

**MULTIGENERATIONAL EFFECTS OF SELENIUM IN RAINBOW TROUT, BROOK TROUT,
AND CUTTHROAT TROUT**

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ABSTRACT

Selenium (Se) is an essential element, toxic at concentrations only slightly above those required. It can become detrimental to fish health when available in excess in the aquatic environment. This study examined the effects of Se on adult female rainbow trout, brook trout, and cutthroat trout fed Se-methionine for five months, and on their larvae. Differences between fish species and between tissues were observed in the accumulation of Se in adults, and dose-dependent effects of Se on plasma thyroid hormone concentrations in brook trout, gonad size in all three species, and indicators of oxidative stress in rainbow trout and cutthroat trout, were detected. Survival of larvae and swim-up success decreased with increasing egg Se concentrations in rainbow trout and cutthroat trout, however, the same effects were not observed in brook trout. Data from this study provide new evidence for species-specific Se accumulation and toxicity in salmonid fish.

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LIST OF ABBREVIATIONS

AB- Alberta
ANOVA- analysis of variance
ACA- Alberta Conservation Association
BC- British Columbia
BK- brook trout
CCAC- Canadian Council for Animal Care
CT- cutthroat trout
DFO- Department of Fisheries and Oceans
dw- dry weight
EC- effective concentration
F1- first generation
GSI- gonadal somatic index
GPx- glutathione peroxidase
GSH- reduced glutathione
GSSG- oxidized glutathione
LPO- lipid peroxidation
MITHE-SN- Metals in the Human Environment-Strategic Network
MS-222- tricaine methanesulphonate
NaCl- sodium chloride
NOEC- no effect concentration
NSERC- Natural Sciences and Engineering Research Council
PVC- polyvinyl chloride
RN- rainbow trout
ROS- reactive oxygen species
S- sulphur
SD- standard deviation
SEM- standard error of the mean
Se- selenium
SeMet- selenium-methionine
SeCys- selenium-cysteine
T3- triiodothyronine
T4- thyroxin
WISE- Water Institute for Sustainable Environments
ww- wet weight

CHAPTER 1
GENERAL INTRODUCTION

Overview of Selenium

Selenium (Se) is an essential element that can be toxic at concentrations only slightly above dietary requirement. It is released in excess into the aquatic environment through anthropogenic activities, which accelerate weathering rates of Se-containing rock or soil (Pressor et al. 1994b, Lemly 1996, Lemly 2004). Selenium is transferred through the aquatic food chain mainly in its organic form, selenium methionine (SeMet), which can accumulate in fish tissues, including muscle, liver, and ovary (Hamilton 2004, Lemly 2004, Chapman et al. 2010). Although not common in the environment, extremely high doses of Se can be fatal to adult fish. Research has been directed towards examining sub-lethal effects in adult fish, but the most common and sensitive end points studied are the malformations caused in larvae by maternal transfer of Se (Lemly 2002, Chapman et al. 2010, Janz 2012). The mechanism of action for Se toxicity has been identified as oxidative stress caused by generation of free radicals when Se cycles with glutathione (GSH) (Palace et al. 2004, Spallholz et al. 2004). Selenium toxicity is species-specific; therefore risk assessment for Se is challenging and it is still not clear how to protect the most sensitive species in an ecosystem. This is especially important for sensitive species such as native Athabasca rainbow trout which are threatened by Se-tolerant, invasive brook trout (ABSWG 2010). This thesis project investigates the differences in species sensitivity to Se using physiological and reproductive end points in rainbow trout, brook trout, and cutthroat trout, both adult females and larvae, in a multigenerational study.

Selenium Chemistry

Selenium can exist in many states. Elemental Se (Se^0) and selenide (Se^{2-}) are not readily bioavailable, and therefore, have fairly low toxicities (Chapman et al. 2010). The inorganic forms selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) are more common and have a relatively high toxicity compared to Se^0 and Se^{2-} . In organic forms, Se exists mainly as selenocysteine (SeCys) and selenomethionine (SeMet). Selenium toxicity follows a U-shaped dose-response curve with adverse effects at low (deficiency) and high (toxicity) concentrations (Köhrle 1999), and has a narrow range of concentrations required to maintain homeostasis. Many enzymes and proteins, such as Se-dependent glutathione peroxidase (GPx) and iodothyronine deiodinase require Se to function and are called selenoproteins (Köhrle 1999, Chapman et al. 2010). Selenium is incorporated into the amino acid cysteine, which is a part of GPx; it catalyzes the reaction of reduced glutathione (GSH) and hydroperoxides into oxidized glutathione (GSSG) and water (Köhrle 1999, Chapman et al. 2010). Iodothyronine deiodinase activates and deactivates thyroid hormones (Köhrle et al. 2005). In other proteins, designated as Se-containing proteins, the selenoamino acid SeMet and Se can be non-specifically incorporated in place of sulphur (S) (Chapman et al. 2010). Many of the functions of Se proteins are not fully understood, however, two important physiological processes, oxidative stress defence and regulation of thyroid metabolism, are known to require Se (Köhrle et al. 2005, Chapman et al. 2010).

Mechanisms of Toxicity

Early literature suggested that Se toxicity was caused by the substitution of Se for S in proteins, disrupting the disulfide bonds when the protein was folded (Lemly 1997, reviewed in Chapman et al. 2010). This hypothesis has not been supported and recent literature has proposed oxidative stress as the primary mechanism of Se toxicity (Palace et al. 2004, Spallholz et al. 2004). It has been suggested that cellular damage occurs when certain Se compounds conjugate with GSH and redox cycle to create superoxide radicals (Palace et al. 2004).

The oxidative stress mechanism of Se toxicity was first proposed by Palace et al. (2004) based on their study of rainbow trout (*Oncorhynchus mykiss*) larvae cells exposed to SeMet and GSH. Concentrations of superoxide radicals increased, as indicated by light emission in an *in vitro* assay, at 216 temperature units after fertilization and remained elevated until 248 temperature units. There was no increase in superoxide radicals in samples that used methionine instead of SeMet.

Misra and Niyogi (2009) studied oxidative stress as a mechanism of toxicity in a series of experiments in which rainbow trout hepatocytes were exposed to selenite *in vitro*. They reported increased reactive oxygen species (ROS) formation and lipid peroxidation (LPO) with increasing selenite concentrations. Moreover, cell viability and the reduced to oxidized (active vs inactive) GSH ratio decreased at higher selenite concentrations. This study provided strong evidence that oxidative damage to rainbow trout hepatocytes was caused by inducing the imbalance of the GSH redox cycle, a result of Se interaction with GSH.

Contamination of the Aquatic Ecosystem

The most common forms of Se in aquatic ecosystems are selenate and selenite, which exist freely in surface waters (Presser 1994, Lemly 1999). Organic selenides are created when Se anions are actively taken up by microbes, algae, and plants, and eventually converted to the selenoamino acids SeMet and SeCys (Presser et al. 1994a, Chapman et al. 2010). Selenium is released into the aquatic system in higher than normal concentrations through anthropogenic activities that accelerate the weathering rates of naturally occurring black shales and phosphate rocks (Presser 1994a, Lemly 2004). Anthropogenic sources include coal and uranium mine runoff, coal-fired power plant fly ash, irrigation drain water in Se-rich areas, and fossil fuel processing operations (Miller et al. 2007).

Selenium methionine is the most prevalent form of Se in the aquatic food web and is associated with bioaccumulation (uptake of Se at a higher rate than can be eliminated) and toxicity in aquatic organisms (Presser 1994a, Hamilton 2004, Chapman et al. 2010). Biomagnification (progressively higher concentrations of a toxicant in successive trophic levels) of Se, occurs at the highest rate at the lower trophic levels (Lemly 1999). Since Se enrichment occurs mainly in lower trophic levels, higher-level consumers may not be exposed to much higher concentrations of Se than organisms slightly lower on the food chain (Chapman et al. 2010). However, higher-order organisms, such as birds and fish, are more sensitive to Se than organisms in lower trophic levels and adverse effects of Se have only been documented in these organisms (Chapman et al. 2010).

Toxicity to Fish

Selenium toxicity in adult fish has not been studied extensively because lethal effects only occur at extremely high Se concentrations, a rare exposure scenario, except as an anthropogenic release of Se-enriched waste (Lemly 2002). One such case occurred in Belews Lake in North Carolina where clarified ash water containing 100-200 µg/L of Se was returned to the lake from a coal-burning power plant over several years (Lemly 2002, Chapman et al. 2010). Lemly (2002) documented symptoms of chronic Se poisoning in various fish in Belews Lake, which included swelling of gill lamellae, elevated lymphocytes, reduced hematocrit and hemoglobin, corneal cataracts, exophthalmus (popeye), and pathological alterations in the liver, kidney, heart and ovary. Similar effects were documented by Sorenson et al. (1982a, b, 1983a, b) in the adult fish community in Martin Lake Reservoir, Texas.

There has been a shift towards examining sub-lethal effects of Se as most exposures are non-lethal at lower Se concentrations and it is important to detect detrimental effects before changes in population dynamics occur. Plasma concentrations of cortisol, a corticosteroid hormone released in response to stressors, increased in rainbow trout during acute exposures to waterborne selenite in the laboratory (Miller et al. 2007). Rainbow trout and brook trout (*Salvelinus fontinalis*) were studied *in vitro* to investigate the sensitivity to Se-induced impairment of cortisol secretion in adrenocortical cells by Miller and Hontela (2011). Selenite impaired cortisol secretion in both species by disrupting the cortisol biosynthesis pathway, but rainbow trout cells were more sensitive (effective concentration which inhibited cortisol

synthesis by 50% compared to controls, $EC_{50}=8.7$ mg/L) than brook trout cells ($EC_{50}=90.4$ mg/L). The organic form of Se, SeMet, did not have an effect on cortisol secretion in either species and cell viability was not affected by Se. However, little is known about the species-specific effect of Se on the physiological stress response in fish exposed chronically to Se in the environment.

Similarly, there are few data on the relationship between excess dietary Se and effects on thyroid hormones in fish, even though Se is a part of deiodinase which activates and de-activates thyroid hormones. Miller et al. (2009) examined rainbow trout and brook trout collected from reference and Se-contaminated sites near Hinton, AB, Canada for differences in plasma cortisol, and thyroid hormones, triiodothyronine (T3) and thyroxine (T4). They found no significant relationship between any of these hormones and Se muscle concentrations, but suggested more research was needed to determine species-specific effects of Se.

A recent study on zebrafish (*Danio rerio*) characterized the effects of Se on critical swimming speed, oxygen consumption, cost of transport, tail beat amplitude, tail beat frequency, and whole body cortisol, triglyceride and glycogen concentrations (Thomas and Janz 2011). Fish fed Se-enriched diets had significantly reduced swimming speed and tail beat amplitudes, but higher whole body concentrations of triglycerides and glycogen, suggesting that substitution of SeMet for methionine causes muscle dysfunction and disruption of metabolism. Furthermore, whole-body cortisol increased in the highest supplemented experimental group.

Teratogenicity of Se

While Se toxicity in adult fish seems to be associated with very high exposures, there is substantial evidence that the most sensitive endpoint of Se toxicity is the occurrence of malformations in larvae, caused by the maternal transfer of Se to developing embryos (Lemly 1997, Janz 2012). Selenium is transferred to the eggs via vitellogenin, the precursor of yolk proteins, which contain SeMet, and are derived from the female's liver (Janz et al. 2010). There is some evidence that oxidative stress caused by excess Se damages developing embryonic cells and leads to malformations in larvae (Palace et al. 2004). These malformations can be classified into four categories: craniofacial, edema, finfold, and spinal (Lemly 1997, Kennedy et al. 2000, McDonald and Chapman 2009). Craniofacial malformations include deformed eyes, head, jaw, or mouth (Holm et al. 2005). Edema refers to fluid surrounding the yolk-sac or eyes (Holm et al. 2005). Finfold deformities include misshapen or missing fins or tail, and spinal deformities refer to curvature of the spine (Holm et al. 2005).

A number of field studies and diet-exposure studies attempted to quantify the effects of maternally transferred Se in fish. Focusing on salmonids, the family of fish studied in this thesis, toxicological endpoints have been documented for rainbow trout (Holm et al. 2005), cutthroat trout (Kennedy et al. 2000, Rudolph et al. 2008, Hardy et al. 2010), brook trout (Holm et al. 2005), brown trout (NewFields unpublished report 2009), and Dolly Varden char (McDonald et al. 2010). Holm et al. (2005) transported fertilized rainbow trout and brook trout eggs from a Se contaminated site in northern Alberta to a laboratory in Winnipeg, Manitoba. They incubated eggs until the yolk had been fully

absorbed and then examined the larvae for malformations. In rainbow trout, there was a significant correlation between the amount of Se in the eggs and incidence of larval malformations was observed for rainbow trout, in the craniofacial, edema, and spinal malformation categories. It was noteworthy that 30% of skeletal, 40% of craniofacial, and 70% of edema malformations could be attributed to Se at a concentration of 12 $\mu\text{g/g}$ ww in eggs (Holm et al. 2005), although there was no relationship for brook trout (Holm et al. 2005). This study provided insight into differences in species sensitivities to Se, but it was criticized by McDonald and deBruyn (2008) for limited sample size, lack of control over experimental design and over exposure to Se, resulting in variable Se burdens in eggs. Therefore, the relationship between rainbow trout Se burdens and incidence of malformations remains unclear.

Kennedy et al. (2000) reported an absence of toxic effects on cutthroat trout (*Oncorhynchus clarkii*) larvae collected from a coal mining area in British Columbia (BC), where Se concentrations ranged from 8.7-81.3 $\mu\text{g/g}$ Se dw. Rudolph et al. (2008) built on this study, collecting eggs from a contaminated site and a reference site, and raising them in the laboratory. The cutthroat trout larvae had significantly lower fertilization and survival rates at high concentrations of egg Se (46.8-86.3 $\mu\text{g/g}$ Se dw). However, no effects were detected at lower egg Se concentrations. Hardy et al. (2010) conducted a long-term (2.5 years) laboratory feeding study to determine the effects of Se on cutthroat trout. They used fairly low Se dietary concentrations (ranging from 1-11.2 mg/kg Se), which resulted in 16.04 $\mu\text{g/g}$ Se dw as the highest tissue burden in the eggs (Hardy et al. 2010). No toxic effects were observed in the larvae. Due to these

inconsistent results, the Se toxicity threshold for cutthroat trout has not yet been defined and there is a gap in egg Se concentrations between 16 and 47 µg/g where effects are likely.

Brown trout (*Salmo trutta*), were studied by NewFields (unpublished report 2009) who described a 10% increase in deformities (EC₁₀) at 17.7 mg/kg Se dw in eggs (Chapman et al., 2010). Dolly Varden char (*Salvelinus malma*) were studied by McDonald et al. (2010) from waterbodies exposed to mining-related Se releases in British Columbia, Canada. Fertilized eggs were raised in the laboratory and larvae deformities were assessed after the yolk was absorbed. No effects of Se were observed on egg or larvae survival, and an EC₁₀ was determined to be 54 mg/kg dw (McDonald et al. 2010).

Taken together, these studies suggest that species sensitivity to Se varies greatly, even between closely related species. However, all of these studies lack evidence of real consequences of larval deformities in the wild. Salmonids bury their eggs under gravel in the riverbed in nests called 'redds' and after hatching and absorbing their yolk, larvae swim through the gravel to the surface of the riverbed. Therefore, research is needed to determine the effect Se caused deformities have on larval swim-up success from redds and potential effects on population recruitment.

Thesis Project

Evidence suggests that rainbow trout are more sensitive to Se than brook trout (Holm et al. 2005). This is a concern because native Athabasca rainbow trout, a

genetically distinct population that live in an area of coal mining in northern Alberta, already face the problem of introduced species such as brook trout (Taylor et al. 2006, ABSWG 2010). Therefore extensive, accurate data are needed to perform risk assessments and ensure native Athabasca rainbow trout are protected. Cutthroat trout are closely related to rainbow trout, but previous studies have shown conflicting results, failing to set a reliable Se toxicity threshold for cutthroat trout. Brook trout are currently considered one of the most tolerant species to Se and are a useful comparison species for this experiment, as they compete for resources with rainbow trout and cutthroat trout in the natural environment (Holm et al. 2005).

The goal of this study is to add to the current knowledge of species-specific Se toxicity in salmonids by using a laboratory feeding study with controlled and continuous levels of Se in the diets of rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and cutthroat trout (*Oncorhynchus clarkii*) to better understand the physiological and reproductive effects of Se.

Hypothesis

Adult rainbow trout and cutthroat trout will have higher levels of oxidative stress than brook trout at similar Se tissue burdens. Rainbow trout and cutthroat trout larvae will have a higher rate of malformations with increasing egg concentration of Se, causing them to be unable to swim to the surface of artificial gravel redds in contrast to brook trout which will not exhibit any detrimental effects.

Objectives

Long Term

- Increase knowledge of species differences in sensitivity to Se toxicity and provide data for use in risk assessments, especially for native species
- Influence anthropogenic practices to use better methods when dealing with Se waste by diagnosis of the detrimental effects of current practices

Short Term

- Compare sensitivity to Se in rainbow trout, brook trout, and cutthroat trout embryos and larvae
- Assess teratogenic potential of maternally transferred Se, comparing the swim-up performance and survival of rainbow trout, brook trout, and cutthroat trout larvae and investigate the relationship between malformations caused by Se and swim-up success
- Document Se effects on adult fish through change in gonad and condition factor, oxidative stress measures and hormonal end points
- Develop and validate a laboratory-based method to assess the impacts of environmental toxicants on the early stages of development in fish by simulating swim up through artificial gravel-bed flumes

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CHAPTER 2

TISSUE ALLOCATION OF SELENIUM AND PHYSIOLOGICAL EFFECTS OF CHRONIC DIET EXPOSURE TO SE-METHIONINE IN ADULT RAINBOW TROUT, BROOK TROUT, AND CUTTHROAT TROUT

Abstract

Selenium (Se), an essential element, is toxic at concentrations only slightly higher than those required for homeostasis and can be a concern in aquatic environments impacted by anthropogenic activities, including coal and uranium mining. Selenium can cause oxidative stress and malformations in salmonids, and toxicity thresholds appear to be species-specific. The aim of this study was to provide comparative data on the tissue-specific allocation of Se and its effects in three species of salmonid fish, to gain a better understanding of species-specific Se toxicity. Adult female rainbow trout, brook trout, and cutthroat trout were chronically exposed to Se-methionine in their diet (15 $\mu\text{g/g}$ and 40 $\mu\text{g/g}$) to characterize Se accumulation in eggs, liver and muscle, and investigate physiological effects. Adults were fed the experimental diets for five months prior to spawning, eggs were collected and fertilized, and adult tissues sampled. Brook trout had a higher ratio of liver and muscle Se to egg Se compared to rainbow trout or cutthroat trout, and a higher ratio of plasma T3:T4 with increasing Se in liver and muscle. There was a slight, but significant, decrease in gonadosomatic index (GSI) related to liver Se in rainbow trout and cutthroat trout. However, brook trout had a bimodal distribution; some brook trout (liver > 19 $\mu\text{g/g}$) failed to produce a viable gonad, whereas others (liver < 13 $\mu\text{g/g}$) were not affected. Lipid peroxidation (LPO) and glutathione (GSH) increased with increasing liver Se in rainbow trout, GSH but not LPO increased in cutthroat trout, and there was no significant relationship between liver Se and LPO or GSH in brook trout. Egg Se burdens were related to a decrease in survival in rainbow trout and cutthroat trout larvae, although no trend was evident for brook trout.

This study provided new information regarding species differences in sensitivity to Se, its tissue allocation patterns and reproductive effects.

Introduction

Selenium (Se) is an essential trace element that follows a U-shaped dose-response curve, with detrimental effects at deficiency and excess levels (Lemly 1999, Chapman et al. 2010). Selenium is an important element in many biomolecules. One of its most important functions is its association with glutathione peroxidase (GPx), the enzyme which catalyzes the reaction of reduced glutathione (GSH) into oxidized glutathione (GSSG), and hydroperoxides into water (Pappas et al. 2008). Selenium is also a component of iodothyronine deiodinase, which activates and de-activates thyroid hormones (Köhrle et al. 2005).

The most common forms of Se in the aquatic ecosystems are inorganic selenate (Se^{+6}) and selenite (Se^{+4}), but Se can also exist in organic forms (Lemly 1999, Lemly 2004). Organic selenides are created when Se anions are taken up by microbes, algae, and plants and eventually converted to selenoamino acids, selenomethionine (SeMet) and selenocysteine (SeCys) (Presser et al. 1994, Chapman et al. 2010). Selenium methionine is the form of Se transferred within the aquatic food web and associated with toxicity in aquatic organisms (Janz 2012). Excess Se may enter the aquatic system through anthropogenic activities, including coal mining and uranium mining that accelerate the weathering rates of naturally occurring black shales and phosphate rocks (Presser 1994, Lemly 2004, Chapman et al. 2010).

The accumulation of Se in fish tissues, including muscle, liver, and ovary, and its toxicity have been investigated in various species (reviewed in Chapman et al. 2010, Janz et al. 2010). One proposed mechanism of Se toxicity is an increase in oxidative

stress (Palace et al. 2004, Spallholz et al. 2004, Misra and Niyogi 2009, Janz 2012), cellular damage occurring when several Se compounds conjugate with GSH to yield reactive oxygen species through redox reactions. Oxidative stress has been detected *in vitro* in hepatocytes exposed to selenite (Misra and Niyogi, 2009). Selenium toxicity also occurs in larvae through maternal transfer of Se to developing embryos (Lemly 1999, Lemly 2002, Janz et al. 2010). Selenium accumulated in the liver is transported to developing larvae via yolk pre-cursor proteins (Palace et al. 2004). Larval malformations and death have been linked to Se-generated oxidative stress, and there is evidence that there are differences among fish species in sensitivity to Se toxicity (Kennedy et al. 2000, Palace et al 2004 Chapman et al. 2010).

Even though the species-specificity of Se toxicity is a characteristic of relevance to environmental managers and the protection of biodiversity, as well as to comparative toxicologists interested in mechanisms of action, comparative data remain limited. For example, exposure to selenite *in vitro* impaired cortisol secretion by adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*), but rainbow trout cells were more sensitive than brook trout (EC_{50} =8.7 mg/L and 90.4 mg/L, respectively; Miller and Hontela, 2011). *In vitro* exposure of SeMet did not have an effect in either species, as it may require further metabolic processing.

Sub-lethal effects of Se in adult fish have not been investigated thoroughly and there is a lack of understanding of the mechanisms underlying differences in sensitivity to Se among fish species. Species-specific sub-lethal effects on liver vitamin E stores, plasma T3 and T4 concentrations, glycemia and plasma cortisol between fish sampled at

a reference site and a coal mining impacted site were reported by Miller et al. (2009a) for rainbow trout and brook trout; however, the relationship between Se muscle concentrations, and these and other key end points such as lipid peroxidation (LPO) were not significant. Recently, Wiseman et al. (2011a, b) reported higher concentrations of plasma sex steroids and basal cortisol concentrations, along with an increase in condition factor and gonadosomatic index, in juvenile rainbow trout fed SeMet.

The present study was designed to compare the sensitivity to Se in three salmonid species, rainbow trout, brook trout and cutthroat trout, exposed to SeMet in a chronic (5 month) feeding study. Tissue-specific allocation of Se and species-specific effects on the hormonal and reproductive status, as well as oxidative stress were examined in adult female fish, to compare the responses in these three fish species under controlled laboratory exposures.

Materials and Methods

Experimental Fish

Adult females were selected from an existing brood stock and fed experimental diets enriched with SeMet at the Allison Creek Trout Brood Station (Coleman, AB, Canada). Five-year-old rainbows (n=36, fork length 64 ± 0.70 cm, body weight 3778 ± 15.00 g) were held in 1000 L tanks (6 fish/tank), three-year-old brook trout (n=50, fork length 40 ± 0.38 cm, body weight 1002 ± 39.19 g, 10 fish/tank), and three-year-old cutthroats (n=60, fork length 36 ± 0.27 cm, body weight 511 ± 11.70 g, 12 fish/tank),

were held in 710 L tanks. Each tank also contained one male, to stimulate ovarian maturation of the females. Fish were fed the experimental SeMet diets for five months during ovarian recrudescence (starting GSI of 1-5 %), until they were spawned and eggs were collected. Fish were fed through the entire period using belt feeders, the amount (expressed as % of total body weight/tank) varying based on months left before spawning, according to standard operating protocols of the hatchery. Specifically, rainbow trout were fed 0.6, 0.5, 0.35, 0.2, and 0.1% in the months from August-December 2009, and brook and cutthroat trout were fed 0.7, 0.7, 0.6, 0.4, and 0.15% from July-November 2010 and December-April 2010-2011, respectively. Water parameters were maintained at 10°C, pH of 8, total water hardness of 250 mg/L and 8-9 mg/L of dissolved O₂.

Diet Preparation and Feeding

Experimental diets were prepared with EWOS® trout chow (EWOS Canada, Ltd.) ground to flour, mixed with deionized water, generic gelatin and selenium methionine (Sigma Aldrich, Catalogue # S3132), as described by Holm et al. (2002). To make the control experimental diet, 3.6 kg ground EWOS® trout chow was mixed in a Hobart® standup mixer (28.39 L) with 2.2 L deionized distilled water containing 80 g of gelatin powder (Holm 2002). Experimental diets were prepared the same way, except SeMet was added to the water mixture at concentrations to obtain low (15 µg/g) and high (40 µg/g) Se diets. The dough was mixed for 20 min, then pressed through a disc with holes (diameter=5 mm) to form long noodles that were dried in a ventilated room. The

noodles were turned over every two hours until the end of the day to allow air circulation and prevent moulding. After drying overnight, the noodles were broken into small pellets by hand and kept frozen at -20°C until required. Actual Se measured in experimental diets was $1.47 \pm 1.00 \mu\text{g/g}$ for control diet, $12.67 \pm 0.48 \mu\text{g/g}$ for the low Se diet and $35.22 \pm 3.78 \mu\text{g/g}$ for high Se diet.

Spawning and Sample Collection

As the expected spawning period neared, females were examined for signs of maturation (a soft underside) and then injected with Ovaprim® (Syndel Laboratories Ltd.) at 0.5 mL per kg body weight. A week later, females were euthanized using MS-222 (0.1 g/L, Sigma Aldrich, Catalogue #E10521) and eggs were collected, fertilized, and transferred to a vertical incubator, as described elsewhere (Pilgrim et al., submitted; Chapter 4). Samples of muscle, liver, eggs, and plasma collected from the adult fish were frozen in liquid nitrogen.

The fertilized eggs were maintained in a vertical incubator until eggs hatched and the yolk sac was absorbed; fry were then euthanized with MS-222 (0.1 g/L), and preserved in Davidson's solution (5 parts 10% formalin, 1 part glycerol, 1 part acetic acid, and 3 parts 95% ethanol) for estimates of survival (present study) and for assessment of larval deformities (Chapter 3). All fish were handled according to protocols approved by the Animal Welfare Committee at University of Lethbridge, in accordance with the Canadian Council for Animal Care guidelines (CCAC).

Tissue Se Concentration

Total Se concentrations were measured in muscle, liver and eggs of adult females and in all three diets at the Department of Fisheries and Oceans (DFO) in Winnipeg, MB, Canada using hydride generation–atomic absorption with a detection limit of 0.05 µg/g (Miller et al. 2009b). All measured Se tissue and diet concentrations are reported per wet weight (ww), unless otherwise stated.

Plasma Hormone Analysis

Plasma T3 (Catalogue #06B-254215), T4 (Catalogue #06B-254011), and cortisol concentrations (Catalogue #07-221102) were measured using radioimmunoassays kits purchased from Mediacorp (Montreal, Quebec). Assays were conducted according to kit instructions and intra- and inter-assay variability was assessed using internal standards (Miller et al. 2007). Plasma estradiol and testosterone were measured with an ELISA kit (Neogen, Catalogue #402110 and #402510 respectively), according to manufacturer's protocols. Plasma samples were extracted with ethyl acetate, dried under nitrogen, re-suspended in 1 mL of extraction buffer, assayed and read at 650 nm using a microplate reader (Varioskan Flash, Thermo Scientific).

Oxidative Stress Analysis

Livers were homogenized in phosphate buffer, and lipid peroxidation (LPO) and glutathione (GSH) concentrations were measured, as previously described (Miller et al. 2007). Lipid peroxidation (Catalogue #21011) and GSH (Catalogue #21012) kits were

purchased from Mediacorp (Montreal, Quebec). Protein was quantified using Bradford reagent (Sigma-Aldrich Inc.) in a spectrophotometric assay (595 nm).

Calculations

To calculate total Se in tissues and total Se body burden, liver and egg mass (extruded eggs and residual ovarian tissue) were weighed at sampling time and muscle mass was calculated as total body mass – (liver + egg mass) for each adult female. Each tissue mass was then multiplied by the Se measured in that specific tissue for each individual, to calculate total Se accumulation in each tissue (egg, liver, muscle). The total Se in each tissue was added to estimate total body Se burden. This value was then expressed as $\mu\text{g/g}$ body weight (total body Se/total body mass) for each individual. Percent accumulation in each tissue was calculated using (total Se in specified tissue/total body Se burden) \times 100. Gonadosomatic Index (GSI) was calculated as $\text{GSI} = [\text{total egg mass spawned} / \text{body weight}] \times 100$ and Condition Factor (CF) of the fish was calculated as $K = \text{weight} / \text{length}^3$.

Statistics

Data were analyzed using the statistic program JMP. Variables (tissue Se concentrations against physiological or morphometric data) were related using regression analysis, with an ANOVA and Tukey test used post-hoc for species comparisons at $p < 0.05$. The type of regression (linear, logarithmic, and exponential)

used to relate the data was based on normality of the residuals generated using ANOVA analysis.

Results

Tissue Se Accumulation

Average Se concentration in each tissue for each treatment group, including the percent of variance (R^2) within each data set attributed to diet, is shown in Table 2.1. The variance in brook trout Se values was mainly due to diet (60-76%), whereas rainbow trout and cutthroat trout were much lower (31-45 and 41-53% respectively). Average GSI, T3:T4, cortisol, LPO, and GSH in each tissue, for each treatment group, and the percent of variance due to diet for each parameter, are shown in Table 2.2. The variance due to diet differed between species and end points; rainbow trout ranged from 4-40%, brook trout from 4-78%, and cutthroat trout from 6-40%.

In Fig. 2.1, Se accumulation in each tissue, represented as percent of total accumulation, is shown for the three fish species. There was no difference in percent Se accumulation in egg tissue between rainbow trout ($58 \pm 3.26\%$) and cutthroat trout ($59 \pm 1.74\%$), and rainbow trout and brook trout ($50 \pm 3.21\%$), however, percent Se accumulation in egg tissue was significantly less in brook trout than cutthroat trout ($p < 0.05$). Accumulation of Se (% of total) in liver tissue of rainbow trout ($7 \pm 0.76\%$), brook trout ($4 \pm 0.47\%$), and cutthroat trout ($2 \pm 0.18\%$) were all significantly different from each other, with the highest level in rainbow trout ($p < 0.05$). There were no significant differences in percent Se accumulation in muscle tissue between cutthroat

trout ($39 \pm 1.72\%$) and the other two species, however the % of total Se was less ($p < 0.05$) in muscle of rainbow trout ($35 \pm 2.87\%$) compared to brook trout ($47 \pm 2.94\%$).

All three trout species had a significant positive linear regression between egg Se and liver Se measured within the same individual (Fig. 2.2a, brook $R^2 = 0.58$, rainbow $R^2 = 0.66$, cutthroat $R^2 = 0.63$, $p < 0.0001$), however, brook trout had a significantly lower regression slope than rainbow trout and cutthroat trout. Liver to egg Se accumulation was approximately 2:1 $\mu\text{g/g}$ in brook trout, 1:1 $\mu\text{g/g}$ in rainbow trout, and 1:1.5 $\mu\text{g/g}$ in cutthroat trout. Muscle Se also increased with increasing egg Se measured in the same individual in all species (Fig. 2.2b, brook $R^2 = 0.64$, cutthroat $R^2 = 0.56$, rainbow $R^2 = 0.49$, $p < 0.0001$), but each species had a significantly different slope ($p < 0.0002$). Brook trout had the highest ratio of Se in muscle to egg tissue, approximately 1:3 $\mu\text{g/g}$, and rainbow trout had the lowest ratio at 1:25 $\mu\text{g/g}$. Cutthroat trout were intermediate at 1:10 $\mu\text{g/g}$ in muscle to egg Se accumulation. In Fig. 2.2c, muscle Se and liver Se are shown as a linear regression (rainbow $R^2 = 0.50$, brook $R^2 = 0.83$, cutthroat $R^2 = 0.63$, $p < 0.0001$). Rainbow trout have a significantly steeper slope than brook trout or cutthroat trout.

The increase in tissue Se was also significant when analyzed as diet treatment groups (Table 2.1, $p < 0.05$). However, although the treatment groups were all significantly different for brook trout, in rainbow trout the low treatment was either statistically similar to control or both control and high treatments. Tissue Se concentrations in cutthroat trout were always significantly different among treatments, except for Se in muscle tissue where control and low were similar. Brook trout had eight mortalities in the high Se treatment, many of which had high muscle Se

concentrations (avg. 2.97 µg/g Se), whereas only one mortality in the high Se treatment occurred for rainbow trout. Cutthroat trout did not have any mortalities.

Reproductive Effects

Condition Factor (CF) at the end of the experiment (after five months) did not vary between SeMet treatments, and females did gain weight (rainbow 259 ± 23 g, brook 279 ± 72 g, cutthroat 71 ± 16 g, corresponding to $6 \pm 0.55\%$ of initial body mass in rainbows, $30 \pm 6.92\%$ in brook trout, and $14 \pm 3.26\%$ in cutthroat). Gonadosomatic Index (GSI) was correlated with egg Se, liver Se, and muscle Se. Rainbow trout and cutthroat trout had a weak negative relationship between GSI and liver Se concentration (Table 2.2, $p < 0.05$, Fig. 2.3, rainbow $R^2 = 0.11$, cutthroat $R^2 = 0.16$, $p = \text{rainbow } 0.0341$, cutthroat $p = 0.0042$). Rainbow trout had a slightly steeper slope than cutthroat trout (rainbow -0.31 , cutthroat slope $= -0.14$). The relationship was distinct in brook trout, as most brook trout had high GSI, but six brook trout females which had liver Se concentrations over 19 µg/g and muscle Se concentrations over 3.6 µg/g did not spawn (undeveloped gonad). All species had a significantly different relationship between liver Se and GSI ($p < 0.0001$).

In addition to GSI, survival of larvae was determined as an indicator of reproductive fitness. Survival of fry ($[\text{number of fry}/\text{number of spawned eggs}] \times 100$) decreased with increased egg Se in rainbow trout and cutthroat trout (rainbow $R^2 = 0.24$, cutthroat $R^2 = 0.21$, rainbow $p = 0.0021$, cutthroat $p = 0.0007$, data not shown), but not in brook trout. Plasma samples taken immediately after spawning were measured for

estradiol and testosterone concentrations, however, no trend was observed between estradiol or testosterone concentrations and liver or muscle Se concentrations.

Thyroid Hormones and Cortisol

There was a linear regression between plasma T3:T4 concentrations and liver Se (Fig. 2.4, $R^2=0.59$, $p<0.0001$), and also muscle Se ($R^2=0.65$, $p<0.0001$, $y = 0.3204x + 0.3512$, data not shown) in brook trout (Table 2.2, $p<0.05$). No significant trend was observed between Se tissue concentrations and T3:T4 in rainbow trout or cutthroat trout when regression analysis was used; moreover, both were significantly different from brook trout (Fig. 2.4, $p<0.006$). When T3:T4 ratios were analyzed as averages among diet treatment groups, rainbow trout had a significantly lower ratio in control and high treatments compared to the low treatment ($p<0.05$), similar to cutthroat trout ($p<0.05$). Plasma cortisol increased with increasing liver Se concentration in cutthroat trout ($R^2=0.14$, $y = 16.978\ln(x) + 139.05$ $p=0.0054$, data not shown) but not in rainbow trout and brook trout when analyzed as a regression. When analyzed as an average for each treatment group, rainbow trout fed the control diet had higher cortisol concentrations than the Se-fed fish ($p<0.05$). There were no differences among treatment groups in the other two fish species.

Oxidative stress

Lipid peroxidation (LPO) and glutathione (GSH) concentrations in the liver were determined in the three salmonid species. Lipid peroxidation increased with increasing liver Se concentration as a logarithmic regression for rainbow trout (Fig. 2.5a,

$y=0.3578\ln(x) + 0.2449$, $R^2=0.27$, $p=0.0464$); however, a significant relationship was not observed for brook trout or cutthroat trout (Table 2.2). This relationship was significantly different from the relationship between LPO and liver Se for brook trout and cutthroat trout. Glutathione concentration in liver tissue was examined for a relationship with increasing liver Se in cutthroat trout (Table 2.2, $p<0.05$, Fig. 2.5b, $y=34.631\ln(x) + 74.611$, $R^2=0.34$, $p<0.0001$) and in rainbow trout ($y=0.1856x^2 - 2.6053x + 79.812$, $R^2=0.23$, $p=0.0495$). A significant trend was not detected for brook trout. When analyzed using the average GSH concentration from each treatment, rainbow trout only had a significant difference between control and low treatments, but not the high treatment ($p<0.05$). A difference was also detected between the control and low treatments for brook trout when analyzed as treatment groups ($p<0.05$).

Discussion

This study investigated the effects of a chronic (5 month) diet exposure to Se (SeMet) in adult female rainbow trout, brook trout, and cutthroat trout. There were species differences in tissue Se accumulation, GSI and survival of larvae, plasma concentrations of cortisol and thyroid hormones, and oxidative stress parameters. Our results provided evidence that adult female brook trout, compared to rainbow trout and cutthroat trout, allocate more Se to muscle tissue and above a specific tissue Se threshold, are vulnerable to systemic toxicity, resulting in mortality. However, compared to the other two species, brook trout transfer less Se to eggs and as a result, exhibit less reproductive toxicity.

To compare our results to other Se diet studies, Se tissue concentrations and the physiological parameters measured were averaged per treatment group (control diet, low Se diet, high Se diet). Variation in tissue Se concentration was not determined strictly by diet treatment, but differed between species. Brook trout Se tissue concentrations mostly depended on diet (60-76%), whereas the relationship was weaker in rainbow trout (31-45%) and cutthroat trout (41-53%), possibly because of species differences in aggression for food, SeMet uptake or metabolism, dietary efficiencies, size, or age. Since variation in Se accumulation can not always be attributed only to diet, grouping individuals by diet treatment may not be appropriate. The same logic also applies to the physiological parameters measured, where variation due to diet differed among species and treatment groups. In some cases, analyzing data by treatment group yielded slightly different results than regression analysis, for example cortisol, when only 4-7% of the variation was due to diet.

Concentrations of Se in eggs, liver and muscle were compared in the three fish species after five months of feeding the experimental Se diets. Based on the calculated total Se body burden, in all three species, the highest proportion of Se accumulated in egg tissue making it the largest 'depot' for Se storage, followed by muscle, then liver as a minor storage tissue. However, brook trout only had 4% more Se accumulation in egg tissue than muscle tissue, whereas rainbow trout and cutthroat trout had 23% and 20% more, respectively. Of the three species, cutthroat trout had the highest Se accumulation in eggs, followed closely by rainbow trout, with Se accumulation in brook trout eggs lower by almost 10%. Accumulation of Se in muscle, expressed as % of total,

was similar in rainbow trout and cutthroat trout, although there was significantly higher accumulation in brook trout. Rainbow trout accumulated a higher percent of Se in liver, followed by brook trout and cutthroat trout. Our results provide new evidence that brook trout store a larger portion of their total Se body burden in muscle tissue compared to rainbow and cutthroat trout, which store a higher portion of the Se in egg tissue. These differences in tissue allocation of Se contribute to a higher risk of systemic Se toxicity in brook trout adults, whereas rainbow trout and cutthroat trout are at risk for reproductive toxicity.

In all three fish species, Se concentration in all three tissues (egg, liver, and muscle) increased with exposure to dietary Se, thus concentrations were positively correlated among tissue depots. Rainbow trout and cutthroat trout had similar ratios of liver to egg Se, whereas brook trout had a significantly higher ratio, allocating more Se into liver or muscle tissue than eggs. Moreover, each fish species had a significantly different ratio of muscle to egg Se; rainbow trout with the lowest ratio, followed by cutthroat trout, and brook trout with the highest ratio. The ratios between muscle and egg Se for each species were comparable to those previously summarized (Chapman et al. 2010). It is important to note that although the three fish species were fed the same diets, brook trout did not accumulate more than ~10 µg/g Se in eggs, whereas rainbow trout and cutthroat trout accumulated up to ~30 µg/g Se in eggs. A species-specific pattern was also observed for muscle Se concentrations, with rainbow trout accumulating up to ~1 µg/g Se, whereas brook trout and cutthroat trout accumulated up to ~5 µg/g Se in muscle. Adult brook trout fed the high Se diet also had higher

mortality than rainbow trout and cutthroat trout fed the same diet, suggesting adult female brook trout experience lethal toxicity instead of offloading Se into eggs.

The mechanisms and biochemical/cellular characteristics underlying these species-specific Se tissue allocation patterns are not clear. Species-specific Se accumulation in tissues might be related to spring versus fall spawning life-history traits and differences in metabolism. Brook trout, fall spawners, feed in the spring and early summer before migrating to spawning grounds, could be using more energy from muscle breakdown to build their gonads during the warm summer months (Warrillow et al. 1997, Schafhauser-Smith and Benfey 2003). Rainbow trout and cutthroat trout, spring spawners, feed in late summer and fall before building gonads during the cold winter months when muscle breakdown is almost impossible (Brown and Mackay 1994, Hilderband and Kershner 2000, Sanderson and Hubert 2009). Therefore brook trout, which build gonads from fat stores and muscle breakdown (which contain lower concentrations of Se than liver tissue), would have a lower concentration of Se in eggs than rainbow trout and cutthroat trout, as they build gonads from fat and liver. In this experiment rainbow trout were under photoperiod manipulation at the hatchery so they spawned in the fall, however the underlying physiology of resource allocation during gonad building could be fixed, whereas the 'trigger' for spawning, photoperiod, can be manipulated. Although cutthroat trout were similar to rainbow trout in proportion of Se burden stored in eggs, and the liver to egg ratio of Se accumulation, they have a slightly different accumulation in muscle tissue, displaying a higher ratio of

muscle Se to egg Se than rainbow trout. Further investigations of the differences in Se tissue allocations between fall and spring spawning salmonids are required.

Another explanation for the species-specific Se accumulation observed in tissues could simply be due to genus differences. Brook trout are *Salvelinus*, whereas rainbow trout and cutthroat trout are *Oncorhynchus*. However, at this time, it is not understood why differential Se accumulation would occur in these closely related genera. Species differences in sensitivity to Se have been reviewed (Chapman et al. 2010) and there is some evidence that sensitivity to cellular oxidative stress may be important. The mechanism, however, may be more complicated, and sensitivity to Se toxicity may be linked to tissue-specific allocation of Se and the use of tissues such as muscle and liver in gonad building. This is an important unknown aspect of Se toxicity.

In the present study, gonadosomatic index (GSI), plasma estradiol and testosterone concentrations, as well as larval survivability were assessed as a measure of reproductive status. Gonadosomatic index decreased slightly for rainbow trout and cutthroat trout when correlated with liver Se, whereas there was a bimodal relationship between GSI and tissue Se was observed in the brook trout. Six brook trout which had liver Se over 19 µg/g and muscle Se over 3.6 µg/g did not develop a functional gonad, whereas below 13 µg/g Se in liver and 2.2 µg/g Se in muscle, brook trout had higher GSI values than rainbow trout and cutthroat trout. Brook trout may have a Se tissue threshold, at least in liver and muscle tissue, linked to normal gonad formation, while rainbow trout and cutthroat trout do not, and their GSI slightly decreases linearly with

increased Se in tissue. It is important to note that all fish gained weight and there were no differences in condition factor among treatment groups.

To evaluate reproductive fitness of the adult females, survivability of larvae was assessed. There was a significant relationship between decreased survival and increasing egg Se concentration for rainbow trout and cutthroat trout, but not brook trout. This finding was in agreement with Rudolph et al. (2008) who reported decreased survival of alevins for cutthroat trout with increased egg Se concentration, although survival of larvae has not been correlated with egg Se in rainbow trout and brook trout (Holm et al. 2005). There were significant relationships between plasma estradiol or testosterone and liver or muscle Se concentrations in our study with adult sexually mature females, in contrast to a study with juvenile rainbow trout fed SeMet for 126 days (Wiseman et al. 2011a).

The present study, in addition to species differences in tissue Se allocation and reproductive effects, detected species differences in other physiological effects of Se. Plasma thyroid hormones were measured since Se is a component of iodothyronine deiodinase, which transforms the precursor thyroxine (T4) into triiodothyronine (T3), the active form (Pappas et al. 2008). Brook trout had increased T3:T4 with increased liver and muscle Se, demonstrating a potentially important difference between brook trout (*Salvelinus*), and rainbow and cutthroat trout (*Oncorhynchus*). Brook trout may up-regulate their metabolism (increase plasma T3) as Se tissue concentrations increase, however the mechanism and consequence of this physiological change requires further research.

The mechanism of Se toxicity is thought to be oxidative stress (Janz 2012), as demonstrated by Misra and Niyogi (2009) in hepatocytes of rainbow trout. In the present study, only rainbow trout had evidence of cellular damage through lipid peroxidation (LPO), as liver LPO was correlated to increasing liver Se. Glutathione (GSH), an antioxidant known to redox cycle with Se and generate free radicals (Palace et al. 2004, Spallholz et al. 2004, Chapman et al. 2010), was also measured in liver. Glutathione increased with increased Se in liver tissue for rainbow trout and cutthroat trout. These results provide further evidence for oxidative stress, through GSH-Se cycling, as the Se toxicity mechanism for rainbow trout, as there is an increase in both LPO and GSH with increasing liver Se. However, cutthroat trout only had an increase in GSH, but not LPO. The GSH redox cycle may not have been altered in this species, as LPO did not increase with increased liver Se. A slight but significant increase in plasma cortisol correlated with increasing liver Se concentration in cutthroat trout, but not in rainbow trout or brook trout. These unique responses in cutthroat trout require further study. Brook trout had no trends in relation to LPO, GSH and Se, although Se toxicity was still observed as mortality in adults, providing evidence that Se-induced oxidative stress may not be the only toxicity mechanism.

This comparative study provided evidence for species-specific Se accumulation in muscle, liver, and egg tissue of rainbow trout, brook trout, and cutthroat trout, with rainbow trout and cutthroat trout storing a higher proportion of Se in egg tissue, and brook trout storing a higher proportion in muscle tissue. Evidence for a link between GSH-Se cycling and oxidative stress was provided for rainbow trout but not the other

two species, indicating mechanisms of Se toxicity other than oxidative stress may be involved. Although adult cutthroat trout accumulated high concentrations of Se in all three tissues there were no detectable detrimental effects. Brook trout, the species expected to be most tolerant to Se, had severe negative physiological effects manifested as increased mortality and failure to develop a gonad above 19 µg/g Se in liver and 3.6 µg/g Se in muscle. Considered the most sensitive species, rainbow trout adult females had evidence of sub-lethal effects oxidative stress in liver tissue. These differences have important consequences for management of fisheries at various anthropogenic impacted aquatic habitats.

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Figures and Tables

Table 2.1 Concentration of Se (mean \pm SEM) measured for each tissue in each dietary treatment group. Diet Se concentrations expressed in $\mu\text{g/g}$ as ww, are the actual concentrations measured by ICPMS. RN-rainbow trout, BK-brook trout, and CT-cutthroat trout.

Species	Diet ($\mu\text{g/g}$)	Muscle ($\mu\text{g/g}$)	Liver ($\mu\text{g/g}$)	Egg ($\mu\text{g/g}$)
RN	1.47 \pm 1.00	0.21 \pm 0.01 ^A	3.77 \pm 0.66 ^A	1.17 \pm 0.14 ^A
	12.7 \pm 0.48	0.51 \pm 0.10 ^{AB}	6.53 \pm 1.67 ^{AB}	4.30 \pm 1.04 ^A
	35.2 \pm 3.78	0.74 \pm 0.15 ^B	17.21 \pm 5.03 ^B	13.0 \pm 2.77 ^B
	R ²	32%	31%	45%
BK	1.47 \pm 1.00	0.23 \pm 0.01 ^a	0.72 \pm 0.23 ^a	0.81 \pm 0.14 ^a
	12.7 \pm 0.48	1.14 \pm 0.09 ^b	7.23 \pm 0.84 ^b	5.01 \pm 0.57 ^b
	35.2 \pm 3.78	3.41 \pm 0.37 ^c	20.4 \pm 3.09 ^c	8.15 \pm 1.11 ^c
	R ²	76%	65%	60%
CT	1.47 \pm 1.00	0.31 \pm 0.07 ^{α}	1.00 \pm 0.07 ^{α}	2.02 \pm 0.24 ^{α}
	12.7 \pm 0.48	0.93 \pm 0.11 ^{α}	6.00 \pm 0.43 ^{β}	9.80 \pm 1.04 ^{β}
	35.2 \pm 3.78	2.05 \pm 0.27 ^{β}	14.4 \pm 1.61 ^{σ}	18.0 \pm 1.95 ^{σ}
	R ²	41%	53%	46%

Letters indicate significant differences between treatments for each species in the specified tissue i.e. A, B, C for rainbow trout, a, b, c for brook trout and α , β , σ for cutthroat trout ($p < 0.05$). R² value represents the amount of variation in the end point that can be accounted for by Se dietary treatment, therefore it is a measure of the 'relatedness' of data points based on the specified treatment.

Table 2.2 GSI, plasma hormones, and liver oxidative stress (mean \pm SEM) measured in each dietary treatment group. Diet Se concentrations are the actual concentrations per ww, measured by ICPMS. RN-rainbow trout, BK-brook trout, and CT-cutthroat trout.

Species	Diet ($\mu\text{g/g}$)	GSI (%)	T3:T4	Cortisol (ng/mL)	LPO ($\mu\text{mol/mg protein}$)	GSH ($\mu\text{mol/mg protein}$)
RN	1.47 \pm 1.00	22.8 \pm 2.0	0.32 \pm 0.03 ^A	216 \pm 27 ^A	0.38 \pm 0.13 ^A	54.2 \pm 9.1 ^A
	12.7 \pm 0.48	16.7 \pm 1.9	0.45 \pm 0.03 ^B	114 \pm 27 ^B	1.08 \pm 0.13 ^B	94.0 \pm 8.6 ^B
	35.2 \pm 3.78	19.3 \pm 1.8	0.33 \pm 0.03 ^A	78.8 \pm 25 ^B	0.90 \pm 0.12 ^B	82.3 \pm 8.2 ^{AB}
	R ²	15%	30%	4%	40%	28%
BK	1.47 \pm 1.00	36.3 \pm 2.5 ^a	0.33 \pm 0.13 ^a	79.7 \pm 30	0.11 \pm 0.03	19.5 \pm 7.8 ^a
	12.7 \pm 0.48	39.6 \pm 1.9 ^a	0.79 \pm 0.11 ^b	109 \pm 24	0.14 \pm 0.02	43.7 \pm 6.0 ^b
	35.2 \pm 3.78	4.29 \pm 2.9 ^b	1.24 \pm 0.13 ^c	68 \pm 29	0.11 \pm 0.03	31.7 \pm 7.2 ^{ab}
	R ²	78%	41%	4%	4%	16%
CT	1.47 \pm 1.00	16.8 \pm 0.75	0.36 \pm 0.05 ^{α}	148 \pm 15	0.10 \pm 0.02	69.1 \pm 15.24 ^{α}
	12.7 \pm 0.48	17.9 \pm 0.57	0.47 \pm 0.04 ^{$\alpha\beta$}	162 \pm 11	0.12 \pm 0.01	134 \pm 11 ^{β}
	35.2 \pm 3.78	17.9 \pm 0.57	0.53 \pm 0.04 ^{β}	182 \pm 11	0.08 \pm 0.01	178 \pm 11 ^{σ}
	R ²	6%	12%	7%	7%	40%

Letters indicate significant differences between treatments for each species in the specified tissue ie. A, B, C for rainbow trout, a, b, c for brook trout and α , β , σ for cutthroat trout. R² value represents the amount of variation in the end point that can be accounted for by Se dietary treatment, therefore it is a measure of the ‘relatedness’ of data points based on the specified treatment.

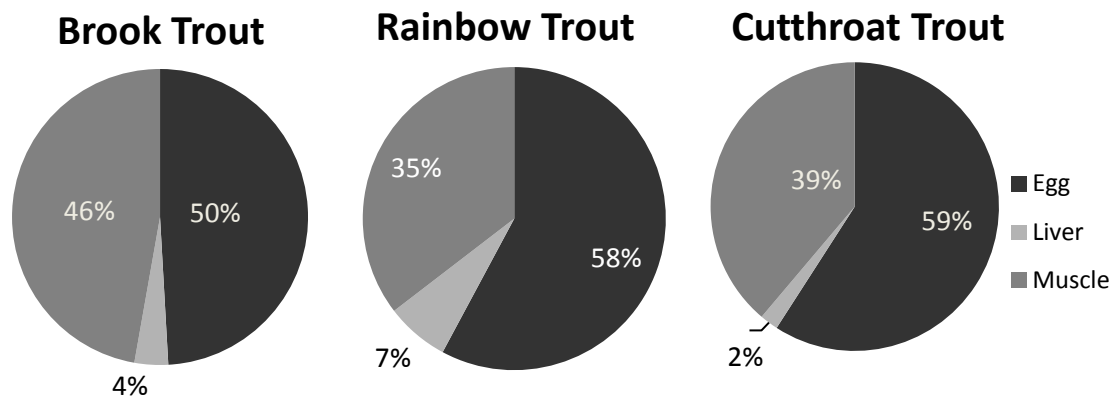
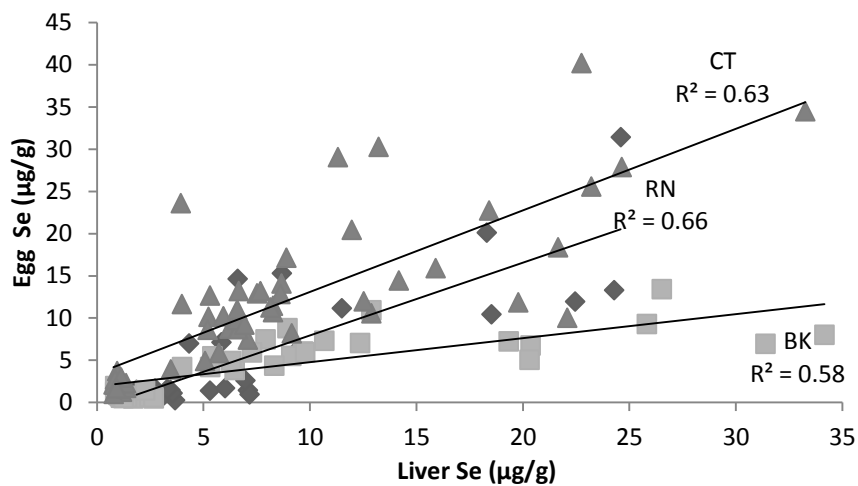
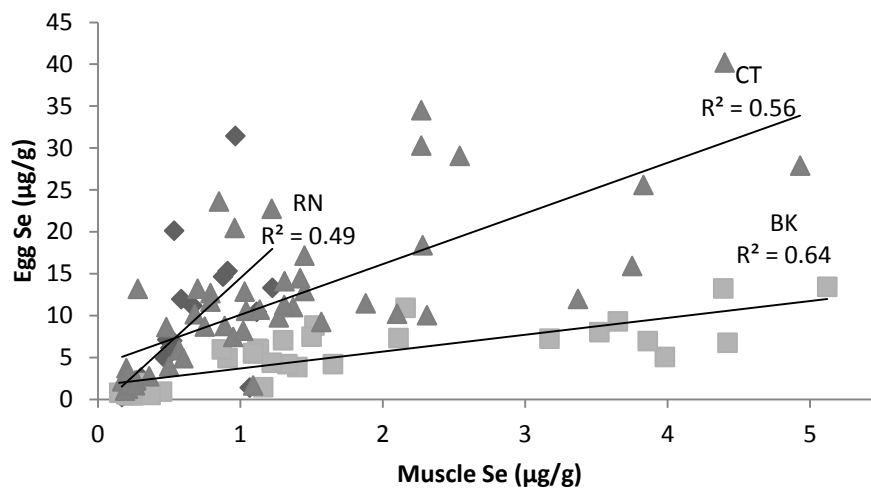


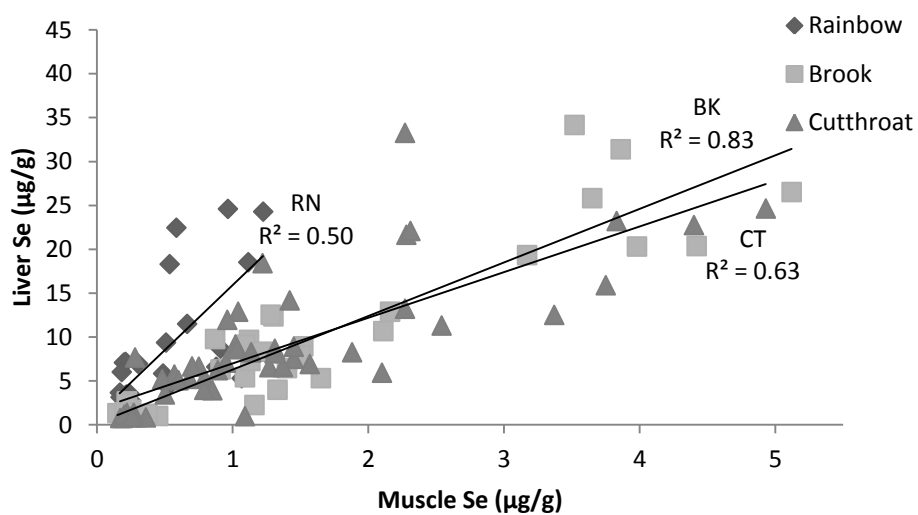
Figure 2.1 Allocation of the Se body burden (expressed as % of total body Se) in eggs, liver and muscle of female rainbow trout, brook trout and cutthroat trout fed SeMet for five months. Egg allocation rainbow=(brook<cutthroat), liver allocation cutthroat<brook<rainbow, muscle allocation cutthroat trout=(rainbow<brook), $p<0.05$.



a)



b)



c)

Figure 2.2 Linear regression a) between egg and liver Se concentrations measured in the same individual fish (RN=rainbow trout, BK=brook trout, CT=cutthroat trout) fed SeMet diet for five months ($p<0.0001$), regression equations for RN: $y=0.8622x - 0.6822$, BK $y=0.2839x + 1.9439$, CT $y=0.9667x + 3.4157$; b) between egg and muscle Se, RN $y=15.576x - 1.0858$, BK $y=2.0168x + 1.6632$, CT $y=6.0524x + 4.0317$, and c) between muscle and liver Se RN $y=14.801x + 1.0897$, BK $y=6.1068x + 0.1785$, CT $y=5.2037x + 1.7704$.

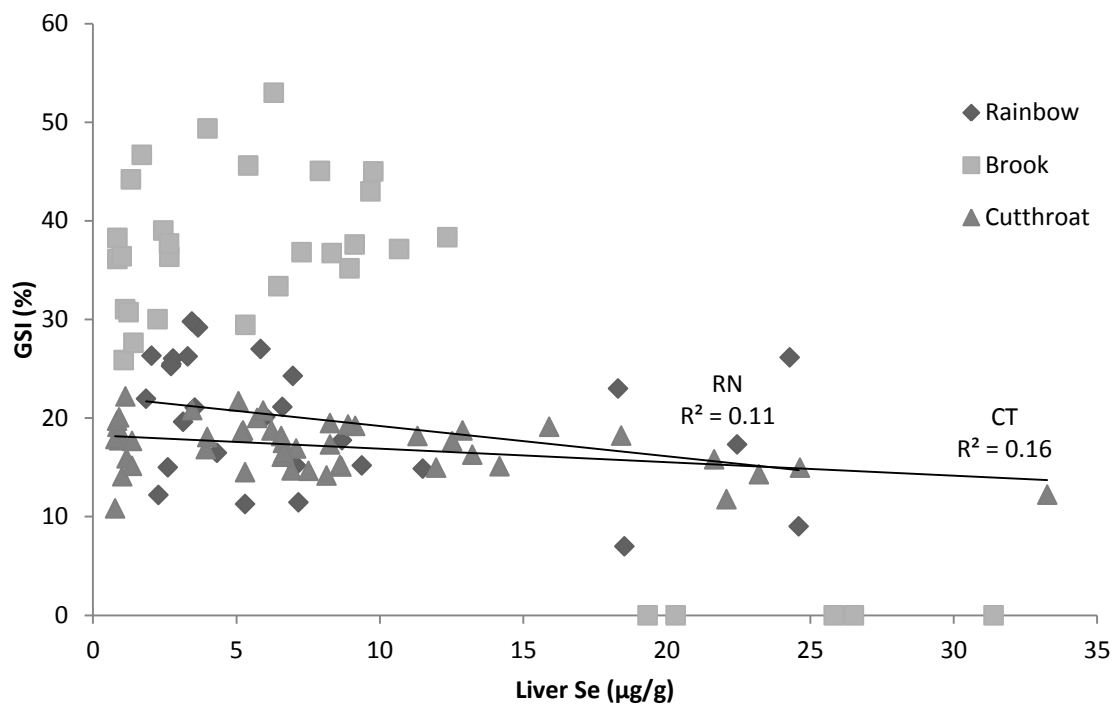


Figure 2.3 Gonadosomatic Index (GSI, [total egg mass spawned/body weight]×100) represented as a linear regression with liver Se for rainbow trout, brook trout, and cutthroat trout fed SeMet diets for five months ($p < 0.04$). Regression equations: RN $y = -0.3062x + 22.261$, CT $y = -0.1369x + 18.262$. RN=rainbow trout, CT=cutthroat trout.

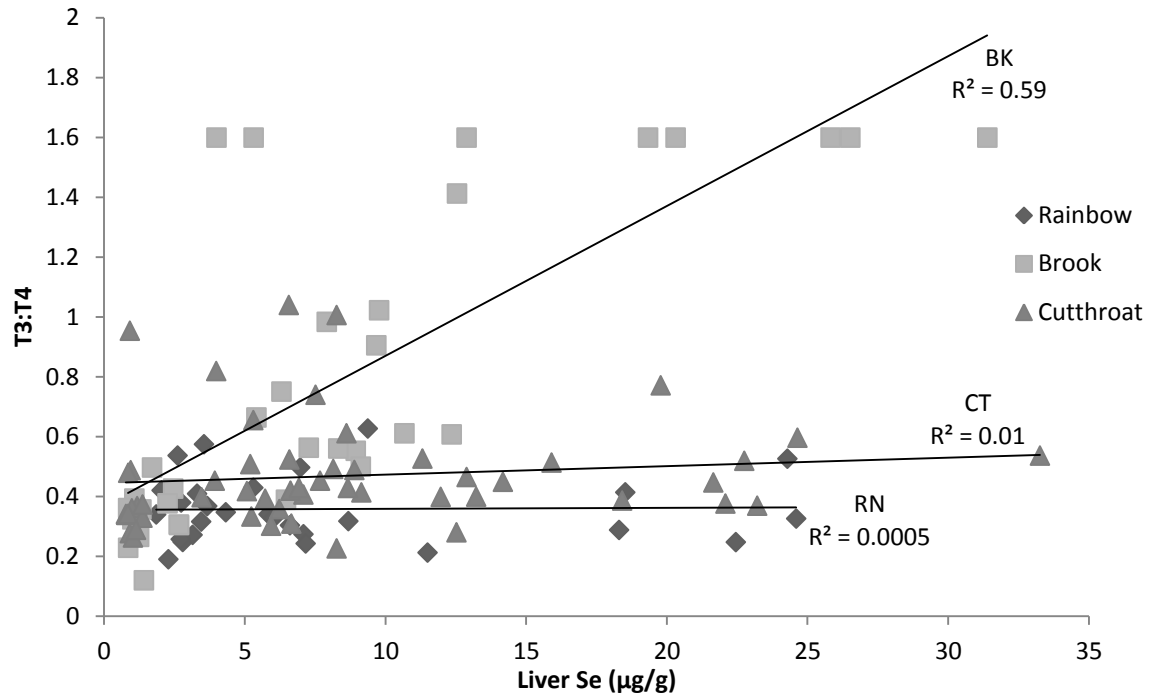
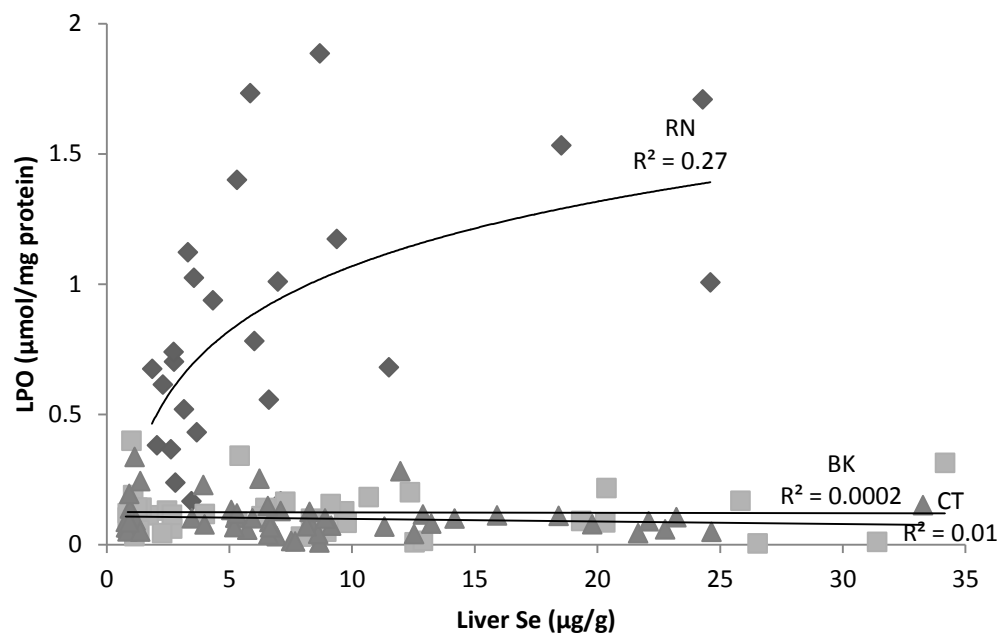
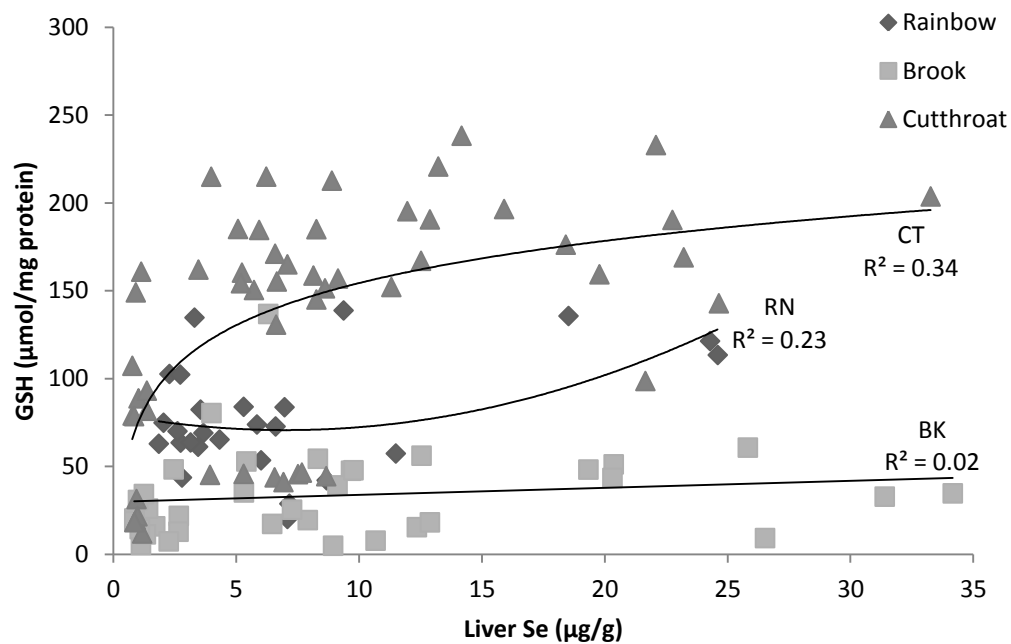


Figure 2.4 Linear regression a) between liver Se and plasma T3:T4 in brook trout fed SeMet diets for five months ($p < 0.0001$). No significant relationship was observed for rainbow trout or cutthroat trout. Regression equations: RN $y = 0.0003x + 0.3562$, BK $y = 0.0501x + 0.3698$, CT $y = 0.0028x + 0.4452$. RN=rainbow trout, BK=brook trout, CT=cutthroat trout. Each symbol is the value obtained for an individual female.



a)



b)

Figure 2.5 Regressions between liver Se concentration and a) liver lipid peroxidation (LPO) ($p=0.0292$), b) liver glutathione (GSH) (cutthroat $p<0.0001$ and rainbow $p=0.0495$). Trout were fed SeMet diets for five months. A significant relationship was not found for brook trout. Regression equations: a) RN $y=0.3578\ln(x) + 0.2449$, BK $y = -0.0001x + 0.1252$, CT $y=-0.001x + 0.1088$, b) RN $y=0.1856x^2 - 2.6053x + 79.812$, BK $y=0.4024x + 29.782$, CT $y=34.631\ln(x) + 74.611$. RN=rainbow trout, BK=brook trout, CT=cutthroat trout.

CHAPTER 3

SURVIVAL, MALFORMATIONS, AND SWIM-UP SUCCESS OF RAINBOW TROUT, BROOK TROUT, AND CUTTHROAT TROUT LARVAE EXPOSED TO MATERNALLY TRANSFERRED SELENIUM

Abstract

Selenium (Se), a required element, has an important function in its association with selenoproteins, including glutathione peroxidase, but it becomes toxic at concentrations only slightly above those required for homeostasis. Fish, especially in early developmental stages, are sensitive to Se toxicity because Se is transferred from the female to developing embryos, causing oxidative stress and malformations. Selenium toxicity varies among closely related species; for example rainbow trout are more sensitive than brook trout. This study examined the effect of Se toxicity on larvae from adult rainbow trout, brook trout, and cutthroat trout fed Se-methionine enriched diets for five months in the laboratory. Larval survival and malformations were compared in the three fish species using a vertical incubator, and swim-up success was estimated in a custom built gravel-bed flume system. Results show decreased survival with increasing egg Se for rainbow trout and cutthroat trout larvae in all developmental stages, while Se decreased survival in brook trout only during the period of development from spawned to eyed eggs. Rainbow trout and cutthroat trout also had decreased swim-up success through gravel in the experimental flumes with increased egg Se, but there was no effect in brook trout. Malformations were evaluated based on a graduated severity index, however the only significant trend detected was an increase in edema for cutthroat trout with increasing egg Se. Survival and swim-up success rather than malformations were identified as highly sensitive endpoints of Se toxicity. This study provides new comparative Se toxicity data for larvae of three salmonids exposed under controlled laboratory conditions.

Introduction

Selenium (Se) can be toxic at concentrations only slightly above those required, and its range of homeostatic concentrations is very narrow (Janz 2012). Selenium is released into the environment through anthropogenic sources such as coal and uranium mining, and irrigation of Se-enriched soils. It reaches the aquatic ecosystem in inorganic form (selenite, selenate), and is converted into organic forms by microbes, algae and plants (Lemly 1999, Hamilton 2004, Chapman et al. 2010). Selenium methionine (SeMet) is the organic form of Se that is transferred through the food chain and can accumulate in fish tissue.

As a required element, Se has important cellular functions, particularly in its association with glutathione peroxidase (GPx). Glutathione peroxidase catalyzes the reaction of reduced glutathione (GSH) into oxidized glutathione (GSSG) allowing GSH to act as an antioxidant by being an electron donor (Pappas et al. 2008). Although GSH is an antioxidant, it has been suggested that oxidative damage occurs when certain Se compounds conjugate with GSH and cycle to create superoxide radicals (Palace et al. 2004, Spallholz et al. 2004, Misra and Niyogi 2009). Palace et al. (2004) detected superoxide radicals increased in rainbow trout (*Oncorhynchus mykiss*) larvae that were homogenized with SeMet and GSH but not in larvae that were homogenized with methionine instead of SeMet. Misra and Niyogi (2009) investigated the oxidative stress mechanism further, exposing rainbow trout hepatocytes to selenite *in vitro*. They provided strong evidence that oxidative damage to rainbow trout hepatocytes was caused by inducing imbalance in the GSH redox cycle, a result of Se interaction with

GSH. Selenium toxicity also occurs in larvae when Se accumulated in the female's liver is transported during vitellogenesis to embryos and developing larvae (Lemly 1999, Janz et al. 2010). Larvae can develop malformations such as a deformed spine, head or jaw, missing or malformed fins, and edema surrounding eyes or heart which has been associated with Se-generated oxidative stress (Holm et al. 2005, Rudolph et al. 2008, Janz et al. 2010).

There is evidence to suggest that Se toxicity is species-specific. Rainbow trout are considered to be highly sensitive to Se, with an EC_{10} (effective concentration for 10% of the population) for skeletal deformities at 21.1 mg Se/kg in eggs, whereas brook trout (*Salvelinus fontinalis*) have a no effect concentration (NOEC) for craniofacial deformities at >20.5 mg Se/kg in eggs (Holm et al. 2005, calculated by Chapman et al. 2010). Cutthroat trout (*Oncorhynchus clarkia*), have a reported NOEC for embryo/larval deformities of >21.2 (Kennedy et al. 2000) and >16.04 mg Se/kg in eggs (Hardy et al. 2010), however another study suggested an EC_{10} for alevin survival at 17-24.1 mg Se/kg in eggs (Rudolph et al. 2008, calculated by Chapman et al. 2010). Even though the available evidence strongly suggests that differences in sensitivity to Se exist among different fish species, the data are not truly comparable since most studies focused on one species, either in the field or laboratory, under various exposures, and measured different toxicity endpoints (see Janz et al. 2010 for review).

This present study was designed to compare the effects of chronic dietary maternal exposures to SeMet, during ovarian recrudescence, on developing larvae of rainbow trout, brook trout, and cutthroat trout. Larval survival rates, rates of

malformations (spinal, craniofacial, edema, and finfold) and for the first time, the swim-up success using experimental gravel-bed flumes, were compared in the three fish species exposed to Se under the same conditions, to better understand Se toxicity in the F1 generation and species differences in sensitivity.

Materials and Methods

Experimental Diets

Experimental diets were prepared from ground EWOS® (EWOS Canada, Ltd.) trout chow, distilled water and gelatin. The control experimental diet was made with 3.6 kg ground EWOS® trout chow and 2.2 L deionized distilled water containing 80 g of gelatin powder, as described in Holm (2002). Selenium methionine (Sigma Aldrich, Catalogue # S3132) was added to the water/gelatin mixture to create the low Se diet (15 µg/g) and high Se diet (40 µg/g). The dough was mixed for 20 min in a 28.39 L Hobard® mixer, then pressed through a perforated disc (diameter=5 mm) to create long noodles. The noodles, left to dry in a ventilated room, were turned over every two hours until the end of the day to allow air circulation and prevent moulding. After drying overnight the noodles were broken into small pellets by hand and kept frozen until required.

Adult Females

Mature females were selected from an existing brood stock and fed the experimental diets at Allison Creek Trout Brood Station (Coleman, AB, Canada) for five months. Five-year-old rainbows (n=36, length=64 ± 0.70 cm, weight=3778 ± 0.15 g)

were held in 1000 L tanks, and three-year-old brook trout ($n=50$, length= 40 ± 0.38 cm, weight= 1002 ± 39.19 g) and three-year-old cutthroats ($n=60$, length= 36 ± 0.27 cm, weight= 511 ± 11.70 g) were held in 710 L tanks until spawning and egg collection. Each tank also held one male to stimulate ovarian maturation. Water temperature was maintained at 10 °C, pH 8, and dissolved O₂ at 8-9 mg/L. Rainbow trout were fed the experimental diets from August - December 2009, brook trout July - November 2010, and cutthroat trout December 2010 - April 2011.

Egg Collection and Incubation

As fish showed signs of maturation (a soft underside) they were injected with Ovaprim® (Syndel Laboratories Ltd.) at 0.5 mL/kg body weight to stimulate spawning. A week later, fish were euthanized with MS-222 (0.1 g/L, Sigma Aldrich, Catalogue #E10521) and each clutch of eggs was extruded into labelled, individual bowls. Milt from 3-5 control males were used to fertilize the eggs and saline solution (0.6% NaCl) was added to aid sperm motility. Fertilized eggs were rinsed after two minutes and placed into a labeled pail containing water for two hours to water-harden.

When eggs were firm to the touch, a subsample from each clutch was placed single file on a 30 cm × 2 cm V-shaped trough and counted. Number of eggs/L and average size of eggs was determined for each adult female using the von Bayer egg chart (von Bayer 1950). Total volume of eggs produced per female was determined using glass beakers before eggs were placed in a vertical incubator tray and sterilized with a 1% Ovadine® (Syndel Laboratories Ltd.) for 5 minutes (iodophor treatment is

common in fish culture) and placed in a vertical incubator. Eggs were incubated at the Allison Creek Trout Brood Station until the eyed stage (visible eye spots) and then a portion from each treatment was shipped to the University of Lethbridge Aquatic Research Facility for the flume swim-up experiment. Eggs were shipped in porous plastic tubes, wrapped in wet foam, in a cooler with ice by ground (150 km).

Tubes containing eggs were soaked in a 1% Ovadine[®] solution for five minutes then rinsed with dechlorinated water at the University of Lethbridge. The volume of eggs from individual females was measured to ensure that a known number of eyed eggs from each female were deposited into individual labeled compartments in the experimental gravel-bed flumes (see Chapter 4 for structural details).

Eggs left at the Allison Creek Trout Brood Station were kept in the vertical incubator until larvae hatched and absorbed their yolk sac. The fish were then euthanized with MS-222 (0.1 g/L) and stored in Davidson's solution (50% formalin, 10% acetic acid, 10% glycerol, 30% ethanol) until morphometric analysis of malformations.

Flume Experiment

Three flumes, made out of plexiglass with 19 screened dividers, were filled with washed round rock (1:1, 14 mm and 28 mm diameter) and used to simulate redds in a riverbed. For more details see Chapter 4 Methods (*Flume Design, Gravel-bed Simulation, and Water Parameters*). Eggs (at eyed stage) were gently poured in an oval-shape formation onto the gravel-bed in each compartment of the flume to simulate a redd. Gravel was then added gently by hand until the eggs were fully covered and then

the rest of the gravel was poured on to reach 20 cm. Eggs were buried under 10 cm (brook trout and cutthroat trout) or 15 cm (rainbow trout) of gravel (Crisp and Carling 1989, Kondolf 2000). Fish larvae (swim-up) that appeared above the gravel were collected every day using a small net and a large pipette. Fish were euthanized with MS-222 (0.1 g/L), counted, and stored in jars of Davidson's solution. Fry collected from each compartment were stored in one jar.

Morphometric Analysis

Preserved larvae were graded in four malformation categories: spinal, craniofacial, edema, and finfold (McDonald and Chapman, 2009). Larvae were given a score from 0-3 based on severity of malformation in each category. A score of 0 would indicate normal, while a 3 would indicate severely malformed. Pictures of 'calibrator' larvae in each category at each severity were used as a reference while grading (see Supplementary Data Fig. 1). A second researcher reanalyzed 20% of the scored larvae to ensure quality control. A hundred larvae from each adult female were scored. Malformation results are based on moderate (2) and severe (3) scores. Total deformities were calculated based on the percentage of larvae that received a moderate or severe score in any category.

Selenium Analysis

Total Se in eggs and experimental diets were measured at the Department of Fisheries and Oceans (DFO) analytical laboratory in Winnipeg, MB, Canada by hydride

generation–atomic absorption (detection limit: 0.05 µg/g) previously described by Miller et al. (2009). All tissue and diet values are expressed as wet weight (ww) unless otherwise stated.

Calculations

Percent survival in the first stage of development (spawned eggs to eyed eggs), was determined using $\% \text{ survival} = (\text{number of eggs at eyed stage} / \text{number of eggs spawned}) \times 100$. In the second stage of larvae development (eyed eggs to yolk-absorbed fry), was calculated using $\% \text{ survival} = (\text{number of fry} / \text{number of eyed eggs}) \times 100$. Lethal concentration for 10% of the population (LC_{10}) values for Fig. 3 were calculated using regression equations obtained from Fig. 3 and setting the intercept to 100% survival to remove the effect caused by factors other than Se. In Fig. 5, LC_{10} s were calculated using regression equations from Fig. 5 with the intercept set at 0% impacted larvae. Swim-up success was determined using the equation $\% \text{ swim-up} = (\text{number of eggs deposited in gravel} / \text{number fish caught above gravel}) \times 100$. The percent of larvae impacted by Se was calculated using $\% \text{ impacted} = (\text{number of eggs spawned} / \text{number of fry} \times 100) + (\text{total number of larvae with at least one malformation graded as a 2 or 3 in any category} / \text{total number of larvae evaluated}) \times 100$.

Statistical Analysis

Linear or logarithmic regressions were used to relate variables in the statistics program JMP. The type of regression used was based on normality of residuals from an

ANOVA. Data was analyzed for significant relationships with an ANOVA, followed by a Tukey or Student's *t*-test post-hoc for species comparisons.

Results

Table 3.1 shows the average egg Se concentration, percent survival to stage 1 (spawned to eyed eggs) and 2 (eyed eggs to fry), percent swim-up success, and total percent malformations measured in each dietary treatment group, including the amount of variance (R^2) that is explained by diet. The amount of variance attributed to diet differed between species and end points measured. The variance in egg Se values was mainly due to diet for all species (45-60%). Variance caused by diet differed between larval end points with rainbow trout ranging from 12-53%, brook trout from 0.5-26%, and cutthroat trout from 19-72%.

A negative regression between survival of eggs up to the eyed stage and egg Se concentration was observed in the three trout species (Fig. 3.1a, rainbow $R^2 = 0.46$, $p < 0.0001$, brook $R^2 = 0.22$, $p = 0.0110$, cutthroat $R^2 = 0.12$, $p = 0.0196$). However, there was no significant difference between regression slopes of the three species (brook -4.55, rainbow -3.12, cutthroat -1.42). When analyzed as dietary treatments there was no difference in the first stage of survival between control, low, and high diet groups for either rainbow trout or brook trout (Table 3.1, $p < 0.05$). The LC_{10} for each species in the developmental stage of spawned to eyed eggs (Fig. 3.1a) was calculated as: brook trout 2.22 $\mu\text{g/g}$ Se in egg tissue, rainbow trout 3.21 $\mu\text{g/g}$, and cutthroat trout 7.04 $\mu\text{g/g}$ (intercept set at 100%).

Rainbow trout and cutthroat trout exhibited a negative correlation (Fig. 3.1b, rainbow $R^2=0.19$, cutthroat $R^2=0.52$) between percent survival in the next developmental stage, eyed eggs to fry, and egg Se concentration (rainbow $p=0.0240$, cutthroat $p<0.0001$). Both species had similar regression slopes (rainbow trout -3.22, cutthroat trout -3.55). Brook trout however did not show a significant correlation between survival and egg Se except when analyzed as diet treatments where low was significantly different from high (Table 3.1, $p<0.05$). No difference was found between rainbow trout treatment groups at this stage. The LC_{10} (Fig. 3.1b) for these species was calculated as: rainbow trout 3.12 $\mu\text{g/g}$ Se in egg tissue and cutthroat trout 2.82 $\mu\text{g/g}$.

Swim-up success in stream-bed flumes was correlated with egg Se concentration (Fig. 3.2). Rainbow trout and cutthroat trout showed a negative regression (rainbow $R^2=0.28$, cutthroat $R^2=0.44$, rainbow slope=-2.89, cutthroat slope=-3.15), while brook trout did not have a significant relationship (rainbow $p=0.0125$, cutthroat $p=0.0010$). Only the high Se treatment was different from control for rainbow trout (Table 3.1, $p<0.05$).

Figure 3.3 shows percent impacted larvae, including total mortality and total malformations of the remaining population. Rainbow trout and cutthroat trout had a logarithmic trend (rainbow $R^2=0.22$, cutthroat $R^2=0.33$, $p=\text{rainbow } 0.0205$, cutthroat $p<0.0001$), whereas brook trout did not show any significant trends. The fitted equation for rainbow trout was $y=7.8498\ln(x) + 69.548$, and cutthroat trout was $y=5.6597\ln(x) + 84.283$. The EC_{10} calculated for rainbow trout was 3.57 $\mu\text{g/g}$ Se in eggs and cutthroat trout 5.85 $\mu\text{g/g}$ (intercept set at 0).

Figure 3.4 shows significant relationships between malformations (evaluated in the categories spinal, craniofacial, edema, finfold, and total) and egg Se concentrations, related on an individual basis (100 larvae analyzed per female). The only significant trend detected was a logarithmic regression for edema malformations in cutthroat trout ($y=1.8344\ln(x) + 0.2237$, $R^2=0.26$, $p=0.0149$). Total malformations, analyzed as dietary treatment groups, were found to be significantly different in rainbow trout high Se (Table 3.1, $p<0.05$). Cutthroat trout also had a significant difference between control and low groups ($p<0.05$).

Discussion

This study investigated the effects of Se on larvae of adult female rainbow trout, brook trout, and cutthroat trout fed experimental diets containing SeMet. Adults were fed control (0 $\mu\text{g/g}$), low (15 $\mu\text{g/g}$), or high (40 $\mu\text{g/g}$) experimental diets for five months before spawning. Fertilized eggs were incubated until the eyed stage, deposited under gravel for the swim-up experiment or maintained in the incubator until the yolk was absorbed. A negative correlation between egg Se and survival up to the eyed stage was observed in all three species, while survival to the fry stage was affected by Se in rainbow trout and cutthroat trout but not brook trout. Moreover, for the first time, using experimental gravel bed flumes, decreased swim-up success correlated to egg Se was observed in rainbow trout and cutthroat trout larvae but not brook trout. Malformations in the larvae were not associated with egg Se concentrations, except for edema in cutthroat trout.

Egg Se and larval fitness endpoints were averaged per treatment for each species as a comparison to other dietary studies where tissue Se has not been measured. This study found that although much of the variance in egg Se values could be explained by diet treatments, this did not hold true for the other endpoints measured where R^2 values differed greatly depending on the species and parameter analyzed. These results suggest that it can be inaccurate to group individuals by dietary treatment as it does not explain the majority of the variance and can generate inaccurate, and sometimes misleading, results.

The present study is the first chronic laboratory investigation where three species of salmonids are exposed to Se through diet under the same controlled conditions. There is evidence that the mechanism of Se toxicity in adult salmonids is oxidative stress caused by Se induced disruption of the GSH redox cycle (Misra and Niyogi 2009). The same mechanism is assumed to cause toxicity in larvae when females transfer Se, in the form of SeMet, from the liver to the developing embryos, as a part of precursors to yolk formation (Lemly 1999). It is thought that larvae are exposed to Se when absorbing the yolk, which causes oxidative stress resulting in deformities and edema (Palace et al. 2004). However, in this study we obtained evidence of toxicity before yolk absorption was completed. Larval survival in the 'first stage' of development (spawned eggs to eyed eggs), before the yolk had been absorbed, decreased with increased egg Se concentrations in rainbow trout, brook trout, and cutthroat trout. Selenium exposure in the early developmental stages could be occurring when vitellogenin is enzymatically cleaved into the primary yolk proteins

lipovitellin and phosvitin, causing oxidative stress in egg follicles. This hypothesis should be tested in future studies; oxidative stress in the larvae was not measured in the present study.

Our results indicate that there are significant differences between species in the susceptibility of larvae to Se toxicity. Rainbow trout and cutthroat trout had decreased survival in both the first stage (spawned to eyed eggs) and second stage of development (eyed eggs to fry), however brook trout only show decreased survival in the first stage at the same egg Se concentrations. Susceptibility to Se toxicity at different points in development could be species-specific, due to differences in the development of antioxidants (Palace et al. 2004). The first developmental stage is a vulnerable period for salmonid eggs, therefore it might be expected, as observed in this study, that Se toxicity would be detected in all three species at this stage (Leitritz and Lewis 1980, Piper et al. 1982, Wedemeyer 2001). In the next stage of development (eyed eggs to fry), brook trout larvae were not affected by Se, while survival of rainbow trout and cutthroat trout larvae decreased. It is possible that in brook trout (genus *Salvelinus*) larvae, antioxidant defense has become more efficient at this stage than in rainbow trout and cutthroat (genus *Oncorhynchus*) larvae, enabling brook trout to detoxify Se-generated radicals and prevent mortality. There is not much information in the literature about timing and development of antioxidants in salmonid embryos, but studies with turbot (Peters and Livingstone 1996), dentex (Mourente et al. 1999), sturgeon (Díaz et al. 2010), and rainbow trout (Aceto et al. 1994, Fontagné et al. 2008) suggest variation in time, concentration during development, and the types of

antioxidants present at each stage of development (Aceto et al. 1994). Sensitivity to Se toxicity could also be dependent specifically on when the GSH becomes available in the developing larvae, because Se is known to redox cycle with GSH to create reactive oxygen species, leading to cellular damage and possibly mortality. There is an urgent need for investigations characterizing the maturation of the antioxidant defenses in fish embryos and larvae.

One of the first challenges salmonid larvae face in the natural environment is swimming through the river-bed where fertilized eggs were buried by the spawning female. There is substantial evidence that Se causes malformations in larvae (Janz et al. 2010, for review), but there have not been any studies that directly link Se-caused malformations and swim-up success. In our study, the swim-up success of larvae of the three salmonid species was evaluated in experimental gravel-bed flumes (Chapter 4) designed to mimic gravel redds and test environmentally relevant effects of Se toxicity in larvae. Our results show that larvae of rainbow trout and cutthroat trout had decreased swim-up success with increasing egg Se concentration, however such a dose-dependent effect of Se on swim-up success was not observed in brook trout larvae. In fact, cutthroat trout had seven clutches all with egg Se concentrations above 10 µg/g where 0-1 larvae appeared above the gravel. These data provide conclusive evidence that Se toxicity in rainbow trout and cutthroat trout larvae causes them to be unfit and unable to overcome environmentally relevant challenges such as swim up through a gravel redd. Such an effect can lead to decreased population recruitment by resident populations and, in the case of threatened species such as Athabasca rainbow trout,

lead to extinction. It is important to note that all fish larvae that successfully completed the swim-up in the gravel bed flumes were morphologically normal, not exhibiting any malformations. Our results imply that the malformed larvae died under the gravel. Future studies could test this hypothesis and terminate the swim-up experiment at various times of development, to determine when the larvae which are not to complete the swim-up die.

Malformation analysis in four categories (spinal, craniofacial, edema, and finfold) was completed on yolk-absorbed fry raised in a vertical incubator. The only significant correlation detected between egg Se load and toxicity was in the edema category for cutthroat trout. Larvae malformations are considered a powerful diagnostic endpoint of Se toxicity, yet in our study we did not observe this effect. Given the major adverse effect of maternally transferred Se on larval survival in this study, we hypothesize that any larvae that would have a Se-induced malformation died before reaching the fry stage. However, we were unable to examine larvae mortalities as they had molded immediately. Mortality data from the entire experiment (spawned eggs to fry) and total malformations were represented as 'impacted larvae' in correlation with egg Se concentrations (Fig. 3.3). Using this analysis, a significant trend was detected for rainbow trout and cutthroat trout larvae, but not brook trout.

The LC_{10} based on larval survival (Fig. 3.1a and b) and larval malformations and survival (Fig. 3.3), for each species were calculated as: rainbow trout 12.44-14.28 $\mu\text{g/g}$ dw (3.11-3.57 $\mu\text{g/g}$ ww), brook trout 8.92 $\mu\text{g/g}$ dw (2.23 $\mu\text{g/g}$ ww, Fig. 3.1a only) and cutthroat trout 11.24-23.72 $\mu\text{g/g}$ dw (2.81-5.93 $\mu\text{g/g}$ ww) Se in egg tissue (using 75%

moisture content in tissue when converting from ww to dw Se concentration). These values are lower and based on a more severe endpoint (mortality), compared to those reported in the literature from field studies (rainbow EC₁₀ skeletal deformities 21.1 mg/kg Se dw, brook NOEC craniofacial deformities >20.5 mg/kg Se dw, and cutthroat EC₁₀ alevin survival 17-24.1 mg/kg Se dw, all in egg tissue). This brings into question the idea of acquired Se tolerance, as first suggested by Kennedy et al. (2000). Little is known about tolerance to Se in fish, however acquired tolerance to other metals has been reviewed (Chapman 2008, Chapman et al. 2010). Since our study used naive hatchery fish and previous studies have focused on fish living in Se contaminated areas, there is the possibility that Se tolerance exists and explains why fish from the field have higher Se tissue accumulation before detrimental effects occur than the naive fish used in this laboratory study.

Se toxicity is species-specific, as suggested by previous studies and confirmed in the present study. Rainbow trout are considered as the most sensitive species and represent the benchmark for comparing the sensitivity of other species. Brook trout have previously been represented as the most tolerant of salmonids (except for the closely related Dolly Varden char) and cutthroat trout as intermediate (Chapman et al. 2010, McDonald et al. 2010). In this study, the most conservative LC₁₀ calculated from survival at either stage or from 'impacted larvae' was similar between the three species (rainbow 12.44, brook 8.92, cutthroat 11.24 µg/g Se dw in eggs). Brook trout had a slightly lower LC₁₀ than the other two species, however Se associated toxicity was only significant in the first stage of development.

This study examined the effects of Se on larvae from adult rainbow trout, brook trout, and cutthroat trout fed SeMet enriched diets in a chronic study. Larval survival, rather than malformations, was identified as a highly sensitive end point of Se toxicity. In all three salmonid species, decreased survival was related to increased Se concentration in eggs in the developmental stage 'spawned to eyed eggs', a trend that persisted for rainbow trout and cutthroat trout in the next stage 'eyed eggs to fry'. Se toxicity to larvae was also assessed in an environmentally relevant experiment using gravel-bed flumes to test swim-up success. Selenium decreased swim-up success in rainbow trout and cutthroat trout but not in brook trout. This comparative study provided new evidence for species-specific sensitivity to Se in early developmental stages and identified important areas for future studies.

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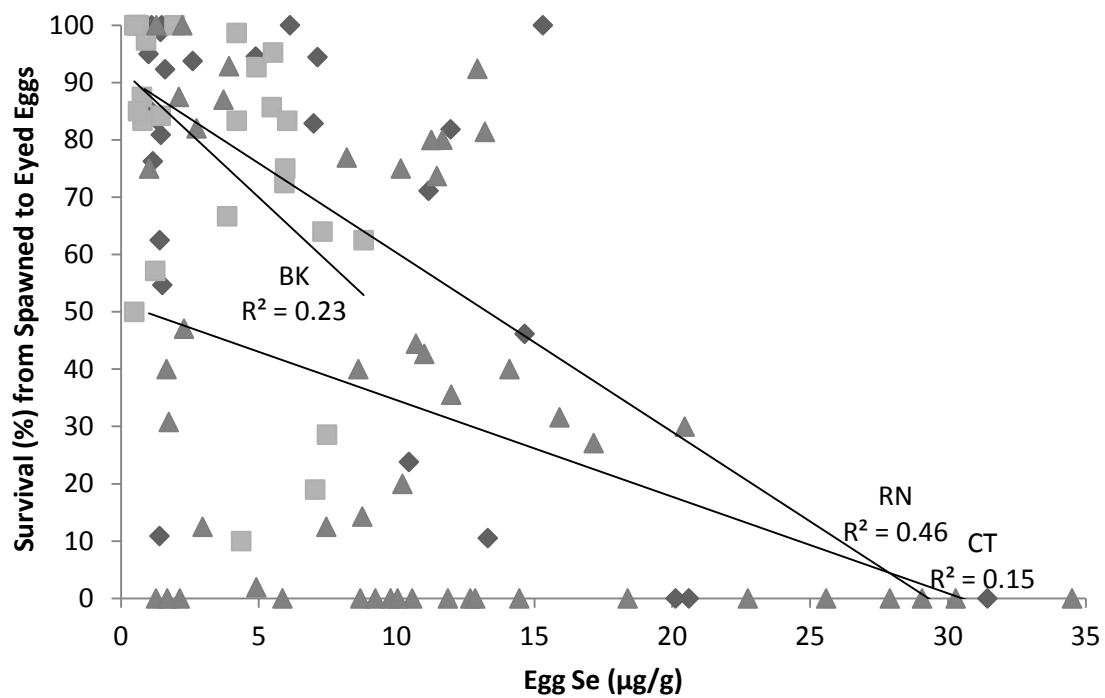
Tables and Figures

Table 3.1 Egg Se, survival in stage 1 and 2 (spawned to eyed eggs, eyed eggs to fry respectively), swim-up success, and total malformations (mean \pm SEM) measured in each dietary treatment group. Diet Se values are the actual concentrations expressed as ww, measured by ICPMS. RN-rainbow trout, BK-brook trout, and CT-cutthroat trout.

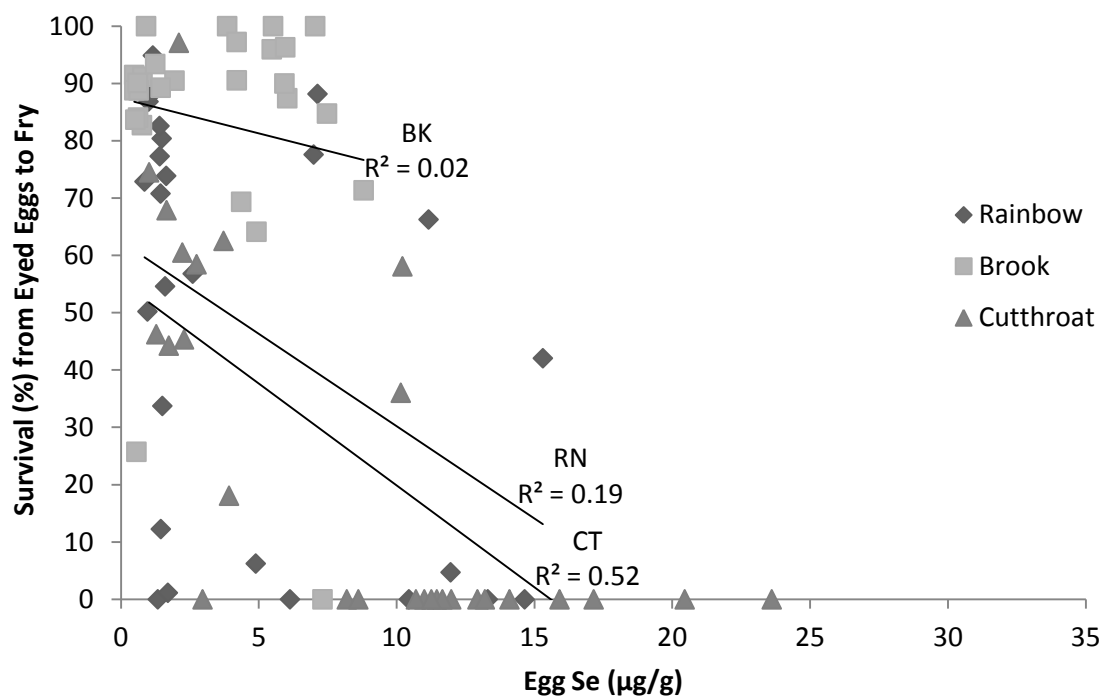
Species	Diet ($\mu\text{g/g}$)	Egg Se ($\mu\text{g/g}$)	Stage 1 survival (%)	Stage 2 survival (%)	Swim-up success	Total (%) malformations
RN	1.47 \pm 1.00	1.17 \pm 0.14 ^A	82.36 \pm 12.25	61.56 \pm 12.35	57.18 \pm 7.70 ^A	10.00 \pm 3.17 ^A
	12.7 \pm 0.48	4.30 \pm 1.04 ^A	77.86 \pm 10.96	48.64 \pm 11.05	73.83 \pm 8.32 ^A	9.86 \pm 3.39 ^A
	35.2 \pm 3.78	13.0 \pm 2.77 ^B	54.72 \pm 10.45	30.33 \pm 12.35	27.45 \pm 7.20 ^B	29.63 \pm 3.66 ^B
	R ²	45%	12%	12%	51%	53%
BK	1.47 \pm 1.00	0.81 \pm 0.14 ^a	86.30 \pm 8.05	82.68 \pm 6.36 ^{ab}	84.00 \pm 6.64	21.30 \pm 6.27
	12.7 \pm 0.48	5.01 \pm 0.57 ^b	71.37 \pm 6.57	88.72 \pm 5.19 ^a	83.42 \pm 6.30	23.93 \pm 5.12
	35.2 \pm 3.78	8.15 \pm 1.11 ^c	71.37 \pm 17.99	44.63 \pm 14.22 ^b	50.11 \pm 19.92	24.23 \pm 14.02
	R ²	60%	8%	26%	14%	0.5%
CT	1.47 \pm 1.00	2.02 \pm 0.24 ^{α}	61.41 \pm 9.64 ^{α}	61.87 \pm 5.62 ^{α}	55.30 \pm 5.49 ^{α}	6.13 \pm 7.10 ^{α}
	12.7 \pm 0.48	9.80 \pm 1.04 ^{β}	30.65 \pm 8.10 ^{β}	14.75 \pm 5.08 ^{β}	21.71 \pm 5.87 ^{β}	48.06 \pm 8.20 ^{β}
	35.2 \pm 3.78	18.0 \pm 1.95 ^{σ}	21.99 \pm 7.12 ^{β}	0 \pm 5.62 ^{β}	0.08 \pm 6.34 ^{β}	n/a
	R ²	46%	19%	72%	72%	55%

Letters indicate significant differences between treatments for each species in the specified tissue ie. A, B, C for rainbow trout, a, b, c for brook trout and α , β , σ for cutthroat trout. R² value represents the amount of variation in the end point that can be accounted for by Se dietary treatment, therefore it is a measure of the 'relatedness' of data points based on the specified treatment.

n/a- There were no surviving larvae from the high cutthroat dietary treatment.



a)



b)

Figure 3.1 Linear regression between percent survival a) in the development stage 'spawned eggs to eyed eggs' $= ([\text{number of eggs at eyed stage} / \text{number of eggs spawned}] \times 100)$ and b) in the stage 'eyed eggs to fry (yolk-absorbed)' $= ([\text{number of fry} / \text{number of eyed eggs}] \times 100)$ with egg Se concentration ($p < 0.03$, BK not significant in b). Regression equations as follows a) RN $y = -3.1237x + 91.514$, BK $y = -4.4836x + 92.385$, CT $y = -1.6865x + 51.473$ and b) RN $y = -3.2206x + 62.41$, BK $y = -1.218x + 87.368$, CT $y = -3.5544x + 55.403$. Selenium tissue concentrations are expressed as wet weight. RN=rainbow trout, BK=brook trout, CT=cutthroat trout.

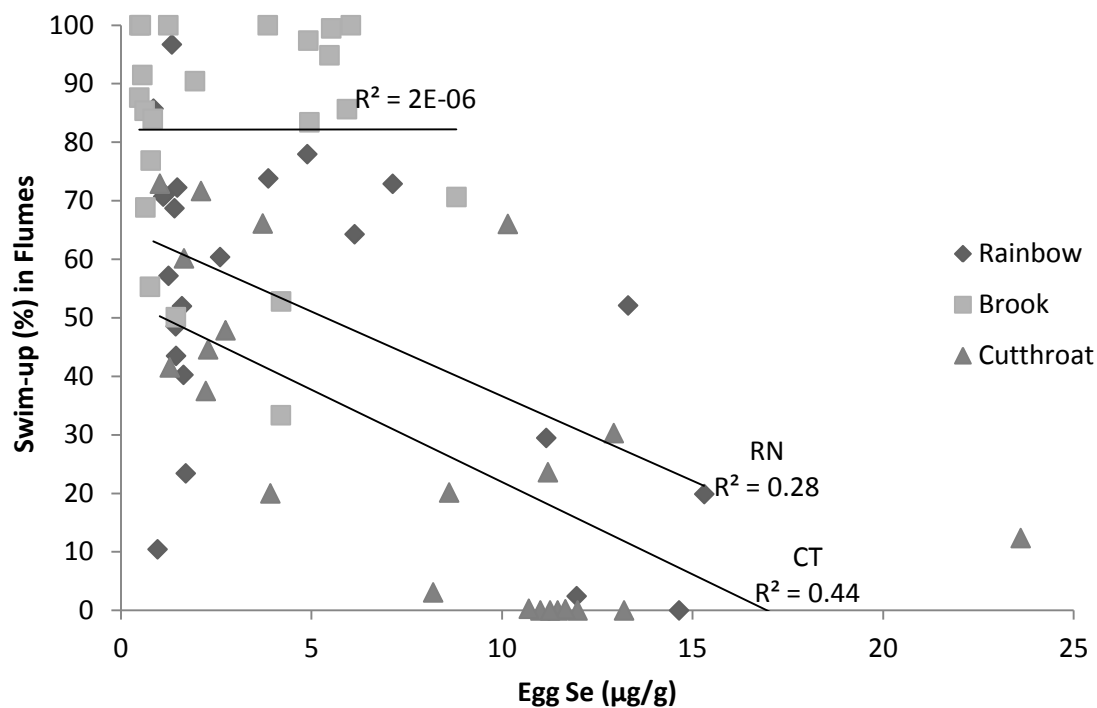


Figure 3.2 Percent swim-up ([number fish collected above gravel/number of eggs deposited]×100) in flumes as a linear regression with egg Se concentration ($p < 0.02$). No significant relationship found for brook trout. Regression equations as follows: RN $y = -2.8875x + 65.506$, BK $y = 0.0108x + 82.138$, and CT $y = -3.1537x + 53.469$. Egg Se concentrations are expressed as wet weight. RN=rainbow trout, BK=brook trout, CT=cutthroat trout.

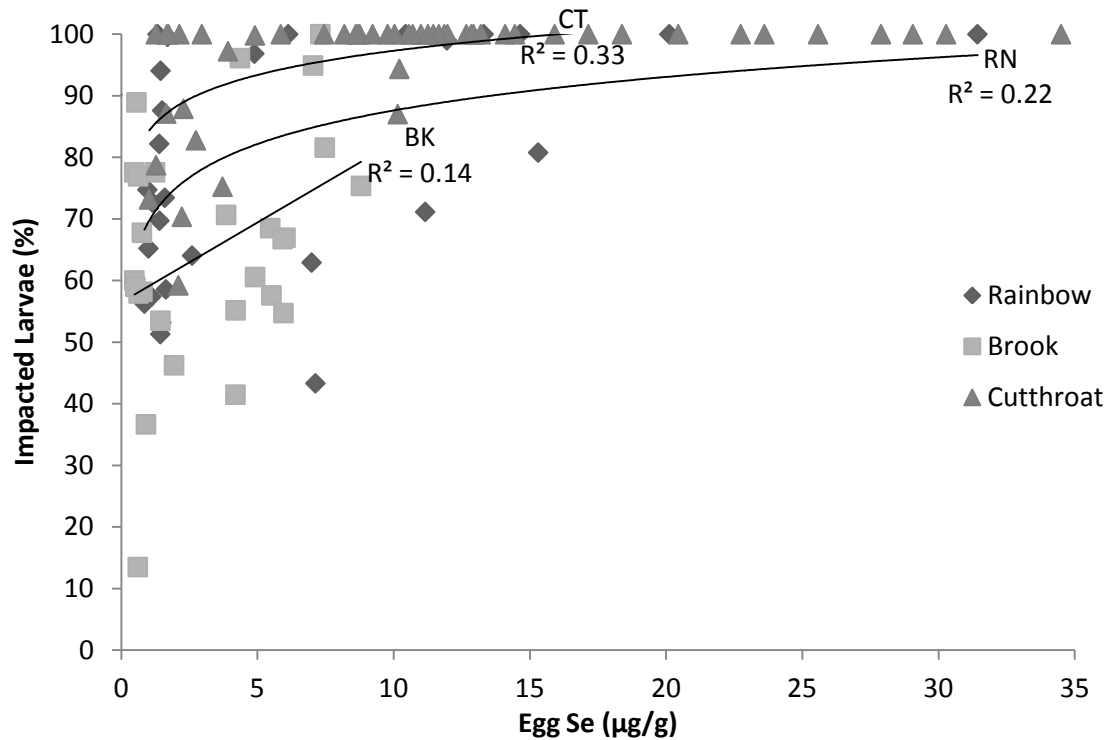


Figure 3.3 Percent impacted larvae, expressed as the sum of % mortality (total mortality of larvae from spawned eggs to fry) and % malformations (larvae with a malformation in any category that scored a 2 or 3 on the severity index) in remaining population, represented as a logarithmic regression with egg Se concentration for rainbow trout and cutthroat trout (p =rainbow 0.0205, cutthroat <0.0001). A significant relationship was not found for brook trout. Regression equations were: RN $y = 7.8498 \ln(x) + 69.548$, BK $y = 5.1425 \ln(x) + 61.405$, and CT $y = 5.6597 \ln(x) + 84.283$. Egg Se is expressed as wet weight. RN=rainbow trout, BK=brook trout, CT=cutthroat trout.

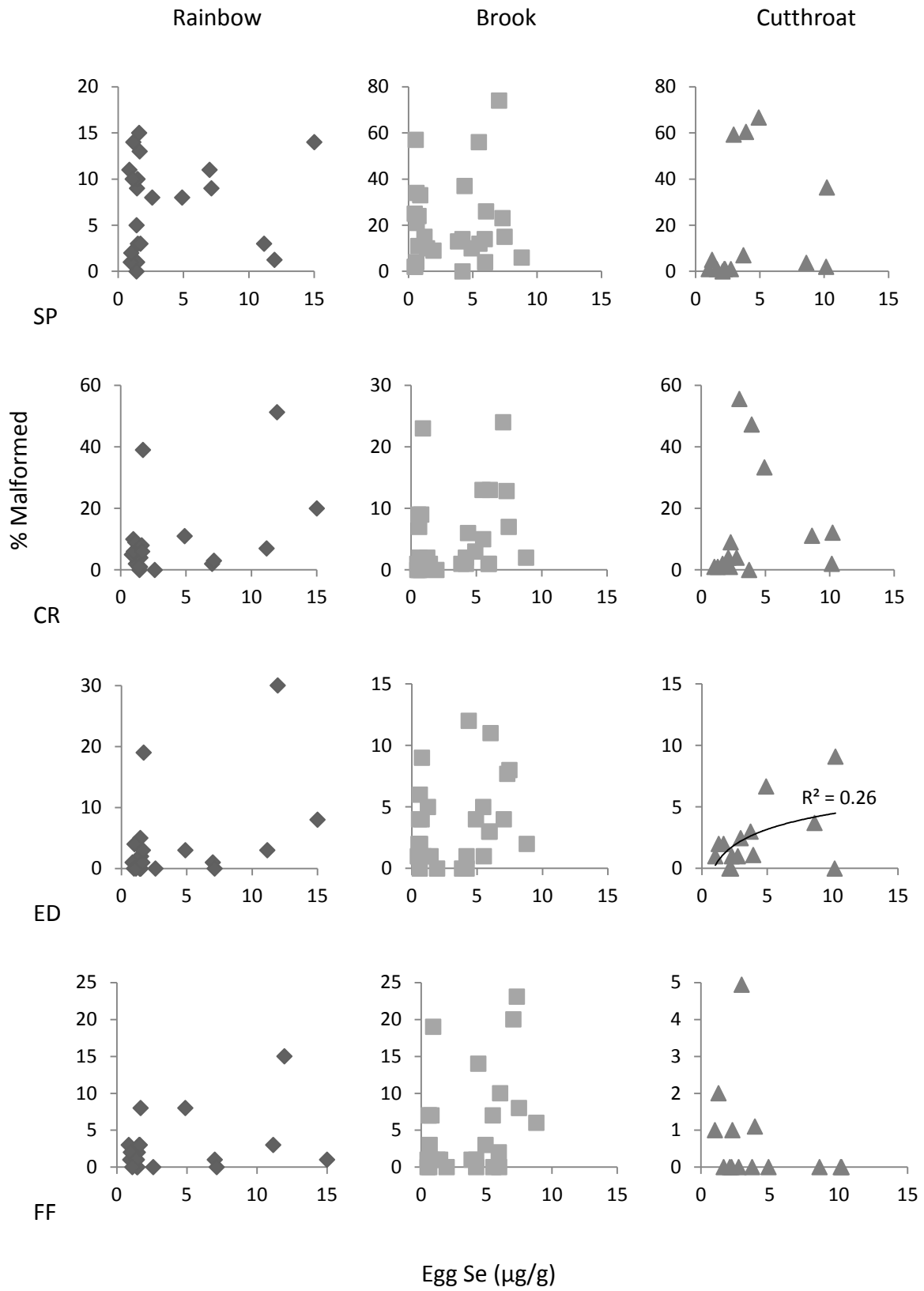


Figure 3.4 Malformations graded as a 2 or 3 for each species in each of the four categories; spinal (SP), craniofacial (CR), edema (ED), and finfold (FF) correlated with egg Se (wet weight, $\mu\text{g/g}$) Y-axis varies between species and within categories. Each data point represents mean value for larvae from an individual female (100 larvae were evaluated per female). The only significant relationship found was in the edema category for cutthroat trout ($y=1.8344\ln(x) + 0.2237$, $p=0.0149$).

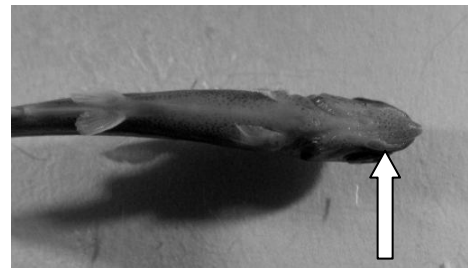
Supporting Information

Score

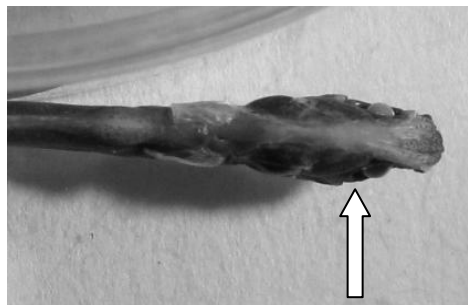
Spinal

Craniofacial

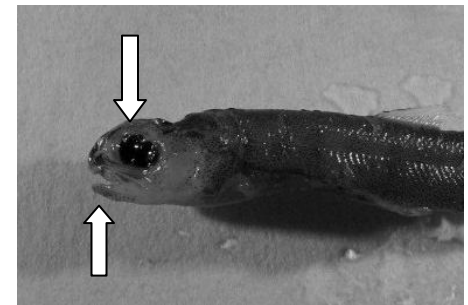
1



2



3



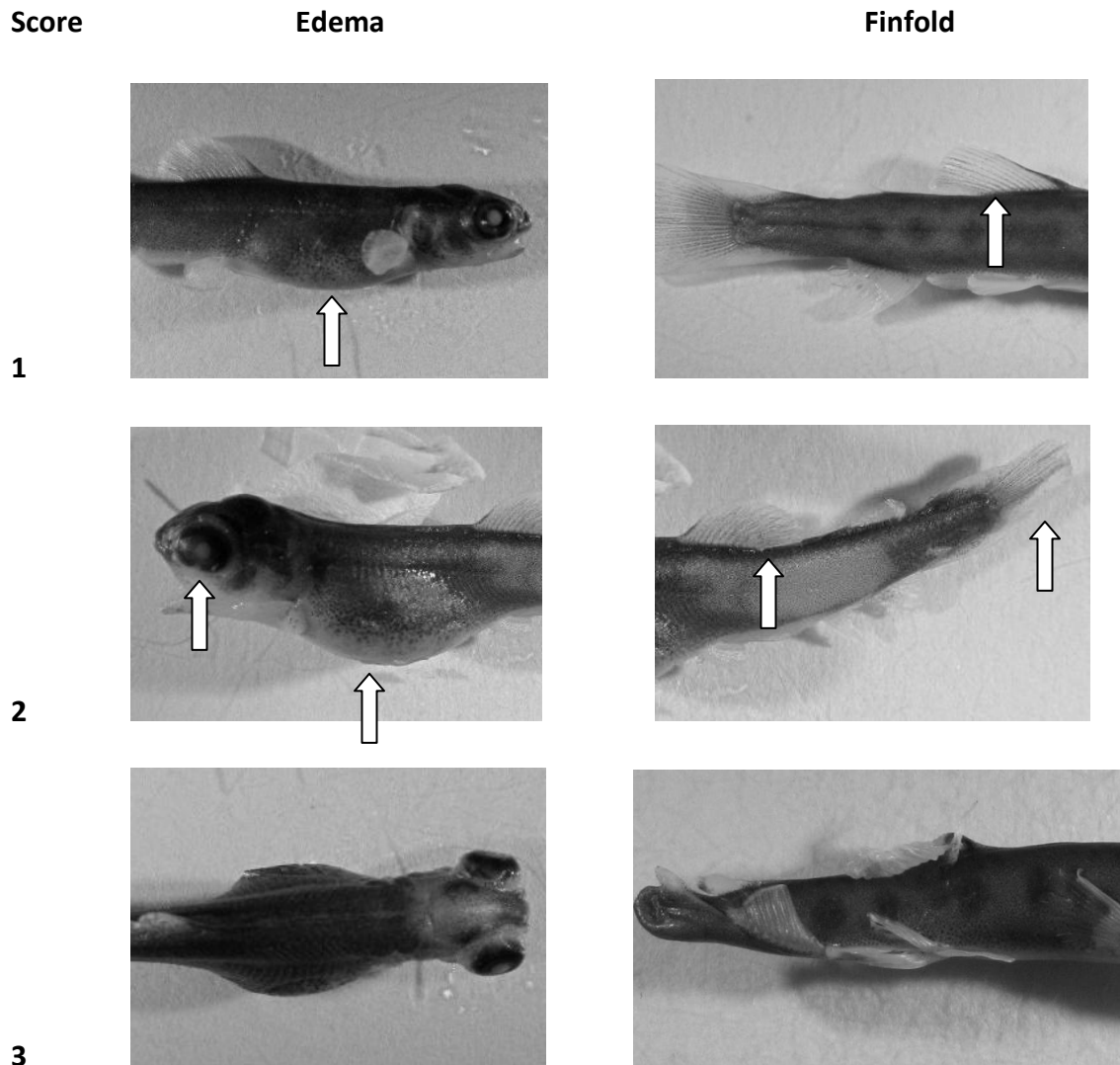


Figure S1 Examples of malformations (scores 1-3) in each of the four categories; spinal, craniofacial, edema, and finfold.

CHAPTER 4

USE OF A MULTI-COMPARTMENT EXPERIMENTAL GRAVEL-BED FLUME TO EVALUATE SWIM-UP SUCCESS OF SALMONID EGG CLUTCHES IN THE LABORATORY

Abstract

Swim-up success, measured as the proportion of fry emerging from a gravel redd, is difficult to quantify in the field and currently available laboratory systems are limited. We simulated redds in the laboratory using multi-compartment gravel-bed flumes and assessed the swim-up success of rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and cutthroat trout (*Oncorhynchus clarkii*) larvae. Gravel-bed flumes were built with compartments to separate egg batches from individual females; eggs were buried under gravel, and oxygenated water was supplied to simulate upwelling through the gravel. Temperature, dissolved oxygen, pH and flow were constant between compartments within the same flume, and flumes operated in parallel. Swim-up success was determined by scoring both the emergence of fry relative to the number of eggs placed in the flume, and simultaneously estimating the hatching success (proportion of eggs that hatch and resorb their yolk sac) of a sample from the same clutch in a vertical incubator. Our results indicate that the experimental flumes can be used in the laboratory to estimate the swim-up success of salmonid larvae.

Introduction

Reproductive endpoints are used in a wide range of studies to compare the performance of individuals, genetic strains or populations, and to evaluate the impacts of environmental stressors, including toxicants (Barlaup et al. 1994, Curry et al. 1995, Rudolph et al. 2008, Granier et al. 2011). For salmonid fish species that bury their eggs under gravel in streams and rivers, leaving them to develop into larvae that swim to the surface of the gravel-bed (Barlaup et al. 1994, Brannas 1995), swim-up success is a key reproductive endpoint and a potential bottleneck for recruitment in populations. It is very difficult to capture newly emerged fry in the field and relate these to the number of eggs laid, in order to assess survival rate from egg to fry.

Spawning salmonids locate a suitable spawning site based on species-specific habitat characteristics, including adequate stream flow, ground water upwelling, and substrate (Snucins et al. 1992, Curry et al. 1995, Malcolm et al. 2003). They must also choose a location with optimum substrate; if the gravel is too large the female will be unable to displace (shift) the substrate to create a nest or 'redd'; if too small, silt could block gravel pores and prevent fry from emerging (Crisp and Carling 1989, Kondolf 2000). Females use their caudal fin to create a depression in the gravel-bed and deposit their eggs, which are fertilized by males and covered by flushed-up gravel (Barlaup et al. 1994, Blanchfield et al. 2003). Eggs hatch under the gravel into larvae (alevins) which remain under the gravel until most or all of their yolk has been absorbed. Such 'buttoned-up' fry swim up to the surface through the interstitial spaces of the gravel (Godin 1979, Brannas 1989, Fraser et al. 1994). This phenomenon, referred to as 'swim-

up', is an important test of larval fitness (Brannas 1989, Steen and Quinn 1999). Many factors can impede swim-up of fry, including fine sediments that can clog interstitial spaces, larval deformities and edema (Kondolf 2000, Sternecker and Geist 2010). Prior to emergence, many eggs and alevins die from lack of oxygen, fungus, predation or disease before even attempting emergence from the gravel-bed (Snucins et al. 1991, Rubin 1995, Dumas and Marty 2006).

Swim-up is challenging to study in the field because fry are difficult to capture, swim-up can occur over an extended period, and the number of eggs buried is usually not known (Rubin 1995). Researchers have simulated redds in the laboratory to provide a controlled setting where a known number of eggs are placed in an artificial redd, and all fry that emerge are captured. One of the first artificial redd designs was a gravel-filled box, with water entering from the top and trickling down through the gravel (Dill 1970). Eggs or alevins were inserted under the gravel using a pipe. Godin (1979) modified this design by switching the inflow and outflow pipes to create upwelling (see also Brannas 1989, Mirza et al. 2001, Sundstrom et al. 2005). Fraser et al. (1994) placed this 'redd box' in a flume with an outflow at the back of the flume and space behind the box to retain swim-up. Other designs covered the gravel with plastic, leaving larvae to swim up through the pipes (Godin 1979, Brannas 1989, Mirza et al. 2001, Sundstrom et al. 2005). Palm et al. (2009) buried eggs under gravel at the bottom of an aquarium and used a propeller at the front of the tank to increase water velocity.

Although an effective early design, the redd box did not allow separation of individual clutches to compare swim-up success of egg batches from different females

under the same conditions. Fudge et al. (2009) divided the box into compartments separated by mesh screens and created upwelling through pipes in the bottom. A pump was installed at one side of the box to increase water flow and an intake at the other end allowed recirculation of water. This design separated egg batches and provided upwelling, but it did not simulate natural stream flow, as the water was funnelled towards the outflows located at the back of the central compartment. Sternecker and Geist (2010) used four-compartment boxes arranged in series in a flume, with water delivered through the front of the flume, entering the boxes through mesh screen at the bottom and exiting through a screen at the back of the box.

The objective of the present study was to create artificial redds in a gravel-bed flume that had separate compartments for multiple egg batches to be tested simultaneously, with constant upwelling and flow through each compartment, flexibility in the size of the compartments, and ease and accuracy of swim-up collection. Cost and ease of dismantling and storage of the system were also considered. To test our artificial redd flume, we compared swim-up success in three species, rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and cutthroat trout (*Oncorhynchus clarkii*). The approach used in this study was to place a known number of eggs from each clutch into a vertical incubator tray to estimate hatching success (proportion of eggs that hatch and resorb their yolk sac, i.e. 'button-up') and simultaneously place a known number of eggs into a compartment of the gravel flume to estimate the proportion that emerged from the gravel (swim-up). Swim-up success then represents the proportion of the eggs hatched and emerged above gravel, divided

by the proportion of fry that hatched and resorbed their yolk sac in the vertical incubator.

Materials and Methods

Flume Design

Two flumes were made from sheets of plexiglass bonded together with methylene chloride. Dimensions of the flumes were 200 cm (l) x 40 cm (w) x 30 cm (h), with 8 mm notches (4 mm deep) cut in the side panel every 10 cm along the length of the flumes, for insertion of removable dividers (Fig. 4.1) to create 20 compartments. Dividers were made by bonding aluminum window screen between two plexiglass frames, 40 cm (l) x 25 cm (h) x 4.76 mm (w) and 3.175 mm (w), with a 5 cm border. The outflow hole (diameter, $d=1.9$ cm) was drilled 44 mm from the top center of the back panel and a bulkhead was placed in the hole to streamline water flow out of the downstream end of the flume (Fig. 4.1).

Treated dechlorinated (City of Lethbridge) water was delivered at 32 psig through a 2.54 cm diameter hose connected to a PVC pipe ($d=6.35$ cm) that ran the length of each flume. From this pipe five PVC pipes ($d=1.27$ cm) ran into the flume (compartments 2, 6, 10, 14, 18) and connected to forks with 4 capped prongs that ran along the bottom of the flume (Fig. 4.1). Five evenly spaced perforations ($d=0.16$ cm) were drilled into each prong running along the bottom of each compartment, ensuring each compartment had its own upwelling water flow (Fig. 4.1). Ball valves were placed in between the pipe connected to the hose and the main pipe running the length of the

flume, and also on each pipe that ran into the flume from the main pipe. Ball valves were partially closed at the top of the flume, closest to the water source, and left open at the bottom of the flume to create uniform water pressure and flow throughout the entire flume, as pressure decreased along the main pipe due to distance from the water source. Flumes were flushed for several days with treated water to ensure any residue from the bonding agents as well as air pockets, which could cause interference in flow, were removed from the pipes. Each compartment was then filled with gravel, flushed with processed water for a minimum of 24 hours, before fertilized eggs were deposited at a specific depth under the gravel (see Gravel-bed Simulation).

Vertical Incubator

A 16 tray MariSource vertical incubator was used in this study to compare hatching success ($[\text{number of buttoned-up fry}/\text{number of eggs deposited in incubator trays}] \times 100$) in the incubator and emergence of swim-up fry in the flumes ($[\text{number of fry emerged from gravel}/\text{number of eggs deposited in flume redd compartment}] \times 100$). The incubator was also used to compare lag-time between development of larvae in the incubator and appearance of fish larvae above the gravel in the flumes.

Water Parameters

Water quality parameters were recorded in three areas of each flume; the first section (top), mid-way (middle), and just before outflow (bottom). Subtle adjustments to flow rate (3.5-4.0 L/min) or ball valve positions were executed in order to maintain

equal flow conditions in all compartments of the flumes. Dissolved oxygen and temperature were measured using a YSI-85 probe, and pH was measured using a CEL850 Sension1 portable pH meter with Platinum Series electrode. Optimal conditions required for swim up were 8-10°C, water flow of 3.5-4 L/min in flume and 18-20 L/min in vertical incubator, pH of 8 and 8-10 mg O₂/L. See Table 1 for water quality data.

Gravel-bed Simulation

Trout spawning redds were simulated in the experimental flumes to create standardized, optimal conditions for the fry to swim up through the gravel. Two sizes of washed round rock (14 mm and 28 mm) purchased from a local landscaping centre were mixed 1:1. These sizes were chosen since similar sizes have been reported for salmonid redds in the literature (Crisp and Carling 1988; Kondolf 2000). The gravel was rinsed with water to remove any fines, disinfected with a 1% Ovadine solution® (Syndel Laboratories Ltd.) and thoroughly rinsed with processed water.

Gravel was added to the flumes to a depth of 5 cm for rainbow trout (see Fig. 4.1) and 10 cm for brook trout and cutthroat trout. Eggs were gently deposited (see Collection and Burial of Eggs) onto the gravel, followed by adding 15 cm of gravel for rainbow trout, and 10 cm of gravel for brook and cutthroat trout for a total gravel depth of 20 cm (Fig. 4.1). Thus rainbow trout eggs were placed deeper in the gravel than the brook and cutthroat trout eggs. The mature rainbow trout were much larger than the other two species, and had larger eggs, and the depth to which we decided to bury the

eggs was based on the work of Crisp and Carling (1988) and Steen and Quinn (1999) who showed that larger sized females dug deeper redds.

Adult Female Fish

Adult females were selected from the existing brood stock at Allison Creek Brood Trout Station (Coleman, AB, Canada) and held at the hatchery for the duration of the experiment. Five-year-old rainbows ($n=9$, average fork length 65.17 ± 1.25 cm, average body weight 3890 ± 33 g), three-year-old brook trout ($n=9$, average fork length 39.33 ± 0.76 cm, average body weight 937 ± 83 g), and three-year-old cutthroats ($n=9$, average fork length 35.23 ± 0.87 cm, average body weight 497 ± 33 g) were used in the experiments. Adult rainbow trout were held from August 2009 to December 2009 until they were spawned and eggs were collected. Brook trout were held from July 2010 to November 2010 until spawned, while cutthroat trout were held from December 2010 to April 2011. All adult females were fed EWOS® (EWOS Canada, Ltd.) Salmonid Brood Feed throughout the entire period. Experimental protocols were approved by the Animal Welfare Committee (University of Lethbridge) in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

Placement of Eggs and Collection of Emerged Fry

As the predicted spawning season neared, fish were anaesthetized and examined for signs of maturation (a soft underside). Females identified as ready for spawning were injected with Ovaprim® (Syndel Laboratories Ltd.) at 0.5 mL per kg body

weight to induce ovulation. A week later fish were euthanized with MS-222 (0.1 g/L, Sigma Aldrich, Catalogue #E10521) and gently squeezed to extrude eggs into labelled, individual spawning pails. Milt pooled from multiple males was used to fertilize the eggs; saline solution (0.6% NaCl) was added to improve sperm motility and aid mixing. Eggs were rinsed after two minutes and placed into a pail of water for two hours to water-harden.

When the eggs were firm to the touch, a subsample from each clutch was placed single file on a 30 cm × 2 cm V-shaped trough and counted. Number of eggs per litre and average size of eggs was determined for each adult female using the von Bayer egg chart (von Bayer 1950). Total volume of eggs produced per female was determined using glass beakers before eggs were placed in an incubator tray. Trays with the eggs were placed into a tub with Ovadine® (1%) for five minutes and then set in the vertical incubator. Iodophor treatment is a standard fish hatchery practice to reduce the bacterial load on the eggs. Eggs were incubated at the Allison Creek Brood Trout Station until the eyed stage (eye pigment spots were visible) and then a subsample from each female was transported to the Aquatic Research Facility at the University of Lethbridge for the flume swim-up experiment. Eggs were transported by ground (150 km) in plastic mesh tubes, wrapped in wet foam, in a cooler with ice.

Upon arrival, the tubes were soaked in a 1% Ovadine® solution for five minutes then rinsed with processed water. Eggs from individual females were placed in a beaker to measure the total volume and ensure that a known number of eyed eggs from individual females were deposited into labelled compartments (~1000

eggs/compartment redd for rainbow and brook trout, 450 eggs/compartment redd for cutthroat due to lower numbers of eggs spawned per female) in the experimental gravel-bed flumes. Eggs were gently poured in an oval-shape formation onto the gravel-bed in each compartment (Fig. 4.1) to simulate a redd. Gravel was then gently added by hand until the eggs were fully covered and the remaining gravel was slowly poured on to reach a total gravel depth of 20 cm. Eggs were not added to compartment 1 or compartment 20, where the gravel was between a flow-through divider and a solid pane (Fig. 4.1).

The remaining eggs at the Allison Creek Trout Brood station were kept in the vertical incubator until alevins absorbed their yolk sac. The fish were then euthanized with MS-222 (0.1 g/L) and stored in Davidson's solution (50% formalin, 10% acetic acid, 10% glycerol, 30% ethanol). Fish that appeared above the gravel were collected every day using a small net and a large pipette. Fish were euthanized with MS-222, counted, and stored in jars of Davidson's solution. Fry collected from each compartment were stored in one jar. Data were analyzed using JMP; ANOVAs with a Student's t-test done post-hoc were used on the data.

Results

There were no significant differences in dissolved oxygen, temperature or pH between different sections (top, middle, bottom) of individual flumes (Table 4.1), or between flumes tested at the same time (November 2009-rainbow, December 2010-

brook, and April 2011-cutthroat). Water quality and flow remained constant even when all flume compartments were filled with eggs.

A significant difference in emergence success (number of swim-up fry collected/number of eggs buried $\times 100$) was detected between species (Fig. 4.2). While rainbow trout and cutthroat trout had emergence success of $58.1 \pm 9.7\%$ and $52.9 \pm 5.6\%$ respectively, the emergence success of brook trout was significantly higher at $85.2 \pm 6.4\%$. The success of hatching and survival to the fry stage (buttoned-up or yolkless fry) in the vertical incubator was similar; $61.5 \pm 9.5\%$ for rainbow trout and $61.9 \pm 4.8\%$ for cutthroat trout, and $82.7 \pm 6.9\%$ for brook trout, and not significantly different from emergence success for any of the species. The swim-up success, calculated as emergence success/hatching success, was $\sim 100\%$ ($85.2/82.7$) for brook trout, 85% ($52.9/61.9$) for cutthroat, and 94% ($58.1/61.5$) for rainbow trout. These estimates were not significantly different from each other or from 100%.

Mean egg diameter was 5.31 mm, 4.45 mm, and 4.32 mm for rainbow trout, brook trout, and cutthroat trout respectively; however, no correlation was observed for swim-up success based on egg size (Fig. 4.3).

The timing of emergence, which closely corresponded to the appearance of buttoned-up fry in the vertical incubator, is illustrated by S-shaped curves (Fig. 4.4) with different slopes and ranges, appears species specific, with differences in the onset of the emergence, the numbers of fish collected each week, and the duration of the emergence period. Cutthroat fry appeared in large numbers above gravel only 3 weeks after placement at the eyed stage. Rainbows started to appear at 3 weeks post burial

but in low numbers, until 7-8 weeks post burial when the emergence noticeably increased. The emergence period lasted the longest for rainbow trout compared to the other two species. Brook trout fry appeared at 7 weeks after placement, but numbers increased rapidly over the next 4 weeks with emergence continuing until week 11 (Fig. 4.4).

Discussion

To quantify swim-up success of fry under controlled conditions in the laboratory, an artificial redd system was built and tested using an experimental gravel-bed flume. The swim-up experiment was done three times, testing three different salmonid species (rainbow trout, brook trout, and cutthroat trout). The flume system had separate compartments for each batch of eggs collected from individual females, each compartment with its own upwelling system to ensure even water flow and oxygen delivery. Developing eggs were placed in the compartments of the flume, on top of a gravel base and underneath 10-15 cm of gravel to simulate a redd.

Two flumes were used each time the experiment was run, and pH, temperature, dissolved oxygen and flow rate were the same in flumes run at the same time and within different sections of the flumes. The similarity of flow and water quality in the two parallel flumes was achieved through the use of ball valves on the inflow lines. Creating evenly spaced holes in the PVC pipes running along the bottom of each compartment also contributed to stable and optimal characteristics of the upwelling water. Adequate temperature and high level of dissolved oxygen, important water

characteristics for salmonid egg incubation (Groves et al. 2008), were maintained in the system, using a flow rate ranging from 3.5-4.0 L/min (Kondolf 2000).

The emergence success ([number of swim-up fry collected/number of eggs buried] × 100) was compared in three salmonid species. Brook trout had significantly higher emergence success (85%) than rainbow trout (58%) and cutthroat trout (53%). Other swim-up studies with salmonids have shown variable rates of success. In laboratory experiments under optimal conditions, 87.5% has been reported for rainbow trout (Fudge et. al, 2008), 85% for brown trout (*Salmo trutta*), and 93.5% for Danube salmon (*Hucho hucho*) (Sternecker et al. 2010), and 60% for Coho salmon (*Oncorhynchus kisutch*) (Sundstrom et al. 2005). Field studies have reported 63.6% swim-up success for brown trout (Dumas et al. 2007) and 66% (Heywood and Walling 2007), 67.2% (Dumas and Marty 2006), and over 90% for Atlantic salmon (*Salmo salar*) (Malcolm et al. 2003). The emergence success rates recorded in the experimental gravel-bed flume in our study are within the range reported in the literature and indicate that species differences exist.

The simultaneous use of the vertical incubator for evaluating hatching success with a sample of eggs from each female provided a unique approach to estimating the true swim-up success, by dividing emergence success in the flume by hatching success in the vertical incubator. Since the estimates for emergence success and hatching success were not significantly different for any of the species (Fig. 4.2), the estimates of swim-up success are not significantly different from 100%. In order to achieve more precise estimates, more clutches would be required. The average SD among clutches for both

emergence and hatching success for the three species was 30%. Thus if two flumes were used at full capacity ($n=36$), then SEM estimates of 5% ($=30/\sqrt{36}$) could be expected for both measures and SEM of $\sim 7\%$ would be expected for swim-up success. If greater precision were required, more flumes could be operated in parallel.

A potentially important aspect of the flume was the depth of gravel in the artificial redds. To simulate natural conditions where larger females dig deeper redds (Crisp and Carling 1989), and since rainbow trout females in this study were larger than brook trout and cutthroat trout, eggs from rainbow trout were placed under 15 cm of gravel while eggs from the other two species were placed under 10 cm of gravel. Since the hatching success and the emergence success were similar for all three species, the depth of egg burial of 10-15 cm appeared to have had little or no influence on either emergence or swim-up success.

Emergence of all three species from the gravel followed an S-shaped curve. Cutthroat trout and rainbow trout fry appear three weeks after egg burial date, although numbers of cutthroat fry increase exponentially while rainbow trout increase slowly. Brook trout fry did not appear until seven weeks after placement. This pattern corresponds to the differences in developmental time between the three species observed in the vertical incubator. Cutthroat trout develop faster to swim-up when raised at the same temperature than rainbow trout or brook trout (Sadler et al. 1986; Wagner et al. 2006, Ripley personal comm.). The length of time for all the emergent fry to appear at the surface of the gravel also varied between species. The majority of cutthroat trout fry appeared at the surface of the gravel within a two week period, at

week 3-5. Brook trout required four weeks, week 7-11, and rainbow trout took the longest at five weeks, week 5-10. It was noted that some of the early emerging rainbow trout and brook trout fry collected above the gravel still had partial yolk sacs. In our experiment there was no food given to entice the fry to the surface, so it is not clear why some fry appeared above the gravel at such an early stage. The environmental cues used by fry in the timing of the emergence could be investigated in the experimental gravel-bed flume described here.

Our results indicate that the experimental gravel-bed flume can be used in the laboratory for a quantitative assessment of the swim-up success in salmonids. Uniform water quality (pH, temperature, dissolved oxygen) was achieved throughout each flume and it was possible to replicate these conditions in different flumes running at the same time. The flumes were affordable and easy to build. At the completion of the experiment, the flumes, pipes and screens were rinsed with 1% Ovadine® solution and dechlorinated water, all the PVC pipes were disconnected, screens were removed, and flumes were stacked for storage.

The design can be improved in future experiments by including an additional drainage outflow at the bottom end of flumes and a sturdy supporting base to hold the flumes at a waist height, rather than the floor as in the current study, to improve ergonomics while collecting swim-up. Stainless steel screens, rather than aluminum mesh, would also improve the durability of the divider mesh screens. Use of flexible plastic tubing (ie. Tygon®) for the forks going into the flumes instead of PVC, would eliminate the need for a large number of 90° elbow connections. Positioning of the

perforated bottom pipes could be also modified to avoid having to cut a corner out of some of the dividers (Fig. 4.1) where foam had to be inserted to block movement of the fry between compartments. The experimental gravel-bed flume described in this study offers exciting possibilities for future experiments to investigate the effects of environmental parameters such as temperature, oxygen saturation, and flow rates on swim-up success of salmonid larvae, species-specific requirements, and studies with eggs collected from wild fish. The experimental system described here would be a valuable tool for experimental work aimed at predicting the impact of climate change on swim-up success of salmonid fish species.

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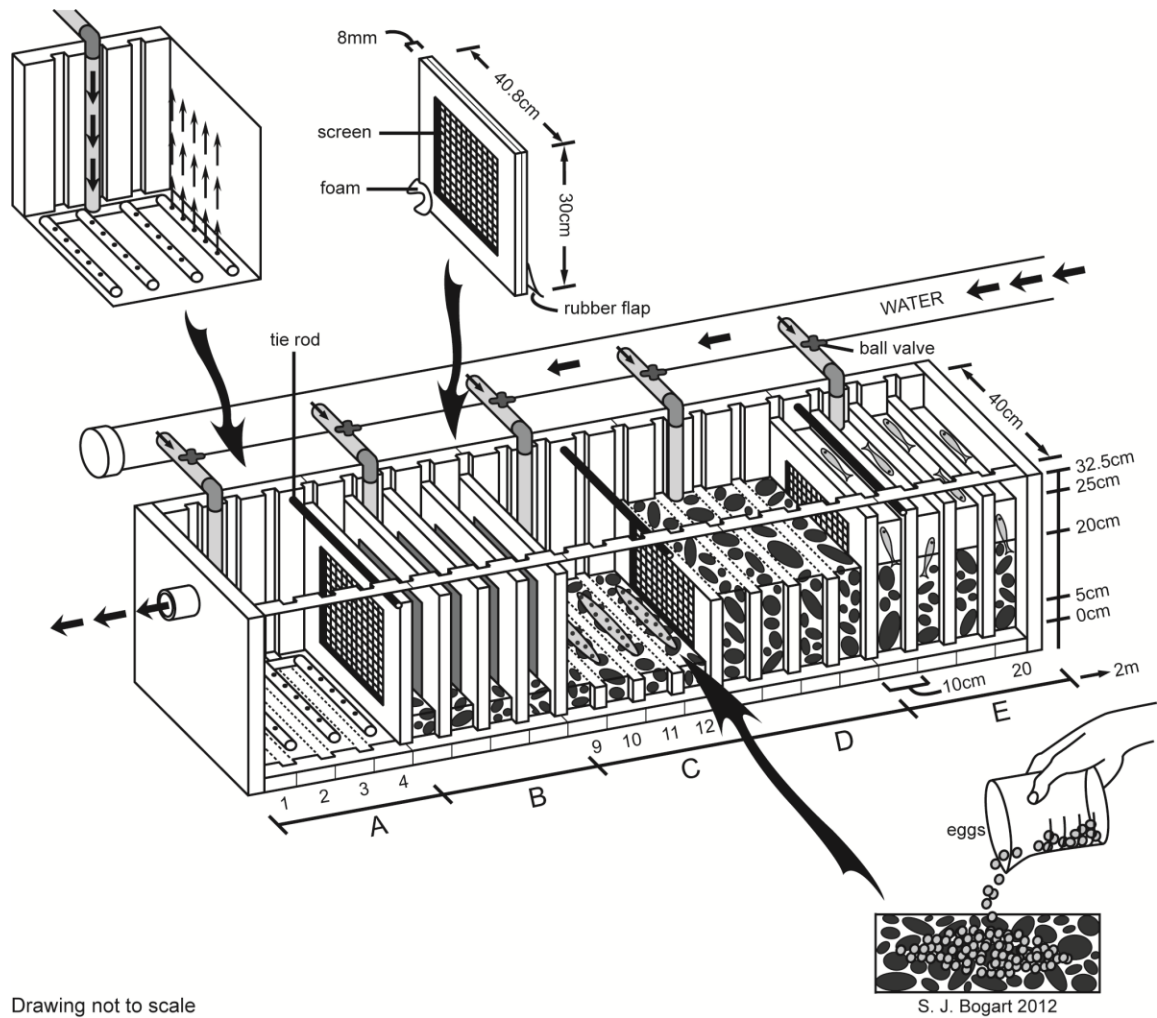
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Figures and Tables

Table 4.1 Water quality in the gravel-bed flume and in the vertical incubator.

Species	System	Flow Rate (L/min)	Area Measured	Temperature (°C)	O ₂ (mg/L)	pH
Rainbow	Flume 1	3.7	Top	8.51 ± 0.34	9.53 ± 0.36	8.02 ± 0.09
			Middle	7.93 ± 0.21	9.72 ± 0.20	8.06 ± 0.08
			Bottom	7.68 ± 0.13	10.00 ± 0.27	8.11 ± 0.06
	Flume 2	3.7	Top	8.06 ± 0.32	9.19 ± 0.30	8.19 ± 0.09
			Middle	7.66 ± 0.17	9.76 ± 0.17	8.24 ± 0.08
			Bottom	7.47 ± 0.15	9.81 ± 0.11	8.26 ± 0.07
	Incubator	18.1	Bottom	7.32 ± 0.10	11.06 ± 0.08	8.24 ± 0.04
Brook	Flume 1	4	Top	9.43 ± 0.02	10.82 ± 0.15	8.34 ± 0.05
			Middle	9.31 ± 0.01	10.79 ± 0.15	8.33 ± 0.05
			Bottom	9.22 ± 0.03	10.75 ± 0.15	8.32 ± 0.06
	Flume 2	4	Top	9.59 ± 0.04	10.76 ± 0.18	8.02 ± 0.11
			Middle	9.38 ± 0.09	10.55 ± 0.18	8.18 ± 0.07
			Bottom	9.22 ± 0.02	10.58 ± 0.18	8.25 ± 0.06
	Incubator	20	Bottom	10.10 ± 0.01	8.53 ± 0.14	8.35 ± 0.02
Cutthroat	Flume 1	3.5	Top	9.75 ± 0.09	7.85 ± 0.50	8.21 ± 0.05
			Middle	9.39 ± 0.09	8.09 ± 0.40	8.19 ± 0.08
			Bottom	9.33 ± 0.08	8.33 ± 0.38	8.20 ± 0.03
	Flume 2	3.5	Top	9.93 ± 0.08	8.66 ± 0.35	8.21 ± 0.09
			Middle	9.56 ± 0.06	8.72 ± 0.35	8.17 ± 0.08
			Bottom	9.14 ± 0.06	8.71 ± 0.34	8.22 ± 0.06
	Incubator	20	Bottom	10.53 ± 0.32	8.96 ± 0.10	8.30 ± 0.10

No significant differences detected between the same areas measured in flumes tested at the same time (Tukey, $p < 0.05$).



Drawing not to scale

Figure 4.1 Flume schematic presented in sections (A-E) to demonstrate chronological time in the experiment: A-empty flume with PVC pipes, B-compartments separated by screens with 5 cm (for rainbow trout) of gravel added to compartments, C-eyed eggs added in oval-shaped 'redd', D-compartments have gravel topped up to 20 cm, E-emerged fry appear above the gravel in each compartment.

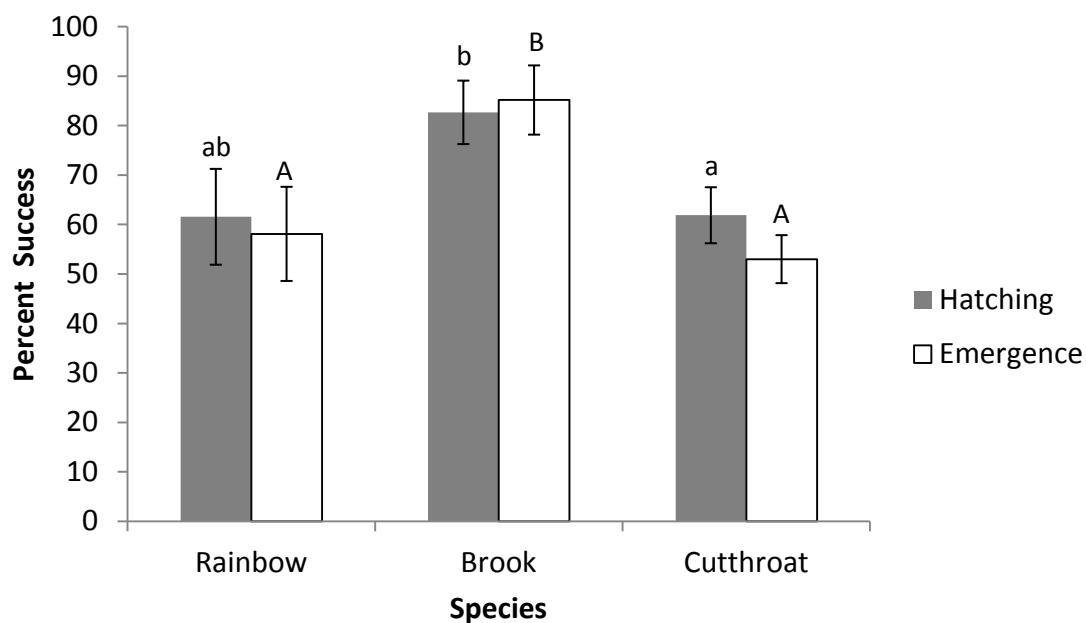


Figure 4.2 Emergence success ([number of emerged fry /number of eggs placed in gravel red compartment]×100; mean ± SEM); hatching success ([number of buttoned-up fry /number of eggs placed in vertical incubator]×100; mean ± SEM) scored for individual females from each species (rainbow trout n=7, brook trout and cutthroat trout n=9). Different letters indicate a significant difference; small letters for hatching success, capital letters for emergence ($p < 0.05$)

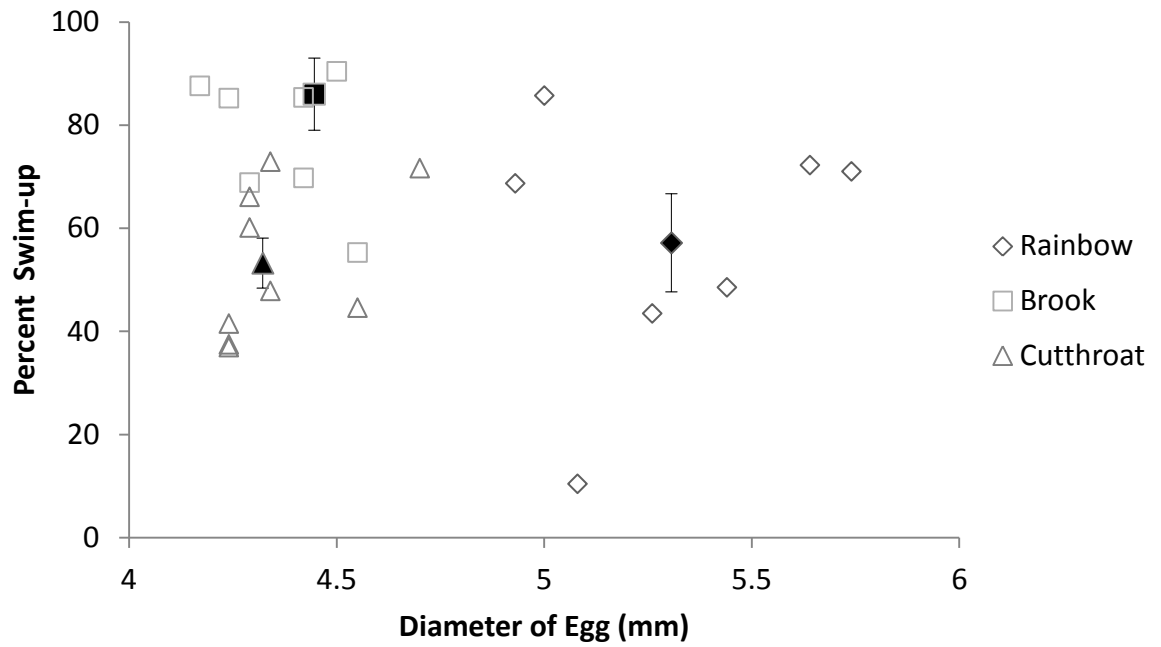


Figure 4.3 Emerged fry collected in relation to average egg size of clutches from individual females for each species. Solid shape indicates species average and SEM.

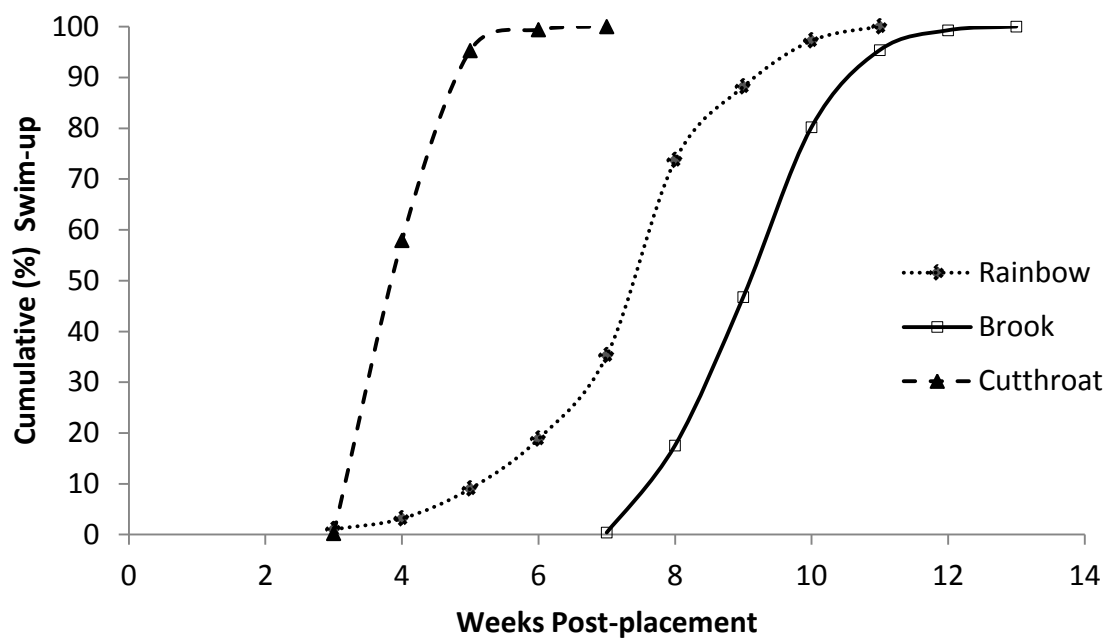


Figure 4.4 Number of emerged fry collected (cumulative % of total) for each egg clutch per week after egg placement.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Chapter 1

Selenium (Se) although essential, can become toxic at doses slightly above required amounts. It is used in the body in association with iodothyronine deiodinase, a key enzyme in thyroid hormone metabolism and with glutathione peroxidase, the enzyme that modifies glutathione (GSH), an antioxidant. Aquatic environments receive excess Se through accelerated weathering rates of black shales and phosphate rocks, a process usually caused by anthropogenic activities such as coal and uranium mining. Selenium can exist in many forms in the aquatic environment, but it bioaccumulates in tissues as selenium methionine (SeMet). Fish are considered to be very sensitive to Se toxicity, similar to other egg-laying vertebrates. Females transfer Se accumulated in the liver to developing embryos in pre-cursors to yolk formation; subsequently larvae can develop malformations or die. The mechanism of Se toxicity is believed to be oxidative stress caused by Se redox cycling with GSH, which disrupts the balance of reduced GSH to oxidized (GSSG). Another aspect of Se toxicity is that it is species-specific. Even closely related species such as rainbow trout and brook trout have very different thresholds for Se toxicity.

Although there is some evidence to suggest that rainbow trout are more sensitive to Se than brook trout, the exposure and resulting Se tissue burdens in the published field studies were variable. It is important to understand sensitivity differences between these two species as native Athabasca rainbow trout that live in a coal mining area in northern Alberta are currently listed as threatened and are competing with invasive brook trout for habitat and food. Cutthroat trout belong to the

same genus as rainbow trout, but published studies reported conflicting results about Se toxicity threshold for this species.

In response to these research needs, this project was developed to assess the effect of Se on physiological and reproductive endpoints in rainbow trout, brook trout, and cutthroat trout using a diet feeding study in a controlled laboratory environment. The experiments were designed to test the hypothesis that rainbow trout and cutthroat trout adult females and larvae are more sensitive to Se toxicity than brook trout, and that oxidative stress is the mechanism mediating these effects. The overall goal of this research was to increase the understanding of species differences to Se toxicity, and to use this new knowledge in risk assessment and to influence management of anthropogenic Se sources.

In the short term, this project compared sensitivity to Se toxicity in rainbow trout, brook trout, and cutthroat trout eggs and larvae, assessing fitness through a flume swim-up study and malformation analysis. In adults, this study determined the effects of Se on hormonal status and oxidative stress.

Chapter 2

To compare the accumulation of Se in tissues and the effects of Se in three species of salmonids, adult female rainbow trout, brook trout, and cutthroat trout were fed control, low (15 µg/g), or high (40µg/g) Se diets for five months prior to spawning. After spawning, adults were euthanized, sampled for plasma and tissues, and spawned eggs were fertilized and raised in a vertical incubator.

Adult brook trout had a higher concentration of Se stored in muscle compared to eggs and liver, whereas rainbow trout and cutthroat trout had a higher Se concentration in egg tissue. It was hypothesized that as spring spawners, rainbow trout and cutthroat trout build gonads mostly from liver tissue that contains high concentrations of Se, resulting in high levels of Se in eggs when Se is transferred through vitellogenesis. However, brook trout, which are fall spawners, can build gonads from muscle breakdown as gonads are formed during the warmer summer months and muscle tissue has lower concentrations of Se and therefore, less may be transferred to eggs.

Gonadosomatic index decreased slightly for rainbow trout and cutthroat trout with increasing tissue Se, but there was a bimodal relationship for brook trout, as six brook trout females did not develop a gonad. Brook trout had an increased ratio of T3:T4 with increased liver and muscle Se, whereas the other species did not have this pattern. Plasma cortisol concentrations increased in cutthroat trout with increased liver Se, but not in the other two species. Rainbow trout had increased concentrations of LPO and GSH with increasing liver Se. Cutthroat trout only had increased GSH, while brook trout exhibited no trends between LPO, GSH, and liver Se.

These differences in hormonal status and oxidative stress suggest that brook trout may have increased metabolism as Se in liver and muscle tissue increase. Oxidative stress, the mechanism of Se toxicity, was surmised to occur in rainbow trout liver as LPO and GSH increased with increased liver Se, however only GSH increased for cutthroat trout and there were no significant relationships for brook trout. Although rainbow trout were predicted to be the most sensitive to Se, adult brook trout

experienced lethal toxicity, while reproductive Se toxicity appeared higher in rainbow trout and cutthroat trout indicating specific-species differences.

Chapter 3

To compare the effects of maternally transferred Se on development and fitness of larvae, eggs from Se-fed rainbow trout, brook trout, and cutthroat trout were raised in a vertical incubator or buried under gravel in the custom-built experimental flumes.

Percent survival of larvae was assessed in the incubator in two stages; spawned eggs to eyed eggs and eyed eggs to yolk absorbed fry. In the first stage all three species had decreased survival with increased egg Se concentration. This trend persisted for rainbow trout and cutthroat trout in the second stage of larvae development but not for brook trout. Moreover, Se also decreased swim-up success in rainbow trout and cutthroat trout larvae in the experimental flumes.

The analysis of malformations revealed that incidence of edema increased in cutthroat trout larvae with increasing egg Se, but no other trends were found in any other category (spinal, craniofacial, finfold) or for the other two species. When total mortality and total malformations of the remaining population were added, rainbow trout and cutthroat trout had an increase in number of larvae impacted by Se with increasing egg Se concentrations.

It was hypothesized that species differences in survival of larvae may be caused by differences in development (timing, levels, types) of antioxidants causing rainbow trout and cutthroat trout larvae to be more sensitive to Se. Since a Se-dependent

decrease in survival also occurred in the spawned to eyed egg stage, it was surmised that Se toxicity is occurring before yolk absorption, probably when yolk precursors are cleaved in developing embryos.

Significant trends were not detected in malformations, in contrast to previous studies, possibly because larvae affected by Se toxicity died. Larvae in this study were more severely affected by Se than those in previous studies (mortality versus malformations endpoint). This was linked to the possibility of Se tolerance as this study used naive hatchery raised fish while earlier studies have used field fish from Se contaminated areas.

Chapter 4

Salmonids bury their eggs under gravel in streams in nests called redds. An important indicator of larvae fitness is swim-up success through the gravel to the surface of the gravel bed. Experimental flumes were built to assess the swim-up success of larvae from rainbow trout, brook trout, and cutthroat trout. These flumes were made out of plexiglass with dividing screens throughout to separate clutches of eggs. Each section had a perforated pipe running along the bottom to allow upwelling of incoming water. A base layer of rocks (5 or 10 cm depending on average egg size) were added to each section, eyed eggs were poured in an oval shape and covered in gravel until the total height of the gravel was 20 cm.

Comparing larvae from females fed control diets, species differences were detected in swim-up success, starting date of appearance of swim-up fry above gravel

and the swim-up rate. Brook trout had higher swim-up success than rainbow trout and cutthroat trout. Emergence patterns for all species followed an S-shaped curve. Rainbow trout and cutthroat trout appeared above the gravel earlier than brook trout, but rainbow trout had the longest period of swim-up time followed by brook trout and then cutthroat trout.

The experimental flumes were useful for salmonid swim-up analysis. Other uses of experimental flumes were suggested and include investigations of the effects of temperature, oxygen saturation, and flow rates on swim-up success, species-specific requirements, and studies with eggs collected from wild fish.

Significance of the Research

This research project was unique in that it provided evidence of Se toxicity in adult and larvae of rainbow trout, brook trout, and cutthroat trout in a controlled laboratory setting. It is a first chronic comparative toxicological study in three species of salmonids, analyzed for effects on the parental and F1 generations. The in-depth analysis of Se toxicity provides unique data for use in future Se risk analysis.

Specific significant findings include:

- Further evidence of differential Se accumulation in tissues between rainbow trout, brook trout, and cutthroat trout.
- New data on sub-lethal effects of Se on adult salmonids, including decreased GSI, and up-regulated thyroid hormones, and cortisol.

- Evidence of lethal Se toxicity to adult brook trout but not adult rainbow trout or cutthroat trout.
- Confirming the oxidative stress mechanism of Se toxicity for rainbow trout but finding the same mechanism did not occur in brook trout and cutthroat trout.
- Se-correlated mortality in rainbow trout and cutthroat trout larvae throughout development, as well as in the first stage of development for brook trout larvae.
- First environmentally relevant data on Se effects on salmonid swim-up success using experimental flumes.
- Finding, contrary to previous literature, Se-linked malformations did not occur, most likely due to the Se correlated decrease in survival.
- Larvae mortality was the most relevant endpoint for Se toxicity, which is more severe than the appearance of malformations that has been used in field studies and provides evidence for acquired Se tolerance.
- Creating a customized experimental flume and developing a protocol to test salmonid swim-up through a gravel redd, which could be studied in a controlled laboratory setting and used for a range of ecology and toxicology based studies.