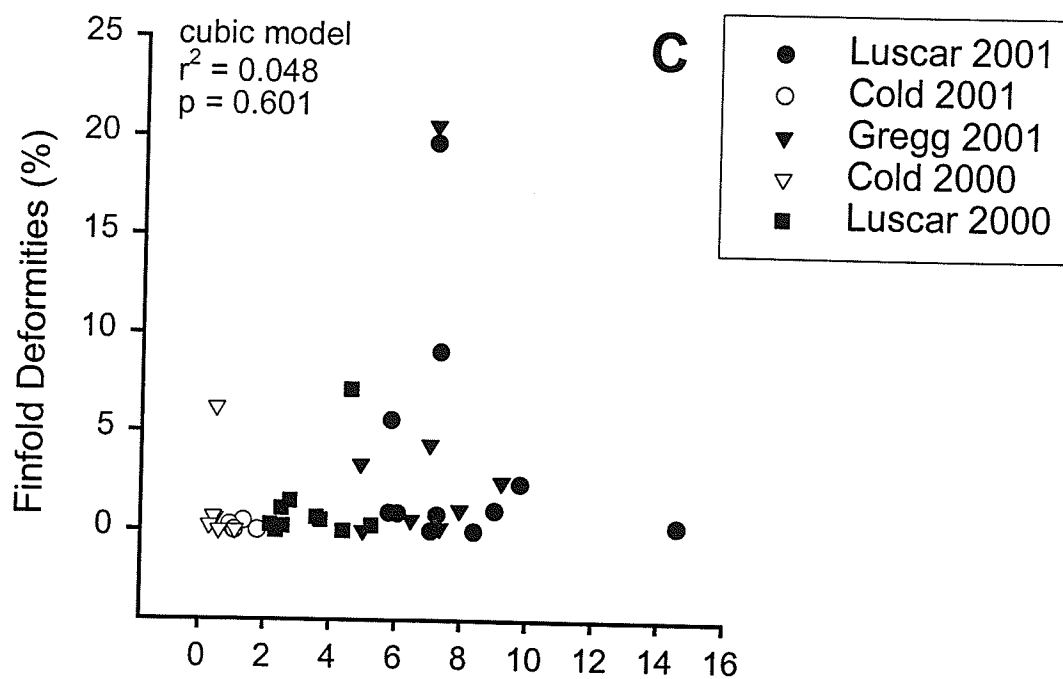


Figure 3.4) Relationship between Se concentrations in subsamples of eggs obtained from adult female brook trout collected over two consecutive years (2000 and 2001) from a high Se site (Luscar Creek), an intermediate Se site (Gregg River) and a reference site (Cold Creek) and deformities in the offspring from each female in each of four categories: (A) craniofacial deformities, (B) skeletal deformities, (C) finfold deformities and (D) edema. Each symbol represents data from one female's offspring.





Chapter 4

**Oxidative stress in laboratory reared rainbow trout fed environmentally relevant
concentrations of selenium**

4.1) Introduction

It has long been recognized that selenium plays a dual role as both an essential trace nutrient and as an environmental toxicant. Both Se deficiency as well as Se toxicity can cause adverse biological effects in fish and wildlife (O'Tool and Raisbeck 1998; Lemly 1996; Shamberger 1983). Nutritionally, Se is an essential component of the catalytic sites of many enzymes, including antioxidant enzymes such as glutathione peroxidase (reviewed in Spallholz and Hoffman 2002). However, whether Se acts as a protective antioxidant or as a potentially toxic prooxidant is dependent on the exposure concentration of Se.

Se-induced terata, such as the defects observed in the previous two chapters in the offspring of rainbow trout and brook trout inhabiting Se-rich waters, have primarily been attributed with the substitution of Se for sulfur during protein synthesis. However, another biochemical mechanism may also explain some of the adverse effects of exposure to elevated Se. Specifically, accumulating evidence has related Se toxicity with oxidative stress (Spallholz 1994). Oxidative stress occurs when reactive oxygen species (ROS) are generated in excess of cellular antioxidant capacity with subsequent oxidative damage of biological macromolecules (Saltman 1989). ROS are produced in all aerobic organisms; but under unperturbed circumstances cells contain enzymatic and non-enzymatic antioxidants, which normally scavenge ROS and prevent cellular damage (Kelly *et al.* 1998). Enzymatic antioxidants, such as superoxide dismutases, glutathione peroxidases and catalases, protect cells by metabolizing potentially damaging ROS (Winston and DiGiulio 1991). Non-enzymatic defenses include α -tocopherol (vitamin E), ascorbic acid (vitamin C), retinoids (vitamin A), and low molecular weight molecules

like glutathione and melatonin. These compounds quench ROS by reducing them or may also stabilize cellular components that have already been oxidized by ROS (Winston and DiGiulio 1991). The condition of oxidative stress and oxidative tissue damage occurs only when the concentration of ROS exceeds antioxidant defenses (Babich *et al.* 1993).

Oxidative damage due to Se metabolism arises when certain Se compounds redox cycle and generate superoxide ($O_2^{\bullet-}$) (Figure 4.1) (Spallholz 1994). Only selenium forms that generate the selenoate (RSe^-) ion are capable of this type of redox cycling. Although selenomethionine, the major dietary form of Se encountered by aquatic organisms (Fan *et al.* 2002), does not generate superoxide in an *in vitro* test system (Spallholz *et al.* 2001). It has been postulated that an endogenous enzyme may metabolize selenomethionine to produce methylseleninic acid (CH_3Se^-), a highly active redox catalyst (Spallholz and Hoffman 2002). To this point, the evidence for oxidative stress as a mechanism of Se toxicosis has primarily come from studies involving mammals and aquatic birds.

The objective of this study was to assess measures of oxidative stress, including non-enzymatic antioxidant concentrations, in the tissues of rainbow trout fed diets containing different environmentally relevant levels of selenomethionine. Se-related indices of oxidative stress in fish may serve as useful bioindicators in assessments of Se exposure and toxicity.

4.2) Materials and Methods

4.2.1) Experimental Diets

Four diets were prepared using 2 parts starter trout chow (Nelson and Sons Inc., South Murray, UT, USA), which contained 48% crude protein, 14% crude fat, 3% crude fiber,

12% ash, and 2% Na, and 1 part deionized distilled water (DDW) containing gelatin and selenomethionine (where applicable). Diets 1-3 contained nominal concentrations of 2, 10, and 20 $\mu\text{g Se/g (dw)}$ and diet 4 was the control to which no additional Se was added. Selenium was added to the diets as seleno-DL-methionine (Se-met; SIGMA Chemical Co., St Louis, MO, USA). Gelatin (Type A, 300 bloom; SIGMA) was added as a binding agent to an amount of 2% of the weight of the dry feed for the experimental and control diets. The dry feed and DDW were then mixed in a commercial stainless steel food preparation mixer. The moist paste was extruded through a No. 4 pellet size screen to produce noodles that were dried in a fan-ventilated chamber at room temperature and stored at -20°C until required for feeding. Analyses by ICP-MS showed that the Se-met supplemented diets contained Se concentrations ($\mu\text{g/g, dw}$) of 3.25 ± 0.23 (nominal 2 $\mu\text{g/g}$), 10.50 ± 0.15 (nominal 10 $\mu\text{g/g}$), and 19.97 ± 0.27 , while the control diet contained $1.59 \pm 0.28 \mu\text{g Se/g}$ (mean $\pm$ SE). With the exception of Se, there were no significant differences in the concentration of other elements in the diets (Table 4.1).

4.2.2) Fish

Three hundred juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained from Stevenson's Rainbow Springs Hatchery (Thamesford, ON). Initial body weights and fork lengths (mean $\pm$ SE) were $98.9 \pm 6.0 \text{ g}$ and $20.3 \pm 0.5 \text{ cm}$, respectively. Fish were acclimated to all experimental conditions for a period of nine weeks prior to feeding the experimental diets. During the acclimation period fish were fed a ration of No. 3 trout pellets (Martin Feed Mills, Elmira, ON) equal to 0.5% of the total body weight per day, six times per week.

4.2.3) Tanks

Rainbow trout were randomly distributed, 25 fish per tank, into 12 fiberglass tanks (200 L). The tanks received dechlorinated Winnipeg City tap water supplied at a rate of approximately 1 L/min maintained at or below 10 ppb Cl_2 by activated charcoal filtration and ozonation (Waggeman *et al.* 1987). Dissolved oxygen was monitored on a weekly basis and the aeration of each tank was individually adjusted to ensure a greater than 80% saturation of dissolved oxygen. During the experiment, water temperatures were maintained between 10.4 and 12.0°C and pH was maintained between 7.27 and 7.65. The photoperiod was controlled by a timed lighting system and was adjusted to the ambient photoperiod in Winnipeg weekly, with a 30-minute period of 'dusk' and 'dawn' with intermediate light intensity. Uneaten food and feces were flushed from the tanks daily via a central stand pipe, which was thoroughly cleaned weekly.

4.2.4) Exposure Procedures

Each tank was randomly assigned to one of four treatment groups, consisting of one of the four dose groups (control, 2, 10, and 20 $\mu\text{g Se/g}$). Thus, each dose group had three replicate tanks. Commencing October 23, 2000 rainbow trout were fed the experimental diets at 0.8% bdwt. 6 times a week for 302 days. Fish were weighed and measured on days 80, 120 and 302 of the exposure period, as well, tissues from seven sacrificed fish from each tank were taken on days 120 and 302.

4.2.5) *Sampling*

On each sampling day, all fish were individually anaesthetized with buffered (pH=7.0) solution of tricaine methane sulfonate (MS-222; SIGMA) at a concentration of 0.4 g MS-222/L until fin movement ceased (~ 3 min). After being weighed and measured, blood was drawn from the caudal vein of each fish using nonheparized 23-gauge needle (120 day sampling) or a 22-gauge needle (302 day sampling) and 10 ml syringe. Blood samples from the 120 day period were then transferred to non additive Vacutainers to obtain serum, whereas blood from the 302 day samples was divided into two aliquots and transferred to Vacutainers containing either EDTA anticoagulant to obtain plasma or clot activator to obtain serum. Serum samples were clotted on ice. All samples were centrifuged at $3000\times g$ for 10 minutes and the serum and plasma was removed and stored at -90°C until analysis. Immediately following blood withdrawal, livers and kidneys were removed for Se and biochemical analyses. The liver and kidney were dissected from the carcass and stored at -90°C until they were analyzed.

4.2.6) *Organ and whole organism measurements*

Liver somatic indices (LSI) were calculated using the formula: $(\text{liver weight}) / (\text{body weight} - \text{liver weight}) \times 100$. Gonado-somatic indices (GSI) were calculated using the formula: $(\text{gonad weight}) / (\text{body weight} - \text{gonad weight}) \times 100$. Condition factor (CF) was calculated using the formula: $[\text{body weight (g)}] / [\text{fork length}^3 \text{ (cm)}] \times 100$.

4.2.7) *Analysis of Se and other elements*

Food samples and livers of rainbow trout were analyzed for Se, Al, Sb, Ba, Be, Bi, B, Cd, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Hg, Mo, Ni, Ag, Sr, Tl, Th, Sn, Ti, U, V, Zn using ICP-MS (Feng *et al.* 1999).

4.2.8) *Blood Electrolytes*

Blood ion concentrations (Na^+ , K^+ , and Cl^-) were measured in thawed serum samples. Na^+ and K^+ concentrations were measured using an Instrumentation Laboratory 943 flame photometer (Instrumentation Laboratory Company, Milano, IT). Cl^- concentrations were measured using a Corning Chloride Analyzer 925 (Corning Medical and Scientific, Medfield, MA).

4.2.9) *Vitamin Analysis*

A reverse-phase HPLC method (Palace and Brown 1994) was used to quantify the concentration of retinoids (Vitamins A compounds) and tocopherol (Vitamin E) in serum (120 day sampling) and plasma (302 day sampling).

4.2.10) *Glutathione Analysis*

Hepatic glutathione concentrations were determined using a modified version of two established glutathione methods (Aréchiga and Hansen 2001; Baker *et al.* 1990). Briefly, liver samples were prepared by homogenizing 0.2g of tissue in 2 ml of 5% sulphosalicylic acid (SSA), to deproteinate the samples. The samples were then centrifuged at $10\,000 \times g$ for 15 minutes at 4°C . After centrifugation, the supernatant was

collected and stored at -20°C prior to being assayed. Samples for total glutathione (GSH) were diluted 1 in 100 with DDW and those for oxidized glutathione (GSSG) were diluted 1 in 10 with DDW. Standards for the total GSH assay were made using glutathione in the reduced form, while standards for the oxidized GSSG assay were made using glutathione in the oxidized form. Stock solutions of 0.04 M GSH or GSSG were made up in SSA and were serially diluted with DDW to make standards containing from 1.56 to 200 pmol of GSH or GSSG. All reagents for the glutathione assay were purchased from SIGMA (St. Louis, MO). The assay is an enzymatic recycling method, using glutathione reductase. Glutathione in the assay reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) to produce the yellow colored TNB (5-thio-2-nitrobenzoic acid). Because both GSSG and GSH produce the same rate of DTNB reduction, 300 μL of the supernatant for GSSG analysis was incubated with 6 μL of 2-vinylpyridine and 7 μL of triethanolamine for 1 hour to conjugate GSH. Fifty μL of standard or sample was added in triplicate to designated wells of the microtiter plate. One hundred μL of freshly prepared reaction mixture (consisting of 5 mL DTNB, 5 mL NADPH, 5.75 mL of 100 mM sodium phosphate buffer with 1mM EDTA, and 0.1 mL of GSH reductase) was then added to each well using an eight channel pipettor. Immediately following, the absorbance of the wells was measured at 405 nm using an EL_x 800 Universal Microplate Reader (Bio-tek Instruments, Winooski, VT) for 10 minutes at 5 minute intervals.

4.2.11) Statistical Analysis

All statistical analyses were conducted with SPSS version 10.0 (SPSS Inc. 1999). One-way ANOVAs were used to test for differences between fish fed control and Se-

contaminated diets. Separate ANOVAs were conducted for each sampling time (120 days or 302 days). If results from the ANOVA were significant ($P \leq 0.05$), Dunnett's multiple comparison test was used to identify significant differences between control and treated fish. Regression analyses were used to test for relationships between Se concentrations accumulated in the liver and other biochemical endpoints at each sampling time. Because Se and glutathione analyses were not performed on the same fish, average values for each dose group were used for the regression analyses. An alpha value of 0.05 was used for all statistical analyses.

4.3) Results

4.3.1) Morphological indices

No change in feeding behavior was observed among treatment groups, with all food pellets being consumed within 5 minutes of feeding. Consumption of Se enriched diets for a 302-day period did not have a significant effect on the weight, length or condition factor (CF) of rainbow trout (Table 4.2). Growth of fish fed elevated levels of Se, calculated as the change in weight from the initial body weight at each of the sampling times, was not significantly different from that of the controls (Figure 4.2). There were small but statistically significant differences in the liver somatic indices (LSI) of trout fed the 2 and 20 μg Se/g diets at the 120-day sampling period. Gonado-somatic indices (GSI) were consistent among the treatment groups and the control (Table 4.2). However, mean GSIs were much higher in fish sampled after 302 days than in those fish sampled after 120 days, indicating that the fish were sexually maturing.

4.3.2) *Se accumulation*

Exposure of rainbow trout to the medium and high dose diets for 120 and 302 days resulted in a significant accumulation of Se in the liver compared to the control group (Figure 4.3). Although the concentration of Se in the livers of fish fed the low dose diet were not significantly different from those of the control, an association between the accumulation of Se in the liver and the dose of Se in the diet was evident.

Although Se was the only element to differ significantly among the Se-laden diets and the control diet, the concentration of several other elements in the livers of Se-exposed fish varied significantly from that of the control group (Table 4.3). Except for Se, the pattern of accumulation of these elements was not consistent. Cadmium (Cd), mercury (Hg) and thallium (Tl) were the only elements that differed significantly at both sampling times. Of these, only thallium showed a consistent pattern of accumulation, decreasing with increasing dietary Se levels. Cadmium exhibited an inconsistent accumulation pattern, decreasing with increasing Se levels after 120 days of exposure and increasing with increasing Se levels after 302 days of exposure. Similarly, mercury increased with increasing Se levels after 120 days of exposure, but showed no clear pattern of accumulation after 302 days.

4.3.3) *Blood electrolytes*

Serum ion levels (Na, K, Cl) were not significantly different in fish fed Se-contaminated diets compared to those fish fed the control diet (Figure 3.4). Sodium and chloride concentrations were also not different between dose groups at both sampling periods. Potassium levels were variable after 120 days of exposure, but were not

statistically different among dose groups. By 302 days of exposure, the potassium levels of all dose groups appeared to increase and variability was reduced.

Of the blood ions measured in rainbow trout fed Se-laden diets or a control diet, only sodium in the serum of fish sampled at day 302 showed a significant relationship (quadratic model, $p = 0.044$) with the concentration of Se accumulated in the liver (Figure 4.5). However, this relationship had a very low coefficient of determination ($r^2 = 0.229$).

4.3.4) *Vitamin concentrations*

No significant difference was observed in the levels of tocopherol or retinol in the serum or plasma of rainbow trout fed Se-laden diets compared to the control groups (Figure 4.6). Mean levels of retinol appeared constant in serum samples from fish sampled after 120 days as in plasma samples from fish sampled after 302 days. However, mean tocopherol levels in plasma sampled after 302 days appeared higher than those in serum sampled after 120 days. At day 120, lowest mean levels of retinol were observed in rainbow trout fed the medium dose diet, whereas tocopherol concentrations were lowest in fish fed the medium and high dose diets. By day 302 of exposure, lowest mean levels of both tocopherol and retinol were observed in fish from the highest dose group. Tocopherol levels in the plasma of rainbow trout sampled at day 302 showed a significant relationship (linear model, $p = 0.031$) to the concentration of Se accumulated in the liver (Figure 4.7). However, hepatic Se concentrations explained very little of the variation in plasma tocopherol levels ($r^2 = 0.174$).

4.3.5) *Glutathione concentrations*

Concentrations of reduced glutathione (GSH) in the livers of rainbow trout fed Se-laden diets were not significantly different from the control groups after 120 or 302 days of exposure (Figure 4.8A). However, significantly higher levels of the oxidized disulfide dimer form (GSSG) and an increased ratio of GSSG:GSH were observed in rainbow trout from the highest dose group at both sampling times (Figure 4.8A and C). At day 120, the lowest mean levels of GSSG and GSSG:GSH in the liver occurred in fish from the 2 μ g Se/g diet. By day 302, mean GSSG concentrations and GSSG:GSH increased with increasing concentration of Se in the diet.

Both the mean concentration of GSSG and ratio of GSSG:GSH in the liver of rainbow trout showed a strong relationship to the level of Se accumulated in the liver at both sampling times. Both GSSG concentration and GSSG:GSH measured at 120 days of exposures were best described by a quadratic model ($p = 0.029$ and 0.024 , respectively), with an inflection point occurring near 14 μ g Se/g (Figure 4.9). However, after 302 days of exposure, they were best described by a linear model ($p = 0.038$ and 0.039 , respectively) (Figure 4.10).

4.4) **Discussion**

Although feed refusal has been reported in a number of dietary Se exposure studies (Hilton *et al.* 1980; Hilton and Hodson 1983; Woock *et al.* 1987; Ogle and Knight 1989), the rainbow trout in the current study did not show any signs of decreased food consumption or altered feeding behavior in response to elevated levels of Se in the diet. In contrast to this investigation, the form of selenium used in many of the studies that

reported refusal was sodium selenite. As well, food refusal in these previous studies was often not observed until dietary doses exceeded 20 µg Se/g.

Rainbow trout fed seleno-DL-methionine-laden diets were found to accumulate Se in the liver in a dose dependent manner. This pattern of accumulation has been reported in dietary Se exposure studies involving a number of other species including bluegill sunfish (*Lepomis macrochirus*) (Coyle *et al.* 1993), fathead minnows (*Pimephales promelas*) (Ogle and Knight 1989), chinook salmon (*Oncorhynchus tshawytscha*) (Hamilton *et al.* 1986) and Atlantic salmon (*Salmo salar*) (Lorentzen *et al.* 1994).

In the present study, Se was not the only element to occur at different concentrations in the livers of fish from different exposure groups. Because Se was the only element to differ in dose among the diets, however, differences in liver concentrations must reflect active regulation by the fish or elemental interactions with Se rather than differences in exposure concentrations. Silver, copper, thallium and selenium were the only elements that were accumulated in the liver to concentrations greater than in the diet (i.e. bioaccumulated). Of these elements, only Se and Tl concentrations were significantly different between fish fed Se-laden diets and fish fed the control diet, although only Se showed a consistent pattern at both sampling times.

The most extensively studied elemental interaction with selenium is that of mercury (reviewed in Cuvin-Aralar and Furness 1991). Although Hg concentrations did not show a consistent pattern of accumulation in the livers of fish across the different dose groups, some of the mechanisms postulated for this specific interaction in the review may help to explain how varying Se concentrations could affect the accumulation of other elements. It is possible that increasing Se levels could modify the distribution of certain elements

amongst organs and tissues. Another possible reason is that Se could compete with certain elements for binding sites, such as those on carrier proteins, which could then affect the rate of uptake, retention, or elimination of the element (Cuvin-Aralar and Furness 1991).

Reduced growth as a result of dietary Se exposures have been previously observed for a number of freshwater fish and waterfowl (Hilton *et al.* 1980; Hilton and Hodson 1983; Heinz *et al.* 1987; Woock *et al.* 1987; Heinz *et al.* 1988; Ogle and Knight 1989; Hamilton *et al.* 1990; Hoffman *et al.* 1992a; Albers *et al.* 1996). However, exposure of rainbow trout to Se-laden diets in the current study did not have any effect on the growth, condition or relative organ size. There are a number of potential reasons for the differences observed in the response of morphological measurements among these studies, including the form of Se, the life stage of the test organism, the dose of exposure, and species specific differences. For example, the form of Se incorporated into the exposure diets of many of the studies that reported a decrease in growth was selenite (Hilton and Hodson 1980; Hilton *et al.* 1983; Heinz *et al.* 1987; Heinz *et al.* 1988; Ogle and Knight 1989). Se in the selenite form is much more toxic to fish and wildlife than organic forms such as selenomethionine (Spallholz and Hoffman 2002). Coyle *et al.* (1993) also observed no effect on the morphological measurements (length, weight, CF or GSI) of bluegill sunfish (*L. macrochirus*) exposed to dietary seleno-L-methionine in combination with waterborne selenate and selenite. Selenomethionine and not selenite is most commonly encountered by organisms in the aquatic environment (Fan *et al.* 2002). Selenomethionine has been shown to be a good model for naturally incorporated Se in food chains (Hamilton *et al.* 1990).

Early life stages are often the most sensitive to toxicant stresses (Rand and Petrocelli 1985). In many of the studies in which growth was affected by Se exposure, the organisms were exposed either as swim-up fry (Hamilton *et al.* 1990) or as ducklings (Heinz *et al.* 1988; Hoffman *et al.* 1992a). Whereas with environmental exposures, selenium is incorporated in the yolk and is assimilated during the most active phase of protein synthesis. Where more mature organisms showed reduced growth (Heinz *et al.* 1987; Albers *et al.* 1996), the dose often exceeded 20 µg Se/g, the maximum dose used in the current study.

Another indicator of fish health is the level of serum ions (Na, K and Cl). Changes in serum ion levels can indicate potential gill or other organ damage (Folmar 1993). In the current study, exposure of rainbow trout to elevated levels of dietary seleno-DL-methionine was found to have no effect on the sodium, potassium or chloride serum concentrations and all concentrations were within ranges for reference or control fish (Folmar 1993). Unlike chloride and sodium, potassium has been reported to show seasonal differences in serum levels. Laidley and Leatherland (1988) found that juvenile rainbow trout had the highest levels of serum K in November and the lowest in February. Correspondingly, in the current study, rainbow trout sampled at day 302, on August 20th, 2001, showed an apparent increase in serum K values compared to those sampled at day 120, on February 19th, 2001.

The only other study to examine the serum ion concentrations of fish inhabiting Se-contaminated waters is that of Lohner *et al.* (2001). The authors collected bluegill sunfish (*L. macrochirus*) from sites receiving coal ash effluent and reference sites in Ohio and found that blood ion levels were increased in fish from the impacted sites. However

it is important to note that concentrations of heavy metals, including arsenic, chromium and copper, were also elevated in fish from the exposed sites. Heavy metals can affect ionic regulation in fish by disrupting the brachial epithelium of the gills or by affecting ion-transporting enzymes such as Na^+/K^+ ATPase (Skidmore and Tovell 1972; Versteeg *et al.* 1988).

Hoffman's (2002) recent review of oxidative stress as a mechanism for the generation of Se-induced biological lesions in aquatic birds observed these studies. To date, the link between oxidant stress and selenium toxicity has not been investigated in fish populations. To address this knowledge gap, the concentration of non-enzymatic antioxidants in rainbow trout exposed to elevated levels of Se in the diet were examined.

Altered antioxidant concentrations of lipid soluble vitamins can be a useful measure of oxidative stress but these have not been measured in studies examining the effects of excess Se on fish and aquatic birds (Palace *et al.* 1998). No significant alterations in antioxidant vitamin concentrations were observed in the blood of rainbow trout fed increased doses of Se. Although not statistically significant, the concentrations of tocopherol and retinol were lowest in the high dose group in the August sampling. Lower vitamin concentrations in the high dose group could represent depletion due to the increased use of tocopherol and retinol as antioxidants; protecting cellular components from oxidative damage resulting from excess dietary Se. It may be that only after 302 days of exposure at the highest Se dose did rainbow trout accumulate sufficient body burdens of Se for Se to exert prooxidant activities. In addition, plasma tocopherol concentrations appeared marginally higher in the August sampling for all dose groups compared to the earlier sampling time. A slight increase in plasma tocopherol levels

could reflect an early exposure phase transport of tocopherol from stores in the liver and kidney to other oxidatively stressed tissues (Palace *et al.* 1996b). Since plasma represents a relatively small proportion of the overall organism's vitamin complement, concentrations in storage organs like the liver and kidney should be examined for potential depletions.

Previous field and laboratory studies have reported manifestations of oxidative stress, including alterations in hepatic glutathione metabolism, in aquatic birds exposed to elevated Se levels (reviewed in Hoffman 2002). Glutathione is an important non-enzymatic antioxidant as well as a cofactor for antioxidant enzymes. It is found at high intracellular concentrations. Glutathione depletion has been associated with lesions in the liver and other organs, with tissue damage often occurring where polyunsaturated lipid concentrations are highest or antioxidant defenses are insufficient (Levine and Kidd 1985).

In feeding studies similar to the current study, mallard ducks (*Anas platyrhynchos*) exposed to elevated levels of selenomethionine as both ducklings (Hoffman *et al.* 1989; 1992a; 1992b; 1996) and adults (Fairbrother and Fowles 1990; Hoffman *et al.* 1991) showed increased plasma and hepatic glutathione peroxidase (GPx) activity as dietary and tissue Se concentrations increased. GPx protects cellular membranes from cytotoxic products of lipid peroxidation by using the reducing power of glutathione (GSH) to reduce lipid hydroperoxides to hydroxy fatty acids (Günzler and Flohé 1986). The mallard studies also demonstrated a dose dependent increase in the hepatic ratio of oxidized glutathione (GSSG) to the reduced form (GSH), as well as an increase in hepatic lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS). The

authors attributed the increase in GSSG:GSH to both an increase in oxidized glutathione (GSSG) and a depletion of the reduced form (GSH).

Similar increases in hepatic GSSG and the ratio of GSSG:GSH were observed in rainbow trout in the current study. However, unlike the mallard studies, no alterations in the levels of the reduced form (GSH) were observed. Therefore, the only factor that can account for the increase in the ratio of GSSG:GSH in the rainbow trout liver is the increase in GSSG. As with the mallard studies, increases in GSSG and GSSG:GSH were dose dependent, showing a strong relationship with hepatic Se concentrations ($r^2 > 0.924$) at both sampling times. However, the mathematical function that best described these relationships was not consistent between sampling times. This difference is likely related to the increased concentrations of Se accumulated by the trout by the later sampling date. The data collected in 120-day best fit a quadratic model, with an inflection point of about 15 $\mu\text{g Se/g}$, while the 302-day data was best described by a linear model. The 302-day data may have appeared linear because hepatic Se concentrations at this sampling time often exceeded 15 $\mu\text{g/g}$, and thus are only representative of the portion of the quadratic curve used to describe 120-day data after the inflection point, which is approximately linear. Data from this experiment indicate that oxidized glutathione and the ratio of oxidized to reduced glutathione may be sensitive indicators of oxidative stress resulting from Se exposure in fish, as previously demonstrated for aquatic birds.

Counter intuitively, many field studies have reported no apparent effects in adult fish inhabiting seleniferous waters in spite of elevated levels of Se in fish tissue that often exceed published toxic effect threshold values (McDonald and Stroscher 1998; Casey and Siwik 2000; Kennedy *et al.* 2000; Lohner *et al.* 2001). This was the case for rainbow

trout and brook trout populations inhabiting Se-laden waters downstream from active coal mining presented in the previous two chapters. However, in light of the findings of the current lab study, the lack of toxic effects in adult fish exposed to elevated Se concentrations naturally in their diet in contaminated habitats is not unexpected if oxidative stress is a key mechanism in the etiology of Se toxicosis. Despite elevated hepatic Se concentrations, the rainbow trout in this study showed little to no variation in growth, condition or biochemical tissue characteristics. Exposure to the highest dietary dose (20 $\mu\text{g Se/g}$) did result in a significant increase of approximately 25% in hepatic GSSG concentrations. This increase is only moderate when compared to previous studies that have reported dose-dependent increases of oxidized glutathione in mallards by as much as 240% (Fairbrother and Fowles 1990; Hoffman *et al.* 1989; 1991; 1992a; b; 1996). These studies also reported a 25% decrease in GSH, as well as other Se-related toxic effects not observed in the current study. Moreover, even the modest increases in GSSG concentrations in the current study were associated with hepatic concentrations of Se (35-110 $\mu\text{g/g}$) which exceeded concentrations observed in the apparently healthy adult brook trout (13 $\mu\text{g/g}$) collected from Se-impacted habitats in the preceding chapters.

However, laboratory studies with selenomethionine have linked Se-related oxidative stress with teratogenesis and embryotoxicity in aquatic birds at lower body burdens than those required to induce subchronic effects in adult birds (Heinz *et al.* 1987; 1989; Hoffman and Heinz 1988). Malformations reported in these studies, including hydrocephaly, microphthalmia, lower bill defects, and foot defects with ectrodactyly (the absence of phalanges), are comparable to the types of deformities observed in the offspring of the evidently healthy adult rainbow trout from Chapters 2 and 3. Thus, it

would appear that Se-methionine is more toxic to developing embryos than to adult organisms. Embryos may be more susceptible to oxidative tissue damage, which could ultimately result in reduced hatching or deformities, because detoxification, cytoprotection and repair processes, which normally occur in the adult liver and other tissues, may be immature or otherwise compromised (Hoffman 2002). For example, antioxidant enzyme activity only develops relatively late in embryonic development (Cowey *et al.* 1985). Therefore it is possible that the rainbow trout in this study, though showing no obvious symptoms of Se-toxicosis, may have accumulated sufficient concentrations of Se to generate oxidative stress in future offspring, which could result in teratogenesis and ultimately in a decrease in reproductive fitness. Additional research is required to clarify the role of oxidative stress in the etiology of Se-related terata in the sensitive embryonic stage.

Table 4.1) Mean trace element concentrations ($\mu\text{g/g}$, ww) in the four treatment diets.

element	Diet			
	control	low	medium	high
Ag	0.015	0.0151	0.0128	0.0113
Al	90.3	90.6	95.6	85.4
As	2.71	2.69	2.54	2.67
B	1.98	1.64	1.95	1.27
Ba	4.05	4.14	4.28	4.00
Be	<0.1	<0.1	0.05	<0.1
Bi	0.0054	0.0037	0.0038	0.0025
Ca	16559	16809	17473	16295
Cd	0.274	0.282	0.273	0.276
Co	0.0790	0.0852	0.0881	0.0841
Cr	0.831	0.813	0.993	0.824
Cu	7.733	7.793	7.963	7.753
Fe	672	663	669	669
Hg	0.0926	0.0970	0.08	0.0817
Li	0.266	0.270	0.338	0.304
Mg	705	694	698	660
Mn	42.0	42.1	42.1	40.1
Mo	0.212	0.221	0.225	0.2
Ni	0.401	0.307	0.358	0.275
Pb	0.459	0.403	0.466	0.437
Sb	0.0462	0.0397	0.0438	0.0466
Se	1.593	3.253*	10.5*	19.967*
Sn	1.163	1.927	1.803	1.943
Sr	58.933	61.7	63.8	60.533
Th	0.0596	0.0387	0.0340	0.0278
Ti	21.267	21.533	23.833	21.367
Tl	0.0066	0.00797	0.0062	0.00617
U	0.0548	0.0489	0.055	0.049
V	0.692	0.707	0.761	0.722
Zn	153	155	158	152

* represents significant differences ($P < 0.05$) between the control and the treatment groups for each sampling time.

Table 4.2) Mean somatic measurements of rainbow trout fed a control diet and three Se contaminated diets (mean $\pm$ SE).

	Weight (g)	Length (cm)	CF ^a	LSI ^b	GSI ^c
Day 80					
Control	230.0 $\pm$ 11.0	25.9 $\pm$ 0.4	1.25 $\pm$ 0.02	NT	NT
Low	227.9 $\pm$ 9.9	25.8 $\pm$ 0.4	1.26 $\pm$ 0.01	NT	NT
Medium	239.1 $\pm$ 8.11	26.5 $\pm$ 0.3	1.23 $\pm$ 0.01	NT	NT
High	232.9 $\pm$ 8.6	26.5 $\pm$ 0.3	1.20 $\pm$ 0.02	NT	NT
Day 120					
Control	299.2 $\pm$ 13.1	27.9 $\pm$ 0.4	1.30 $\pm$ 0.02	0.817 $\pm$ 0.06	0.184 $\pm$ 0.1
Low	289.1 $\pm$ 12.0	27.7 $\pm$ 0.4	1.29 $\pm$ 0.02	0.996* $\pm$ 0.03	0.060 $\pm$ 0.01
Medium	304.2 $\pm$ 9.4	28.3 $\pm$ 0.3	1.31 $\pm$ 0.04	0.862 $\pm$ 0.06	0.070 $\pm$ 0.01
High	303.2 $\pm$ 10.0	28.4 $\pm$ 0.3	1.28 $\pm$ 0.02	1.003* $\pm$ 0.03	0.075 $\pm$ 0.01
Day 302					
Control	701.1 $\pm$ 25.5	36.8 $\pm$ 0.5	1.37 $\pm$ 0.01	0.934 $\pm$ 0.05	0.879 $\pm$ 0.5
Low	668.2 $\pm$ 24.4	36.4 $\pm$ 0.4	1.36 $\pm$ 0.01	0.976 $\pm$ 0.05	1.176 $\pm$ 0.4
Medium	645.2 $\pm$ 18.0	36.0 $\pm$ 0.4	1.36 $\pm$ 0.01	0.956 $\pm$ 0.04	0.900 $\pm$ 0.3
High	684.1 $\pm$ 19.3	36.8 $\pm$ 0.3	1.36 $\pm$ 0.01	0.953 $\pm$ 0.05	0.994 $\pm$ 0.4

^a CF = condition factor = weight (g) / length (cm)³ $\times$ 100

^b LSI = liver somatic index = liver weight / (body weight – liver weight) $\times$ 100

^c GSI = gonado-somatic index = gonad weight / (body weight – gonad weight) $\times$ 100

NT = not taken

* represents significant differences ($P < 0.05$) between the control and treatment groups for each sampling time

Table 4.3) Mean trace element concentration ($\mu\text{g/g}$, ww) in the livers of rainbow trout fed diets containing nominal concentrations of 0 $\mu\text{g Se/g}$ (C), 2 $\mu\text{g Se/g}$ (L), 10 $\mu\text{g Se/g}$ (M), or 20 $\mu\text{g Se/g}$ (H).

element	Day 120				Day 302			
	C	L	M	H	C	L	M	H
Ag	0.0545	0.0560	0.0598	0.0530	0.133	0.101	0.134	0.139
Al	0.439	0.3462	0.547	0.301	1.100	0.248	0.585	3.676*
As	0.251	0.276	0.175	0.170	0.171	0.211	0.150	0.134
B	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Ba	<0.01	<0.01	<0.01	<0.01	0.0093	0.0077	0.0833	0.0180
Be	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Bi	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Ca	55.0	77.0	61.8	57.2	59.9	58.9	52.4	51.5
Cd	0.0477	0.0341*	0.0240*	0.0187*	0.0288	0.0469*	0.0412*	0.0430*
Co	0.0114	0.0089	0.0054*	0.0056*	0.0073	0.0125	0.0121	0.0121
Cr	0.0423	0.0429	0.0607	0.0623	0.0503	0.0369	0.0621	0.0318
Cu	48.0	74.4	67.7	55.2	84.8	68.1	118.6	167.0*
Fe	124.2	125.0	142.4	108.3	284.7	260.2	331.2	343.9
Hg	0.0277	0.0209	0.0428*	0.0408*	0.0524	0.0340*	0.0700*	0.0478
Li	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Mg	184.3	205.5*	191.7	197.7	200.3	199.3	206.3	185.1
Mn	1.241	1.211	1.205	1.170	1.161	1.010	1.261	0.978
Mo	0.219	0.259	0.244	0.244	0.219	0.225	0.238	0.213
Ni	0.0613	0.0458	0.0576	0.0296	0.0201	0.0101	0.0211	0.0331
Pb	0.0118	0.0196	0.0050	0.0067	0.0053	0.0085	0.0190	0.0161
Sb	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Se	2.47	6.81*	23.80*	34.66*	4.1	15.3*	53.0*	110.8*
Sn	0.0337	0.0225	0.0164	0.0238	0.0131	0.0126	0.0210*	0.0173
Sr	0.0632	0.213	0.0837	0.0507	0.0948	0.0714	0.0703	0.1924
Th	0.0019	0.0046	0.0024	0.0016	0.0018	0.0017	0.0020	0.0018
Ti	4.80	5.18	4.82	5.10	4.84	4.30*	4.19*	3.75*
Tl	0.0174	0.0101*	0.0109*	0.0063*	0.0122	0.0166*	0.0101	0.0056*
U	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
V	0.120	0.108	0.104	0.128	0.107	0.048*	0.048*	0.050*
Zn	24.0	24.1	23.9	22.3	22.8	22.7	26.6	23.5

* represents significant differences ($P < 0.05$) between the control and treatment groups at each sampling time.

Figure 4.1) The generation of superoxide by redox cycling of selenides (RSe^-) (after Chaudiere *et al.* 1992). GSH = reduced glutathione and GSSG = oxidized glutathione.

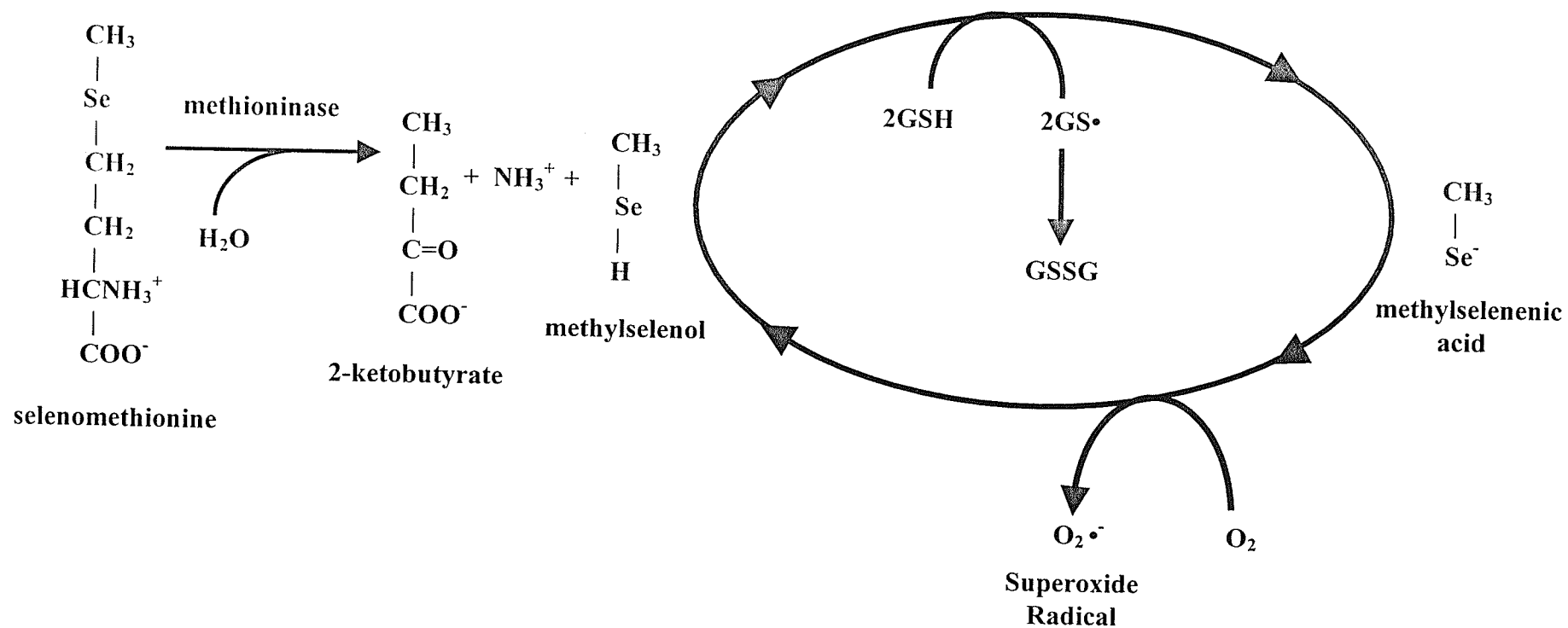


Figure 4.2) The weight gain of rainbow trout fed a control diet and three Se contaminated diets for 120 and 302 days. Data are expressed as the mean final body weight – mean initial body weight for each treatment group.

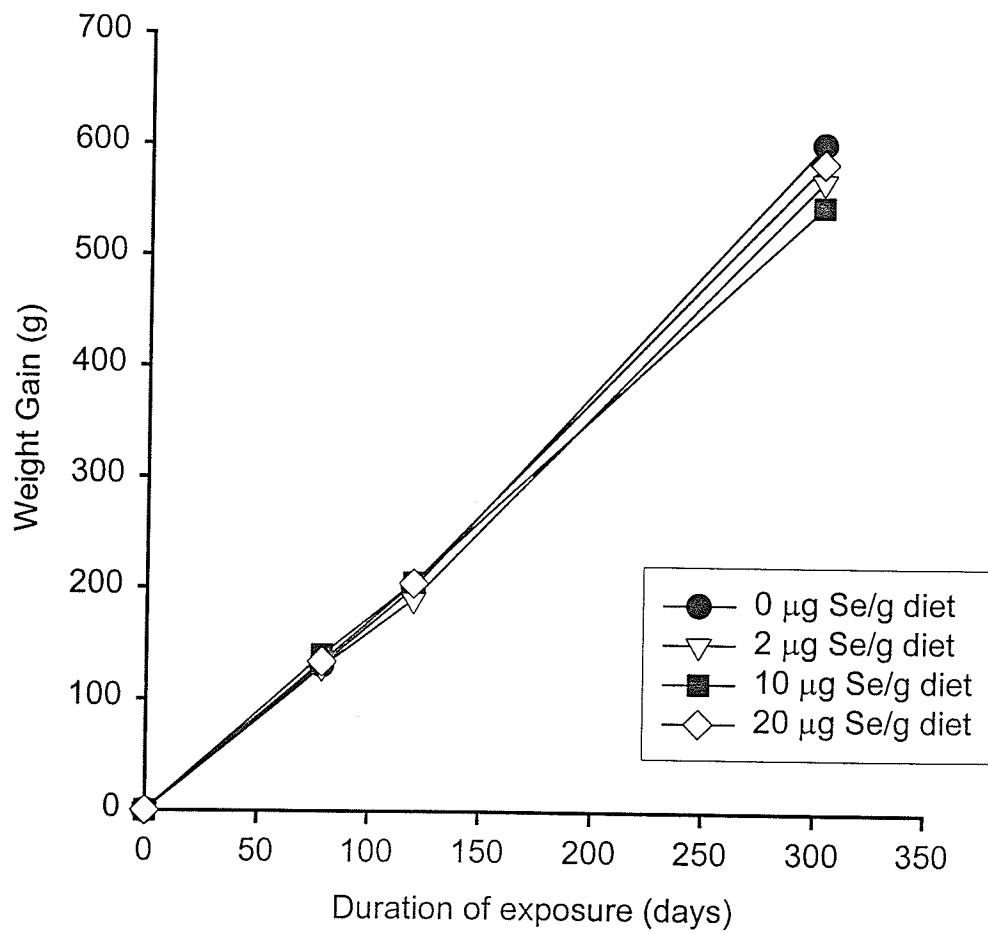


Figure 4.3) Se accumulation in the liver of rainbow trout fed diets containing 0, 2, 10, and 20 $\mu\text{g Se/g}$ for 120 and 302 days. Data are expressed as mean $\pm$ SE. Asterisks represent significant differences ($P < 0.05$) between the control and treatment groups for each sampling time.

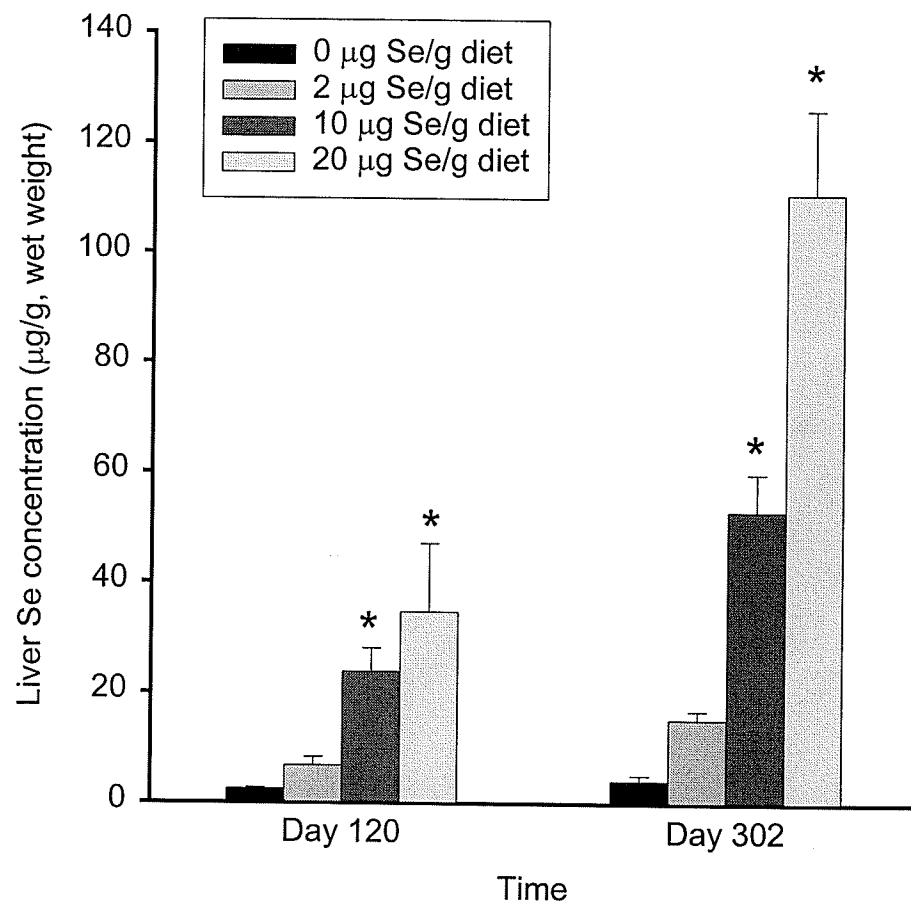


Figure 4.4) Serum ion concentrations of rainbow trout fed diets containing 0, 2, 10, and 20 $\mu\text{g Se/g}$ for 120 and 302 days. (A) sodium concentration, (B) potassium concentration, (C) chloride concentration. Data are expressed as mean $\pm$ SE.

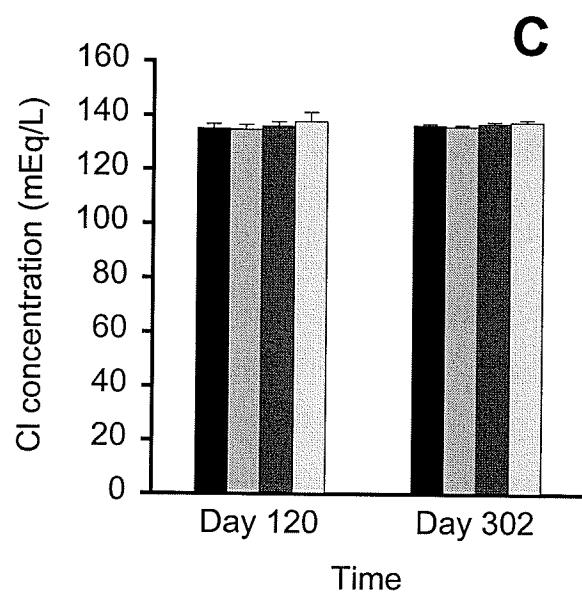
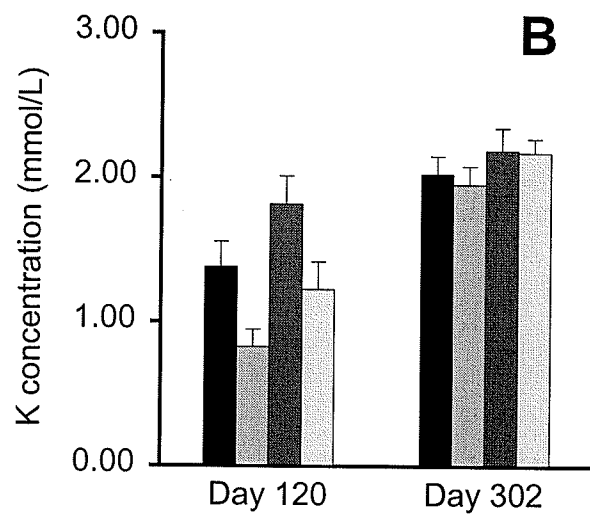
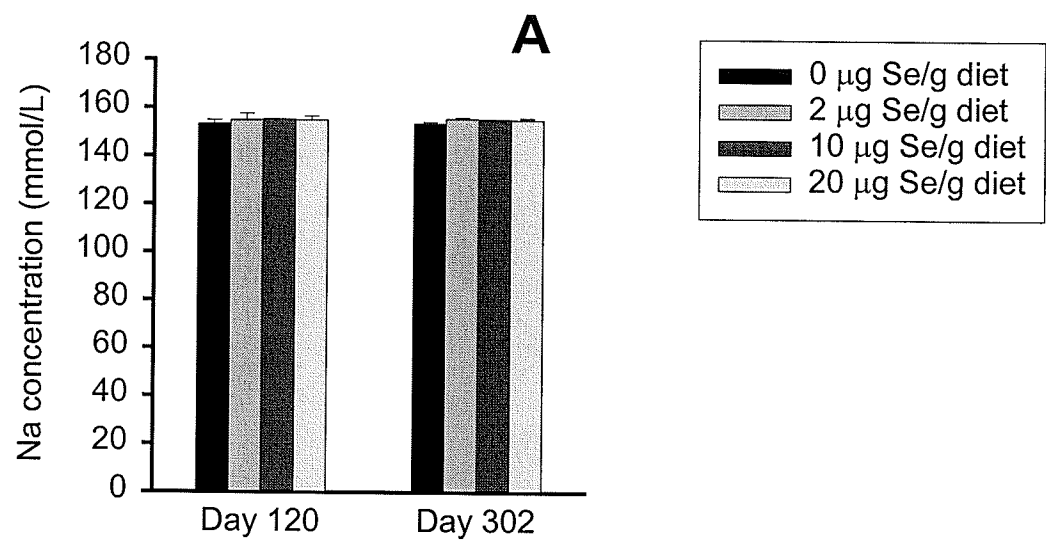


Figure 4.5) Relationship between liver Se concentrations and levels of sodium in the serum of rainbow trout fed diets containing 0, 2, 10, and 20 μg Se/g for 302 days. Each symbol represents data from one fish.

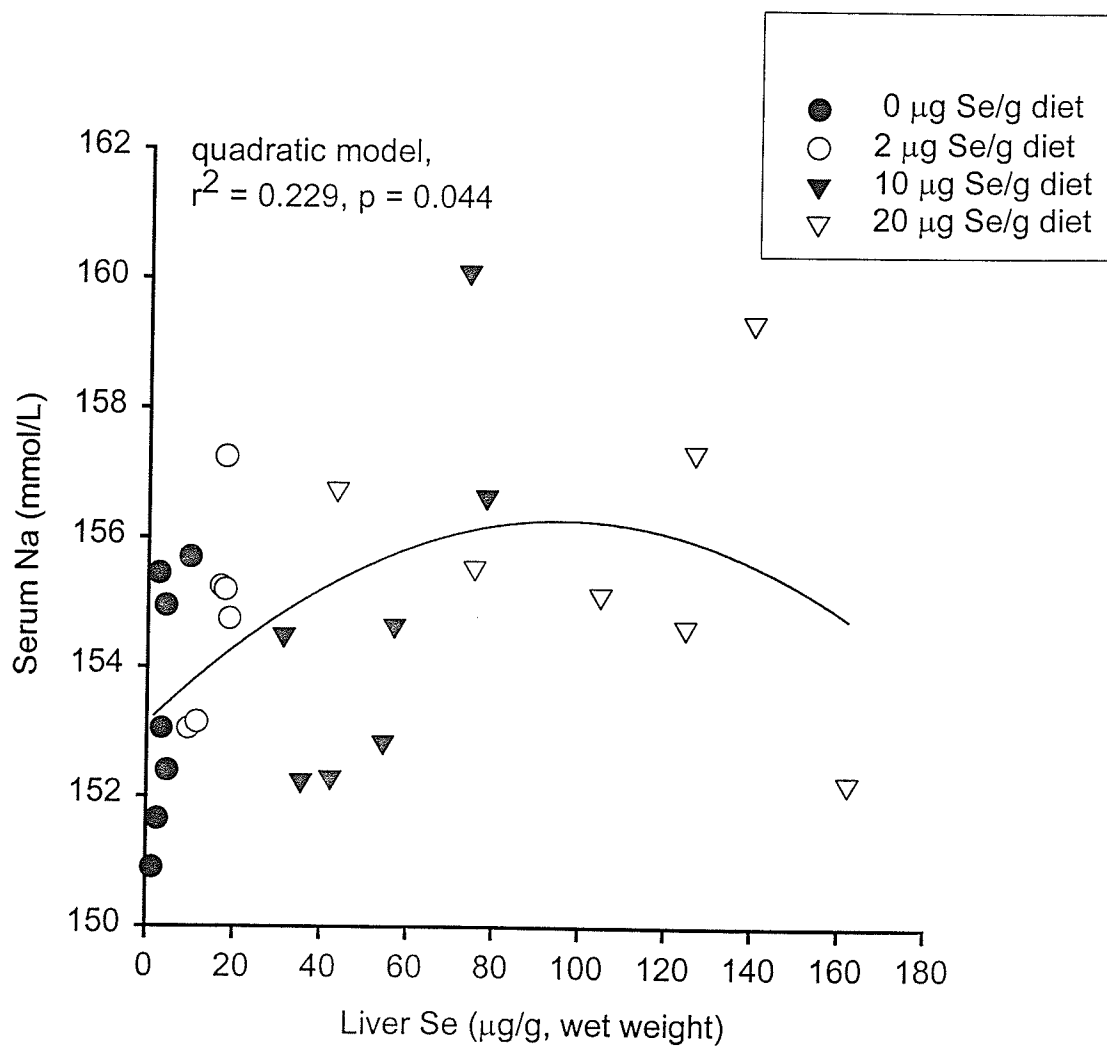


Figure 4.6) Concentrations of (A) retinol and (B) tocopherol in the serum (after 120 days of exposure) and plasma (after 302 days of exposure) of rainbow trout fed diets containing 0, 2, 10, and 20 $\mu\text{g Se/g}$. Data are expressed as mean $\pm$ SE.

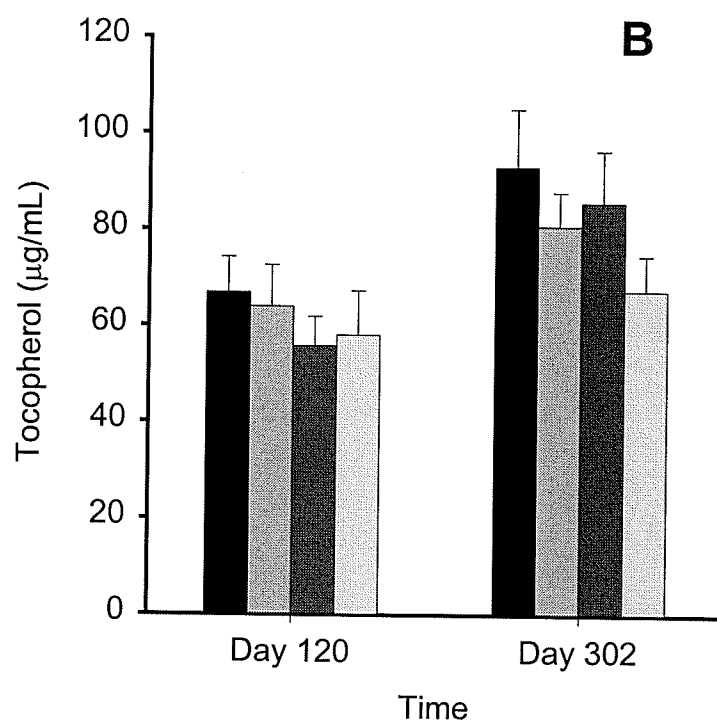
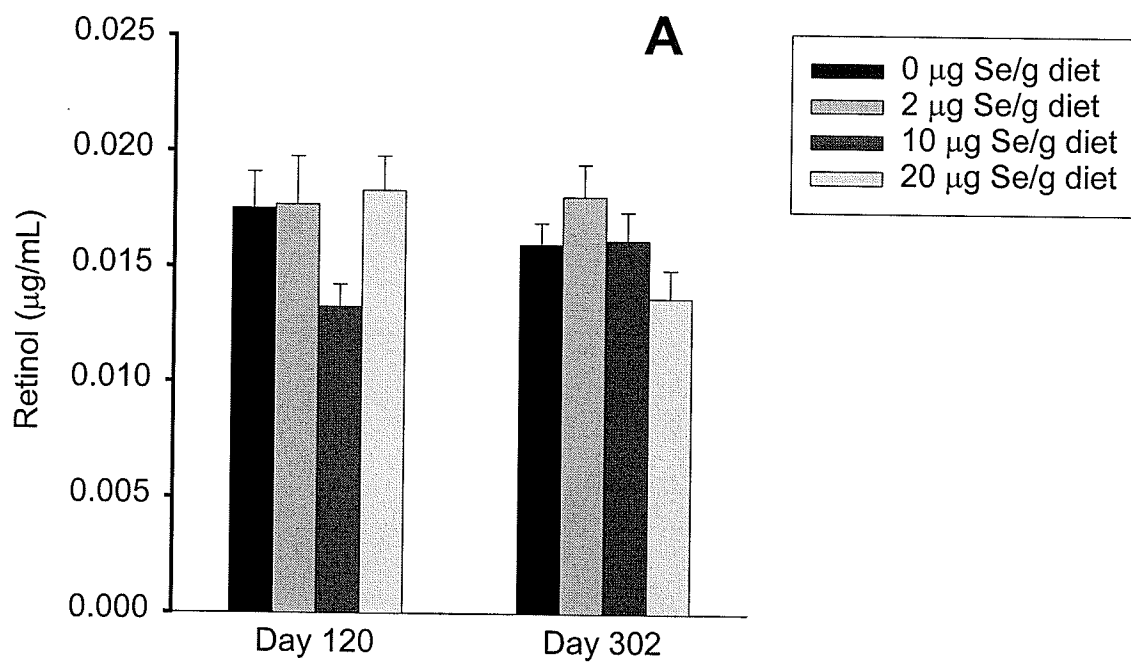


Figure 4.7) Relationship between liver Se concentrations and levels of tocopherol in the plasma of rainbow trout fed diets containing 0, 2, 10, and 20 $\mu\text{g Se/g}$ for 302 days. Each symbol represents data from one fish.

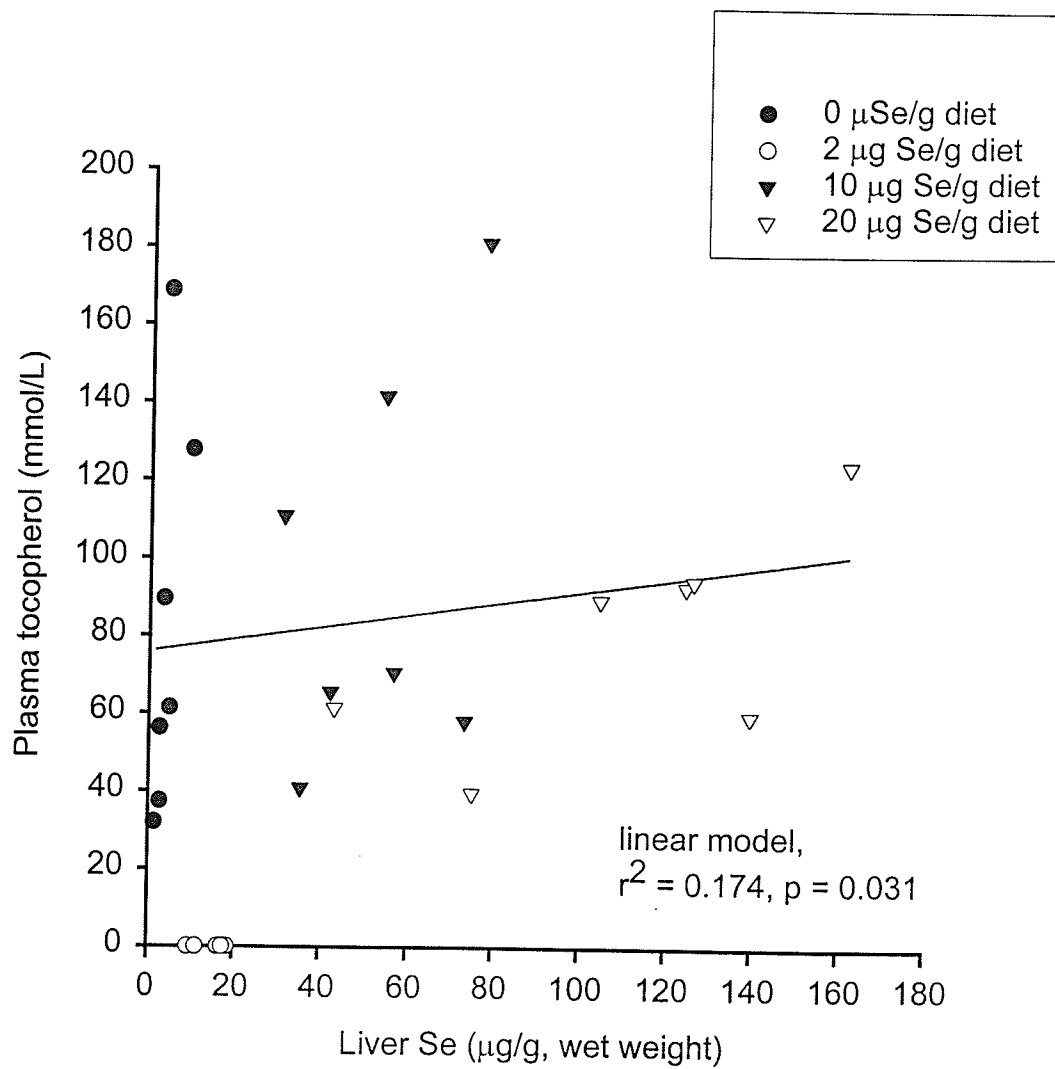


Figure 4.8) Concentrations of (A) reduced glutathione (GSH), (B) oxidized glutathione (GSSG) and (C) ratio of GSSG:GSH in the livers of rainbow trout fed diets containing 0, 2, 10, and 20 $\mu\text{g Se/g}$. Data are expressed as mean $\pm$ SE. Asterisks represent significant differences ($P < 0.05$) between the control and treatment groups for each sampling time.

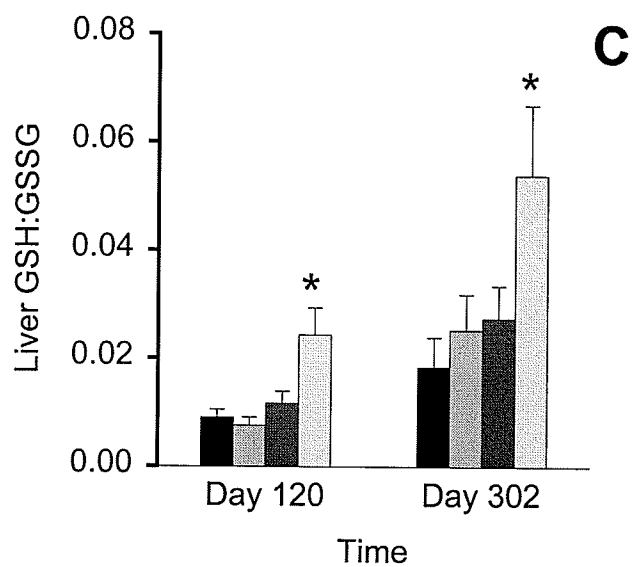
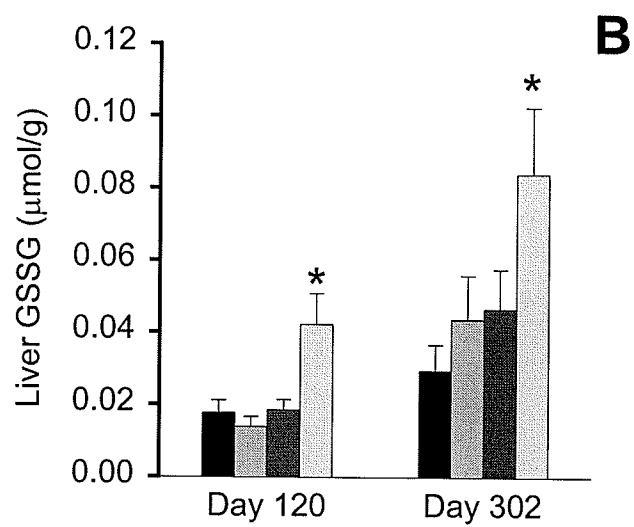
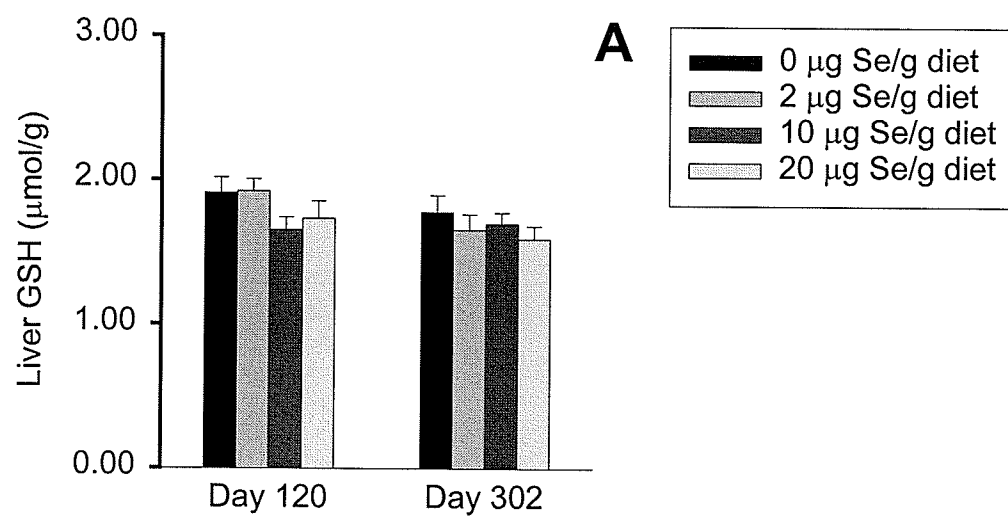


Figure 4.9) Relationship between liver Se concentrations and (A) oxidized glutathione and (B) GSSG:GSH of rainbow trout fed diets containing 0, 2, 10, and 20 μg Se/g for 120 days. Each symbol represents the mean for each treatment group.

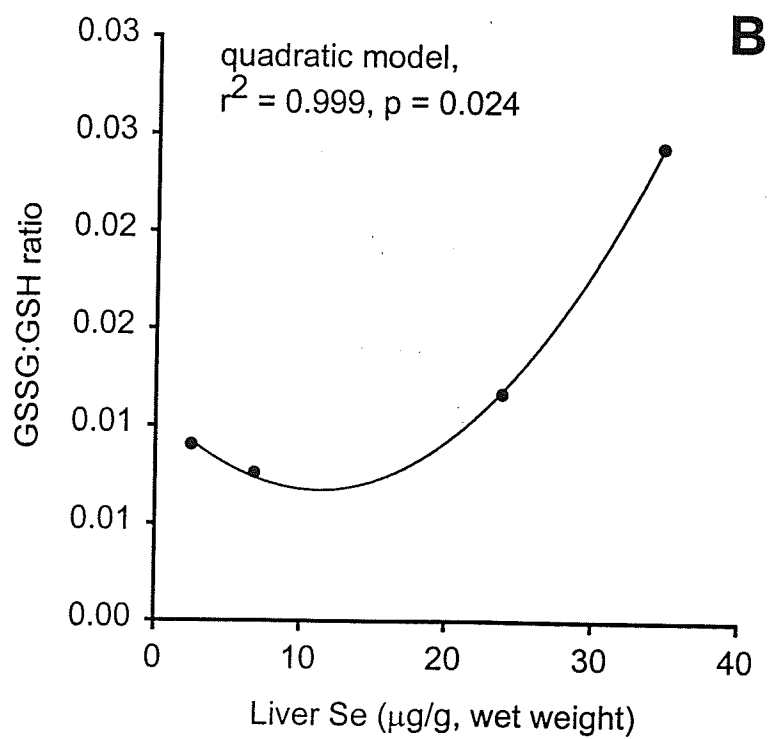
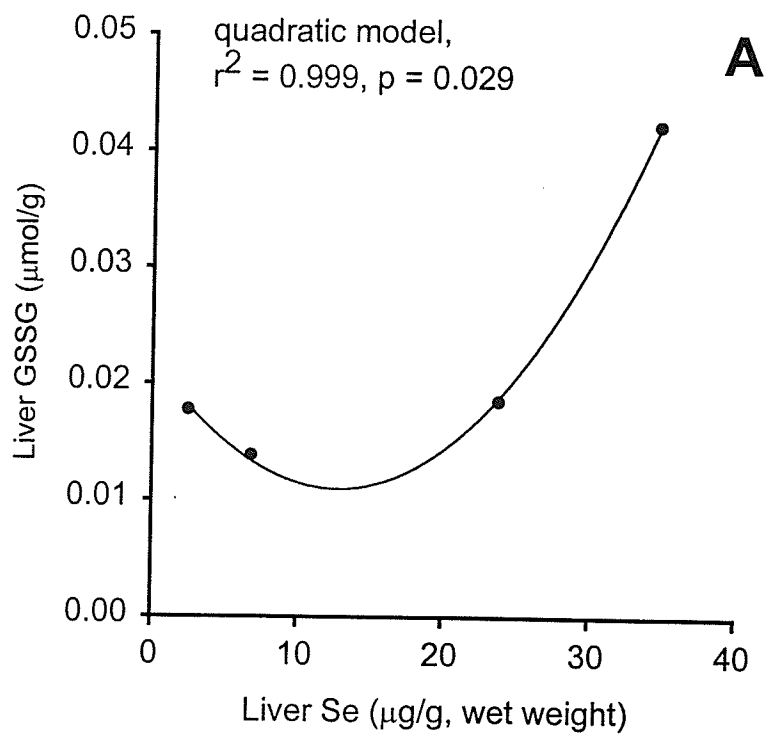
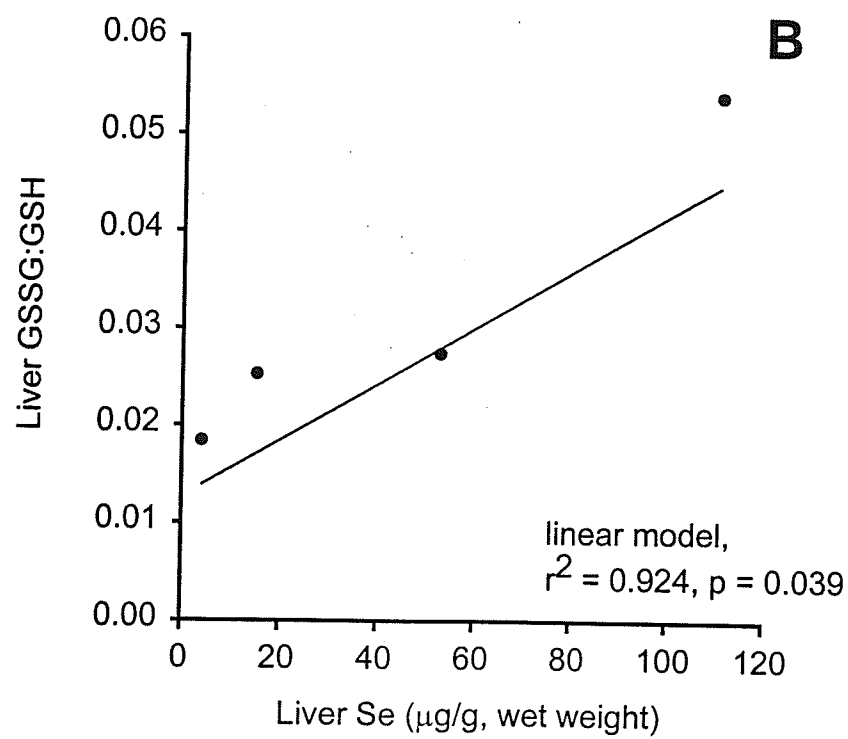
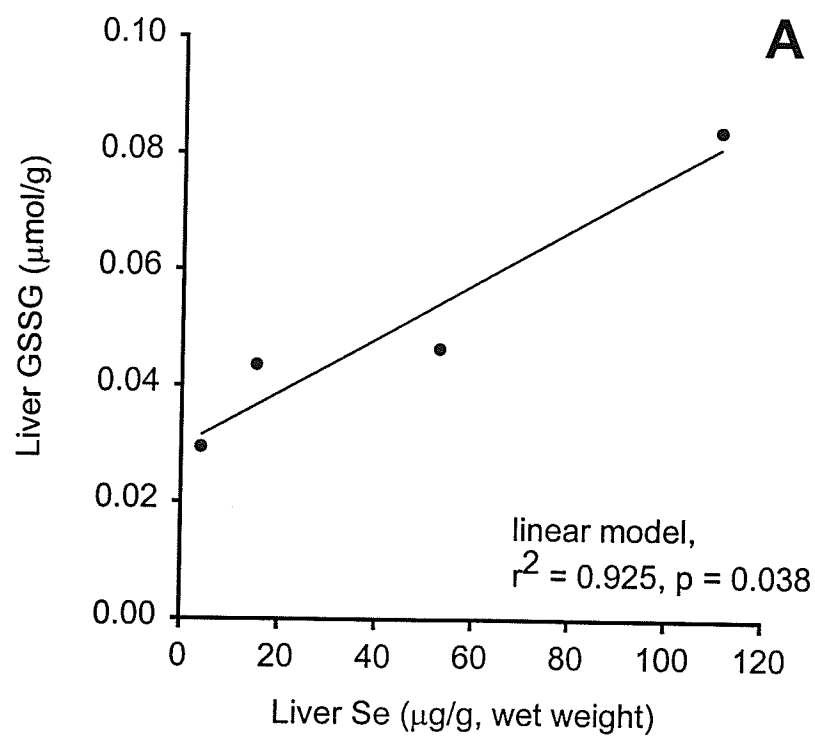


Figure 4.10) Relationship between liver Se concentrations and (A) oxidized glutathione and (B) GSSG:GSH of rainbow trout fed diets containing 0, 2, 10, and 20 μg Se/g for 302 days. Each symbol represents the mean for each treatment group.



GENERAL CONCLUSIONS

Chapter 1: Toxic effects of selenium on fish

- While Se is an essential trace element in the diet of all vertebrates, there is a narrow range between dietary requirements and toxic effect threshold concentrations.
- The earliest indicators of Se toxicity are effects on embryonic development and survival of offspring.
- The existing literature regarding Se poisoning in natural fish populations is based largely on fish that inhabit standing warm water habitats. As a result, the applicability of toxic effect thresholds and guidelines derived from these systems to flowing cold water habitats is a matter of contention in the literature.
- To date, there has been no assessment of potential effects on reproductive fitness of salmonids inhabiting seleniferous waters downstream from “end pit lakes” left by deep pit coal mining operations as part of their reclamation strategy.

Chapter 2: An assessment of three methods for detecting developmental abnormalities in fry from brook trout and rainbow trout exposed to elevated Se levels

- Of the three methods used to assess developmental abnormalities, frequency assessment was the most time- and cost-effective method. As well, the data generated was the most useful to derive site specific toxic effect thresholds. As a result, this method was chosen to evaluate larvae in the field study presented in Chapter 3.
- Although the SI method had comparable equipment and analysis requirements as in the frequency assessment method, it required complicated statistical analysis.
- Morphometric analysis required specialized equipment, considerably more time than the other two methods, and a complicated statistical analysis. Although the results of the analysis were less subjective than simple visual inspection, the measurements were not as robust at detecting the wide variety of deformities observed in the other two analyses.

Chapter 3: Development and survival of fry from wild rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) exposed to elevated selenium in an area of active coal mining

- Rainbow trout and brook trout in Se-contaminated streams accumulated elevated levels of Se in their eggs compared to fish from the reference sites.

- In both species, Se concentrations in muscle tissue were positively correlated with Se concentrations in the eggs.
- Fertilization, mortality and development time did not differ between Se-contaminated sites and the reference sites.
- There is a significant relationship between the occurrence of edema in rainbow trout fry and Se levels in eggs.
- The types of deformities were not consistent between the two species, with rainbow exhibiting primarily spinal deformities and generalized edema and brook trout exhibiting primarily craniofacial and finfold deformities.
- A significant relationship was found between the concentration of Se in the eggs and the incidence of skeletal and craniofacial deformities and edema for rainbow trout. The inflection point for all three regression curves occurred around 6µg Se/g. No significant relationships were evident between Se concentrations and deformities in brook trout.
- Selenium downstream from coal mining operations may be impacting the reproductive fitness of rainbow trout.
- Relationships between Se and deformities require additional study in brook trout.

Chapter 4: Oxidative stress in rainbow trout fed environmentally relevant concentrations of selenium

- Exposure of rainbow trout to diets with elevated levels of selenomethionine had no effect on feed consumption or feeding behavior, growth, or condition.
- Rainbow trout were found to accumulate Se in a dose dependent manner in the liver.
- GSSG concentrations and the ratio of GSSG:GSH were significantly elevated in trout fed the highest dietary dose (20 µg Se/g) after 120 and 302 days of exposure. Increases in GSSH and the ratio were dose dependent and showed a strong relationship with hepatic Se levels.
- Environmentally relevant dietary doses of Se were sufficient to produce oxidative stress in adult rainbow trout. This mechanism of toxicity warrants further investigation as oxidative stress type lesions have been observed in offspring of trout inhabiting seleniferous waters.

Future studies

- In order to establish the ecological relevance of the impact of increased incidences of deformities on population dynamics, a survival study should be conducted to assess the survival of the offspring of fish inhabiting seleniferous waters.
- To validate the results of my field studies, fish in a controlled laboratory feeding experiment should be spawned, and the resulting offspring reared to the swim-up stage and examined for deformities.
- Measurements of additional indices of oxidative stress, including enzymatic defenses and membrane breakdown by-products, should be made in the tissues of fish exposed to elevated levels of Se through the diet.
- In order to establish a connection between oxidative stress and the development of terata, the eggs and developing larvae of Se-laden fish should be analyzed for biochemical indices of oxidative stress.

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APPENDIX I. Acute toxicity (96-hour LC50 in mg/L) of selenite and selenate for various species and life-stages of fishes.

Species	Selenite (Se IV)	Selenate (Se VI)	Reference
Arctic grayling (<i>Thymallus arcticus</i>)			
alevin	76	100	Buhl and Hamilton 1991
juvenile	34.3	180	"
Bluegill sunfish (<i>Lepomis macrochirus</i>)	20.3	63	Canton 1999 ^b
Bonytail (<i>Gila elegans</i>)			
fry	19	55	Hamilton 1995
juvenile	17	246	"
older juvenile	15	217	"
Brook trout (<i>Salvelinus fontinalis</i>)	10.2	ND	Cardwell <i>et al.</i> 1976
Chinook salmon (<i>Oncorhynchus tshawytscha</i>)			
eyed egg	>560	>1000	Hamilton and Buhl 1990
alevin	104	>320	"
swim-up fry	27.3	114	"
juvenile	13.1	ND	"
swim-up fry	13.9	112	"
juvenile ^a	23.3	154	"
Coho salmon (<i>Oncorhynchus kisutch</i>)			
swim-up fry	7.8	32.5	Hamilton and Buhl 1990
juvenile ^a	13.6	39.0	"
alevin	80	329	Buhl and Hamilton 1991
juvenile	7.83	74	"
Colorado squawfish (<i>Ptychocheilus lucius</i>)			
fry	14	66	Hamilton 1995
juvenile	36	286	"
older juvenile	32	331	"
Fathead minnow (<i>Pimephales promelas</i>)	1.601	5.5	Canton 1999 ^b

appendix I continued

Species	Selenite (Se IV)	Selenate (Se VI)	Reference
Rainbow trout (<i>Oncorhynchus mykiss</i>)			
alevin	11.8	470	Buhl and Hamilton 1991
juvenile	9	32.3	"
adult	6.45	ND	Hodson <i>et al.</i> 1980
Razorback sucker (<i>Xyrauchen texanus</i>)			
fry	15	48	Hamilton 1995
juvenile	8.9	36	"
older juvenile	16	25	"
Walleye (<i>Stizostedion vitreum</i>)			
juvenile	11.7	ND	Mauk and Brown 1999

ND = no data

^a exposed in brackish water

^b average values reported from studies cited in US-EPA 1987.

APPENDIX II. Selenium tissue concentrations associated with toxic effects in various species and life-stages of fish exposed to elevated Se in dietary, waterborne or field studies.

Species	Exposure & Life-stage	Tissue	Se concentration (µg/g, wet weight)	Effect	Reference
Bluegill sunfish, (<i>Lepomis macrochirus</i>)	field adult	ovary	7	reproductive failure	Gillespie and Baumann (1986)
	field adult	carcass ovary	5.8-6.7 9.9-11.8	declining populations	Baumann and Gillespie (1986)
	field adult	whole- body	2.5 ^a	no effects reported	Saiki <i>et al.</i> (1992)
	waterborne adult	ovary	4.5	reproductive failure	Hermanutz <i>et al.</i> (1992)
		liver	7.3		
		muscle	4.2		
		whole- body	4.6		
	field	eggs	1-34 ^a	teratogenic defects	Lemly 1997b
	waterborne + dietary juvenile	whole- body	1.3-2 ^a	blood changes, gill damage	Lemly (1993b)
	waterborne + dietary adult	whole- body	4.8 ^{a,b}	reproductive failure	Coyle <i>et al.</i> (1993)
		ovary	10.5 ^a		
Chinook salmon, (<i>Oncorhynchus tshawytscha</i>)	dietary juvenile	whole- body	2.9	kidney damage, reduced smolting	Hamilton <i>et al.</i> (1986)

appendix II continued

Chinook salmon (continued)	waterborne swim-up larvae	whole-body	1.0 ^a	reduced growth, mortality	Hamilton and Weidmeyer (1990)
	dietary swim-up larvae	whole-body	1.0 ^a	mortality reduced growth	Hamilton <i>et al.</i> (1990)
	dietary ^c juvenile	whole-body	3.6 ^a	reduced growth	Hamilton <i>et al.</i> (1990)
Colorado Pikeminnow (<i>Ptychocheilus lucius</i>)	field adults	muscle plugs	2.4 ^a	declining populations	Osmundson <i>et al.</i> 2000
Common carp (<i>Cyprinus carpio</i>)	field adult	whole-body	2.5 ^a	no effects reported	Saiki <i>et al.</i> (1992)
	field	eggs	1.3-30 ^a	teratogenic defects	Lemly 1997b
Cutthroat trout (<i>Oncorhynchus clarki lewisi</i>)	field adult	eggs muscle liver	5.3 ^a 3.1 ^a 9.2 ^a	no effects reported	Kennedy <i>et al.</i> (2000)
	field adult	muscle liver ovary	1.5-3.8 ^a 3.5-12.5 ^a 7-12 ^a	no effects reported	McDonald and Stroscher (1998)
Fathead minnow (<i>Pimephales promelas</i>)	dietary swim-up larvae	whole-body	13-15 ^a	reduced growth	Bennet <i>et al.</i> (1986)
	dietary adult	whole-body	1.4 ^a	reduced growth	Ogle and Knight (1989)

appendix II continued

Fathead minnow (continued)	waterborne adult	ovary	5.9	reproductive failure	Schultz and Hermanutz (1990)
Green sunfish (<i>Lepomis cyanellus</i>)	field adult	liver muscle	21.4 12.9	blood changes, gill, kidney, liver and ovary damage	Sorenson <i>et al.</i> (1984)
Lake chub (<i>Couesius plumbeus</i>)	field adult	ovary carcass	11 11	edema, fin deformities	TAEM (1996)
Lake chubsuckers (<i>Erimyzon sucetta</i>)	polluted sediments adult	whole-body	1.4 ^a	mortality, decreased growth, fin erosion	Hopkins <i>et al.</i> (2000)
Largemouth bass (<i>Micropterus salmoides</i>)	field adult	carcass ovary	4 7.4	declining populations	Baumann and Gillespie (1986)
	field adult	whole-body	2.4 ^a	no effects reported	Saiki <i>et al.</i> (1992)
	field	eggs	1-40 ^a	teratogenic defects	Lemly 1997b
Mosquitofish (<i>Gambusia affinis</i>)	field adult	whole-body	2.8 ^a	no effects reported	Saiki <i>et al.</i> (1992)
	field adult	whole-body	>25 ^a	reproductive failure	Saiki and Ogle (1995)

appendix II continued

Mosquitofish (continued)	field	eggs	1-30 ^a	teratogenic defects	Lemly 1997b
Northern pike (<i>Esox lucius</i>)	field adult	whole- body	2.5 ^d	no effects reported	Besser <i>et al.</i> (1996)
Perch (<i>Perca fluviatilis</i>)	exper. ponds adult	ovary muscle	7 5.6	reproductive failure	Crane <i>et al.</i> (1992)
Rainbow trout (<i>Oncorhynchus mykiss</i>)	waterborne adult	whole- body	2 ^a	mortality	Gissel Nielsen and Gissel- Nielsen (1978)
	dietary juvenile	liver kidney carcass	12.3 ^a 2.7 ^a 1.1 ^a	kidney damage	Hilton and Hodson (1983)
	dietary juvenile	liver kidney	51.3 ^a 11.2 ^a	mortality, decreased growth, kidney damage	Hicks <i>et al.</i> (1984)
	field adult	eggs muscle	19.5 3.3	no effects reported	Casey and Siwik (2000)
Razorback sucker (<i>Xyrauchen texanus</i>)	field larvae	larvae	1.1-1.5 ^a	declining populations	Hamilton <i>et al.</i> (2000)

appendix II continued

Razorback sucker (continued)	field adult	muscle-plugs eggs	1-8 ^a 1-2.8 ^a	declining populations	Hamilton and Waddell (1994)
Redear sunfish (<i>Lepomis microlophus</i>)	field adult	liver	7.6	ovary, kidney, liver, and gill damage	Sorenson (1988)
	field adult	liver	19.6	blood changes	Sorenson and Bauer (1983)
Spottail shiners (<i>Notropis hudsonius</i>)	field adult	whole-body	3.8 ^d	no effects reported	Besser <i>et al.</i> (1996)
Striped bass (<i>Morone saxatilis</i>)	dietary adult	muscle	3.8	liver and kidney damage	Coughlahan and Velte (1989)
	field juvenile	whole-body	2 ^a	declining populations	Saiki and Palawski (1990)
White suckers (<i>Catostomus commersoni</i>)	field adult	whole-body	1.5 ^d	no effects reported	Besser <i>et al.</i> (1996)

^a reported as dry weight and converted to wet weight assuming 75% moisture

^b derived from Figure 2 in Coyle *et al.* (1993)

^c exposed in brackish water

^d derived from Figure 2 in Besser *et al.* (1996)