

**SPECIES DIFFERENCES IN SELENIUM TOXICITY: LINKING CELLULAR RESPONSES TO
POPULATION EFFECTS**

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ABSTRACT

Model organisms are often used in ecotoxicological studies and environmental risk assessments; however, species differences in responses to toxicants exist. A meta-analysis identified normal biomarker ranges for rainbow trout (RT) and brook trout (BT), and showed that RT had greater whole-body lipids and plasma T4 levels than BT. Exposure to selenium inhibited cortisol secretion of trout adrenocortical cells; however, RT were more sensitive than BT. To investigate species vulnerability at the individual level, RT and BT were stocked into reference and selenium-contaminated pit lakes. Fish accumulated more Se from selenium-contaminated than reference lakes, and selenium accumulation was similar between species. Chronic selenium exposure had a greater energetic cost for RT than BT, but this was mitigated by food availability. Chronic selenium exposure decreased plasma T3 and T4 levels, but did not alter other endocrine or oxidative stress biomarkers. This project highlights the need for both species- and site-specific risk assessments.

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LIST OF ABBREVIATIONS

AC = adenylyl cyclase
ACTH = adrenocorticotrophic hormone
ANOVA = analyses of variance
ANCOVA = analyses of covariance
ATA = 3-amino-1,2,4-triazole
BSO = buthionine-[*S,R*]-sulfoximine
BT = brook trout
cAMP = cyclic adenosine monophosphate
CCME = Canadian Council of the Ministers of the Environment
cP450_{c21} = cytochrome P450 21-hydroxylase
cP450_{c11} = cytochrome P450 11 β -hydroxylase.
cP450_{c17} = cytochrome P450 17 α -hydroxylase-17,20 lyase
cP450_{scc} = cytochrome P450 side chain cleavage
DETCA = sodium diethyldithiocarbamate trihydrate
df_M = degrees of freedom (model term)
df_E = degrees of freedom (error term)
EC₅₀ = concentration that inhibits 50% of cortisol secretion
F = female
GSH = reduced glutathione
GSI = gonadal somatic index
h = hour
HSI = hepatosomatic index
3 β -HSD = 3 β -hydroxysteroid- Δ 5-steroid dehydrogenase
HPLC = high pressure liquid chromatography
I = immature
LDH = lactate dehydrogenase
LPO = lipid peroxidation
M = male
MEM = minimal essential medium
min = minutes
MS-222 = 3-aminobenzoic acid ethyl ester
M2R = melanocortin 2 receptor
MITHE-SN = Metals In The Human Environment Strategic Network
NAC = N-acetyl -L-cysteine
NOEC = no observable effect concentration
NS = not significant
NSERC = Natural Science and Engineering Research Council
PKA = protein kinase A
QA/QC = quality assurance/quality control
RT = rainbow trout
ROS = reactive oxygen species
SE = standard error

Se = selenium

Se-Met = selenomethionine

SOD = superoxide dismutase

SGR = specific growth rate

StAR = steroidogenic acute regulatory protein

T3 = triiodothyronine

T4 = thyroxine

USEPA = United States Environmental Protection Agency

Vit A = retinol

Vit E = tocopherol

**CHAPTER 1. REFERENCE VALUES FOR OXIDATIVE STRESS, PHYSIOLOGICAL
STRESS RESPONSE, AND CONDITION BIOMARKERS IN JUVENILE RAINBOW
TROUT AND BROOK TROUT – A META-ANALYSIS**

Abstract

Fish are commonly used bioindicators of aquatic health and a large number of different biochemical and physiological parameters have been measured using different model species. This paper defines means and ranges for reference values of oxidative stress biomarkers (lipid peroxidation, LPO; reduced glutathione, GSH), energy reserves (glycogen, lipids, glucose), physiological stress response parameters (plasma cortisol, T3, and T4, gill Na⁺/K⁺ ATPase), and condition and growth indices (K, HSI, specific growth rate) of juvenile rainbow trout and brook trout reported in the scientific literature. It also compares the mean values of the two species (*t*-test) and investigates the effect of water temperature and fish size (linear regression) on the parameters of interest. Rainbow trout had greater whole-body lipids and plasma T4 levels than brook trout, but no other differences between species were identified. The means and ranges reported may be used to determine if an effect of anthropogenic disturbances has occurred in areas with little or poor quality reference data, to confirm that reference groups are within the normal range, and to predict species-specific vulnerability to toxicants if their mode-of-action depends on parameters that vary among species.

Key words

rainbow trout, brook trout, reference, biomarkers, energy reserves, stress, condition, growth

Introduction

Many aquatic health and risk assessment programs use fish as bioindicators, monitoring various physiological parameters to determine exposure or effects. In the Canadian Environmental Effects Monitoring Program gonadal somatic index, hepatosomatic index, and condition factor are often measured to determine the effects of pulp and paper mill and metal mine effluents on fish health (Barrett, Lowell et al. 2010). Other parameters that may be used in such programs include acetylcholinesterase inhibition for pesticide exposure (Quinn, Rasmussen et al. 2010), metallothionein induction for metal exposure (Ait-Aissa, Ausseil et al. 2003), and other non-specific health or damage indicators such as the physiological stress response (Levesque, Dorval et al. 2003; Vijayan, Prunet et al. 2005; Miller, Wang et al. 2007), energy reserves (Kennedy, Sweeting et al. 1995; Levesque, Moon et al. 2002; Teh, Deng et al. 2005), condition (Alves and Wood 2006) and growth indices (Teh, Deng et al. 2005), and oxidative stress biomarkers (Miller, Wang et al. 2007; Ramsden, Smith et al. 2009).

Oxidative stress is the underlying mechanism of action for many different pollutants, including selenium-induced teratogenesis (Palace, Spallholz et al. 2004). Oxidative stress results from an imbalance between oxidative radicals and cellular antioxidant defences (Kelly, Havrilla et al. 1998). When unchecked, oxidative radicals damage proteins, lipids, and DNA, affecting many different physiological systems. Lipid peroxidation is frequently measured as an indicator of oxidative damage (Kelly, Havrilla et al. 1998) and elevated levels have been observed in fish exposed to agricultural chemicals (Dorval, Leblond et al. 2005), heavy metals (Roméo, Gnassia-Barelli et al. 2000; Roberts and Oris 2004), pulp and paper effluent (Oakes, McMaster et al. 2003), and pharmaceuticals (Laville, Ait-Aïssa et al. 2004). Cellular antioxidant defences, such as vitamins (Ndayibagira, Cloutier et al. 1995), glutathione (Miller, Rasmussen et al. 2009), superoxide dismutase (Puangkaew, Kiron et al. 2005), catalase (Ferrari, Venturino et al. 2007), EROD (Gonzalez, Shaikh et al. 2009), and glutathione peroxidase (Wang, Han et al. 2007) are

also used as biomarkers of oxidative stress as they may be depleted or induced by exposure to pollutants (Kelly, Havrilla et al. 1998). However, reference values for these cellular responses need to be defined to determine effect of pollutants. Additionally, such data could be used to determine relative species-specific sensitivities of toxicants, if the toxicant acts on parameters that vary among species.

Effects of pollutants can be also detected at the organism level. When a fish perceives an alteration in its environment as a stressor, cortisol (the primary stress hormone in fish) is released from adrenocortical cells in the head kidney (Barton 2002). Cortisol maintains homeostasis by promoting gluconeogenesis, altering lipid metabolism, and suppressing the immune system and sex steroid secretion (Hontela 2005). Elevated cortisol levels have been documented in response to toxicants (Miller, Wang et al. 2007), changes in water quality (Wagner, Bosakowski et al. 1997) and habitat alterations (Sloman, Wilson et al. 2002). In addition to the effects listed above, cortisol increases the clearance of thyroid hormones from the plasma (Mustafa and MacKinnon 1999), increases plasma glucose through glycogen mobilization, and stimulates osmoregulation, including the activity of gill Na^+/K^+ ATPase (Mommsen, Vijayan et al. 1999). Fish with chronically elevated cortisol levels may also have depleted energy reserves (Vijayan, Ballantyne et al. 1991), as the energy acquired through food is allocated to maintenance of homeostasis. Compiling reference values will determine the normal variability associated with these biomarkers, including energy reserves, and allow comparison to new laboratory and field data.

Ultimately, the energy reserves of a fish determine its evolutionary fitness. Without sufficient energy stores, fish will be unable to survive the winter or successfully reproduce (Hemre, Mommsen et al. 2002). If anthropogenic stressors decrease food sources or increase costs of obtaining the food, energy reserves may decline (Rasmussen, Gunn et al. 2008). They may also decline as energy is used to maintain homeostasis during exposure to a toxicant (McGeer, Szebedinszky et al. 2000). Energy reserves commonly measured include liver

glycogen levels (Teh, Deng et al. 2005), muscle or whole-body triglyceride levels (Bennett and Janz 2007), whole-body lipid content (Weber, Higgins et al. 2003), and plasma glucose levels (Becker, Moraes et al. 2009). Some toxicants may also impair the ability of organisms to properly use energy they acquire. For example, yellow perch from metal contaminated lakes were unable to use energy stored as glycogen and triglycerides (Levesque, Moon et al. 2002). Energy reserves may also be depleted if a fish is chronically stressed by a pollutant (McGeer, Szebedinszky et al. 2000; Teh, Deng et al. 2005) or experiences a change in its environment (Driedzic and Short 2007). When energy is limited, growth and condition of fish may also be impacted. Fish exposed to pollutants, or another stressor, may have lower condition factors (Rasmussen, Gunn et al. 2008), altered hepatosomatic indices (Wilson and Wood 1992), and decreased growth (Teh, Deng et al. 2005). Compiling reference data will define the natural variability within measures of energy reserves and provide an indication of what is physiologically “normal” for a healthy fish.

Biochemical and physiological endpoints are currently used as markers of aquatic animal health and such use may increase in the future; thus, the normal range of values needs to be defined for species of interest. Rainbow trout are often used in toxicology as they are considered sensitive to toxicants, are present in many aquatic systems in North America, and are easily cultured (Rexroad, Palti et al. 2008). Model species can be very useful, but care must be taken when extrapolating results to other species, as species-specific vulnerabilities to toxicants exist. For example, rainbow trout exhibit higher rates of selenium-induced teratogenesis (Holm, Palace et al. 2005) and are more sensitive to selenite toxicity *in vitro* (Miller and Hontela 2011) than brook trout. Species-specific vulnerabilities to toxicants may be identified by different baseline or normal physiological parameters. For example, the different sensitivity of yellow perch (tolerant) and rainbow trout (sensitive) to cadmium toxicity can be partially explained by different Ca^{2+} maximum uptake rates of their gills (Niyogi and Wood 2004). If the mechanism of

action is known, normal ranges (e.g. Ca^{2+} maximum uptake rates) may be used to predict species vulnerability to a toxicant (e.g. cadmium). Additionally, normal or reference values may be used in effects assessments to determine if the fish is being impacted by anthropogenic activities. This may be especially useful in areas where baseline or reference data is missing or of poor quality.

The objective of this meta-analysis was to collect normal or reference values for common physiological biomarkers and condition indices from the scientific literature. The analysis focuses on juvenile rainbow trout and brook trout from control treatment groups to provide a range of values that can be used in future ecotoxicological studies, as normal or reference. It also determines if physiological differences between rainbow trout and brook trout explain their different sensitivities to toxicants such as selenium.

Materials and methods

Two hundred and two studies published from 1972 to 2010 were used to collect a data set of reference or control group values of common physiological biomarkers and condition indices used in ecotoxicology (Table 1.1). Only studies that used juvenile, freshwater rainbow trout and brook trout were considered. Means or medians (if a range of values were reported) from the control groups (laboratory studies) or reference sites (field studies) were collected for energy reserves (plasma glucose, liver glycogen, and whole-body lipid concentrations), liver oxidative stress biomarkers (tocopherol, retinol, reduced glutathione, and lipid peroxidation levels), physiological stress response (plasma cortisol, plasma T3, plasma T4 levels, and gill Na^+/K^+ ATPase activity), and condition indices (liver somatic index, condition factor, specific growth rate). Water temperature and fish weights reported in the publications were also collected as fish size (Miller, Rasmussen et al. 2009) and temperature (Quinn, Rasmussen et al. 2010) may affect

the biomarkers of interest. No other co-variants (e.g. diet, time of sampling, etc.) were included in order to maximize the number of papers that met the requirements for analyses.

All statistical analyses were run using JMP 7.0.1. software. Data was log transformed to respect normality and non-parametric tests were used as needed. Levene's test was used to determine if the variances were equal between rainbow trout and brook trout. If variances were equal, the normal values of the parameters listed above in juvenile rainbow trout and brook trout were compared using a two-tailed *t*-test assuming equal variances. If variances were not equal, a two-tailed *t*-test assuming unequal variances was used. If the data was not normal, the VanderWaerden test was used to compare species. An analysis of covariance (ANCOVA) was used to investigate the influence of fish weight and water temperature on the parameters of interest. ANCOVA models used were: weight x species x weight*species and temperature x species x temperature*species. If there were less than three observations for a species, the data set was excluded from the analyses.

Results

There were no significant differences between the normal values of juvenile rainbow trout and brook trout (Table 1.2) for plasma glucose (VanderWaerden test; $p = 0.8154$), liver glycogen (VanderWaerden test; $p = 0.4827$), plasma cortisol (*t*-test; $p = 0.8194$), plasma T3 (*t*-test; $p = 0.9068$), gill Na^+/K^+ ATPase (*t*-test; $p = 0.1476$), hepatosomatic index (*t*-test; $p = 0.5844$), condition factor (*t*-test; $p = 0.1846$), or specific growth rate (VanderWaerden test; $p = 0.5645$). Rainbow trout had highly significantly greater whole-body lipid levels (Table 1.2) than brook trout (*t*-test; $p = 0.0377$; $\alpha = 0.05$). They also had greater plasma T4 levels (Table 1.2) than brook trout (*t*-test = 0.0638; $\alpha = 0.10$). There were not enough juvenile brook trout observations to compare liver retinol, tocopherol, GSH, and LPO levels to rainbow trout (Table 1.1).

Weight had a significant influence on rainbow trout liver glycogen levels ($R^2 = 0.2824$; $p = 0.0281$; Figure 1.1), but there were not enough brook trout paired glycogen and weight observations to test species differences. Weight did not have a significant influence on gill Na^+/K^+ ATPase ($p = 0.5764$; only rainbow trout), plasma glucose ($p = 0.4951$), whole-body lipids ($p = 0.1651$), liver GSH ($p = 0.6176$), liver LPO ($p = 0.4027$), plasma cortisol ($p = 0.9654$), plasma T3 ($p = 0.5902$), plasma T4 ($p = 0.3147$), hepatosomatic index ($p = 0.6041$), condition factor ($p = 0.3837$) or specific growth rate ($p = 0.4818$). Water temperature did not have a significant influence on any of the parameters tested: condition factor ($p = 0.1263$), plasma glucose ($p = 0.5471$), liver glycogen ($p = 0.5472$), whole-body lipids ($p = 0.2396$), liver GSH ($p = 0.9661$), liver LPO ($p = 0.0965$), plasma cortisol ($p = 0.6931$), plasma T3 ($p = 0.9185$), plasma T4 ($p = 0.1550$), gill Na^+/K^+ ATPase ($p = 0.3052$), hepatosomatic index ($p = 0.3787$), or specific growth rate ($p = 0.4060$).

Discussion

This paper presents normal values of commonly measured energy reserves, oxidative stress biomarkers, physiological stress response parameters, and condition and growth indices for juvenile rainbow trout and juvenile brook trout. This data can be used by ecotoxicologists and risk assessors to determine if a potential effect is occurring. If the rainbow trout and brook trout in their studies or monitoring programs fall outside of this normal range, then an adverse effect may be occurring and mitigation efforts or an investigation of cause is needed. Similar approaches are beginning to be applied to effects-based biological assessments. The Canadian Environmental Effects Monitoring Program defines a warning level effect on a fish sentinel species as a change or difference greater than 10% in condition factor or a greater than 25% change in gonad size, liver size, growth, or age (Barrett, Lowell et al. 2010). A similar approach

could be used with the normal values presented in Table 1.2. If the exposed or impacted group of fish has a difference greater than 25% from the mean value presented in this study, then a significant impact may be occurring. The data summarized in this paper could also be used to ensure that control/reference groups of fish are behaving normally and are true controls.

For several of the parameters investigated in this paper, knowledge gaps were identified for juvenile brook trout; only a limited number of juvenile brook trout studies have been published. Thus, species comparisons were not possible for oxidative stress biomarkers as only one study (Miller, Rasmussen et al. 2009) reported values for liver vitamins, GSH and LPO juvenile brook trout. The difference in number of observations may be due to the widespread use of rainbow trout as a model species in toxicology and its prevalence in the aquaculture industry (Rexroad, Palti et al. 2008). While brook trout are also occasionally studied, experiments with this species are not as common as those with rainbow trout. Additionally, rainbow trout mature later than brook trout (Scott and Crossman 1973), giving researchers a longer window of time to work with juvenile rainbow trout than juvenile brook trout. More research on juvenile brook trout would enable researchers to investigate mechanisms underlying species-specific vulnerabilities to toxicants in a manner similar to the experiment that investigated rainbow trout and yellow perch sensitivities to cadmium (Niyogi and Wood 2004).

Juvenile rainbow trout had significantly greater whole-body lipid reserves than brook trout. This pattern was not detected in any of the other energy reserves, or condition and growth indices investigated in this paper. This difference may reflect the different life history strategies exhibited by these salmonids. Brook trout mature as early as one to two years, while rainbow trout mature between three and five years of age (Scott and Crossman 1973). This suggests that rainbow trout allocate large amounts of energy to growth and energy storage during their first few years, but brook trout begin to allocate energy to reproduction earlier. Lipid reserves are used for over-winter survival (Morgan, McCarthy et al. 2002); thus, the difference in whole-body lipids

suggests juvenile rainbow trout allocate more energy to over-winter survival than juvenile brook trout. This difference in resource allocation may be a function of different spawning seasons. Rainbow trout spawn in the spring (Scott and Crossman 1973); thus, large whole-body lipid reserves may be advantageous for rainbow trout as it increases their chance of over-winter survival (Morgan, McCarthy et al. 2002) and also increases their chance for reproduction in the spring. Conversely, brook trout spawn in the fall (Scott and Crossman 1973) and can reproduce before winter; thus, over-winter survival may be less important as they have already reproduced. It has been shown that reproduction does decrease the over-winter survival of brook trout (Hutchings 1994) due to lipid depletion (Hutchings, Pickle et al. 1999).

While the difference in lipid reserves between juvenile rainbow trout and brook trout may reflect their different life-history strategies, it may also alter their susceptibility to winter stress syndrome. Winter stress syndrome occurs when the combination of decreased over-winter energy intake and exposure to a toxicant that exerts a metabolic cost results in a decreased over-winter survival (Lemly 1996). Winter stress syndrome has been investigated in bluegill (Lemly 1996), pike (Bennett and Janz 2007), and sculpin (Bennett and Janz 2007); however, little is known about the susceptibility of rainbow trout and brook trout to this phenomenon. Adult brook trout may be more vulnerable to winter stress syndrome because they have lower whole-body lipids than rainbow trout and their over-winter survival may be already decreased by reproduction (Hutchings 1994). However, the occurrence of winter stress syndrome in the field depends on energy intake. Brook trout feed over the winter (Sweka and Hartman 2001); thus, they should be able to meet their over-winter energy demands if sufficient food is available. Site-specific factors will have a large impact on the prevalence of winter stress syndrome in trout populations and may mask the effect of different whole-body lipid reserves observed in this study.

Glycogen, the storage form of glucose, is another energy store often investigated (Hemre, Mommsen et al. 2002; Levesque, Moon et al. 2002) as it is important for over-winter and hypoxia

survival (Hemre, Mommsen et al. 2002). While there were no species differences in liver glycogen reserves, there was a significant negative relationship between liver glycogen reserves and weight for juvenile rainbow trout. Smaller fish had greater liver glycogen stores than larger fish, reflecting an age or size specific energy allocation strategy. Larger fish may have allocated their acquired energy to growth, not building up glycogen stores as quickly as the smaller fish; however, this may not result in higher over-winter survival rates for smaller fish as smaller fish also have higher metabolic rates and deplete energy stores at a faster rate (Nilsson and Ostlund-Nilsson 2008). Other research has shown that the survival probability increases with size due to increases in total energy stores and decreased predation risk (Fonseca and Cabral 2007), and that total glycogen mass increases with body size (Heermann, Eriksson et al. 2009). Even though there was more glycogen per gram in the liver of smaller fish, larger fish have a greater amount of total glycogen stores (Heermann, Eriksson et al. 2009); thus, fish size should be controlled for in both field and laboratory experiments to remove this source of variation.

In contrast to liver glycogen levels, a species difference was detected in plasma T4 levels. Normal plasma T4 levels were significantly greater in juvenile rainbow trout than juvenile brook trout; however, there were no significant species differences in the other physiological stress response parameters investigated. Thyroxine (T4), the inactive form of 3,5,3-triiodo-L-thyronine (T3), is important for fish development, growth, osmoregulation, and reproduction (Blanton and Specker 2007). The species difference in plasma T4 levels may be due to differences in the basal secretion rate of T4 from the thyroid follicles or due to different conversion rates to T3. T4 synthesis and release is controlled by the thyroid stimulating hormone secreted by the pituitary gland (Blanton and Specker 2007), and conversion to T3 occurs primarily in the liver by deiodinases. A conclusion regarding the importance of this species difference is difficult to make without also investigating the entire thyroid cascade including the thyroid hormone receptors (Blanton and Specker 2007); however, it is unlikely that the difference in T4 levels will have

large implications for species-specific vulnerability to toxicants, as no significant difference between juvenile rainbow trout and brook trout plasma T3 levels (active form) was observed.

Conversely, no species differences were detected in plasma cortisol levels, suggesting juvenile rainbow trout and brook trout respond to stressors in a similar manner. While the range of values for both species was large, the standard error was small, suggesting the majority of the values included in the analyses were similar. Some research has documented species differences between rainbow trout and brook trout plasma cortisol levels (Benfey and Biron 2000; Miller, Rasmussen et al. 2009), but this does not appear to be a global pattern. Additionally, water temperatures ranging from 2.5 to 16 °C also had no effect on cortisol levels; however, this was expected as these temperatures are well below the critical upper temperature for both species (Eaton, McCormick et al. 1995; Eaton, McCormick et al. 1995; Bear, McMahon et al. 2007).

Water temperature did not significantly influence any of the other parameters tabulated in this study. The upper critical temperature for rainbow trout is approximately 26°C (Eaton, McCormick et al. 1995; Eaton, McCormick et al. 1995; Bear, McMahon et al. 2007) and estimates for upper critical temperature of brook trout range from 21°C (Martin and Petty 2009) to 25.3°C (Eaton, McCormick et al. 1995). The range of water temperatures documented in this study was wide (brook trout: 3 – 18 °C; rainbow trout: 1 – 21°C); however no studies came within 2°C of the upper critical temperatures. Increases in growth rates and condition may occur with increasing temperature up to the maximum growth temperature (Robinson, Josephson et al.). The present study, used 119 different estimates of growth rate over a temperature range of 1 to 21°C, but an influence of temperature on growth rate was not observed. However, other factors that influence growth rates, such as diet (Pereira, Rosa et al. 2002) and stocking density (Marchand and Boisclair 1998), may have masked the temperature effect.

This study has identified and compared the normal ranges of, energy reserves, physiological stress parameters, and condition and growth indices for juvenile rainbow trout and brook trout. Rainbow trout had higher levels of whole-body lipids than brook trout suggesting that brook trout may be more vulnerable to winter stress syndrome if food availability is low. Rainbow trout also had higher plasma T4 levels than brook trout, but this may not have large physiological implications as no species difference in the active form (plasma T3) was observed. Additionally, the information tabulated in this paper can be used to determine if an effect has occurred in environmental monitoring programs with little or poor quality reference data, to check the control or reference groups are within the normal range, and to predict species-specific vulnerability to toxicants if their mode-of-action depends on parameters that vary among species.

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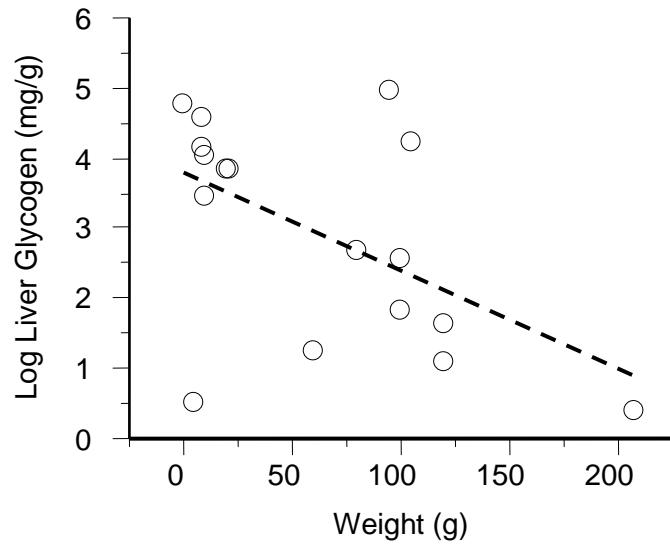


Figure 1.1. Influence of weight on liver glycogen levels in rainbow trout. (ANCOVA: $R^2 = 0.2824$; $p = 0.0281$).

Table 1.1. Number of observations (n) and the papers used to define the normal range of energy reserves, oxidative stress biomarkers, physiological stress response parameters, and condition indices for juvenile rainbow trout and brook trout.

| Parameter | Rainbow Trout | | Brook Trout | |
|------------------------|---------------|--|-------------|---|
| | n | References | n | References |
| <i>Energy Reserves</i> | | | | |
| Plasma Glucose (mg/ml) | 32 | (McCormick, Sakamoto et al. 1991; Davies, Cook et al. 1994; Johansen, Kennedy et al. 1994; Powell, Speare et al. 1994; Vijayan, Pereira et al. 1994; Kennedy, Sweeting et al. 1995; Barton and Grosh 1996; Bleau, Daniel et al. 1996; Hontela, Daniel et al. 1996; Wood, Johnston et al. 1996; Sanchez, Speare et al. 1997; Procarione, Barry et al. 1999; Ruane, Nolan et al. 2000; Sugiura, Dong et al. 2000; Aydin, Erman et al. 2001; De Boeck, Alsop et al. 2001; Galvez, Hogstrand et al. 2001; Campbell, Handy et al. 2002; Kirchner, Kaushik et al. 2003; Rogers, Richards et al. 2003; Toa, Afonso et al. 2004; Jentoft, Aastveit et al. 2005; DiBattista, Levesque et al. 2006; Gaylord, Teague et al. 2006; Landman, van den Heuvel et al. 2006; Miller, Wang et al. 2007; Miller, Rasmussen et al. 2009; Aluru, Leatherland et al. 2010) | 4 | (Hyndman, Kieffer et al. 2003; Hyndman, Kieffer et al. 2003; Rehulka and Minarik 2007; Miller, Rasmussen et al. 2009) |
| Liver Glycogen (mg/g) | 17 | (Gordon and McLeay 1978; Haux and Norberg 1985; Ewing, Barratt et al. 1994; Johansen, Kennedy et al. 1994; Kennedy, Sweeting et al. 1995; Bleau, Daniel et al. 1996; Hontela, Daniel et al. 1996; Gregory and Wood 1998; Ricard, Daniel et al. 1998; Mattsson, Tana et al. 2001; Yamamoto, Konishi et al. 2001; Kirchner, Kaushik et al. 2003; Viant, Werner et al. 2003) | 3 | (Whiting and Wiggs 1977; Whiting and Wiggs 1978; Vijayan, Ballantyne et al. 1991) |

| Parameter | Rainbow Trout | | Brook Trout | |
|---|---------------|---|-------------|---|
| | n | References | n | References |
| Whole-body Lipids (%) | 34 | (Vandenheuvel, McCarty et al. 1991; Riley, Higgs et al. 1993; Teskeredzic, Higgs et al. 1995; Teskeredzic, Teskeredzic et al. 1995; Dockray, Reid et al. 1996; Lauff and Wood 1996; Fisk, Yarechewski et al. 1997; Burel, Boujard et al. 1998; D'Cruz and Wood 1998; Dockray, Morgan et al. 1998; Kieffer, Alsop et al. 1998; Linton, Reid et al. 1998; Dabrowska, Fisher et al. 1999; Vielma, Ruohonen et al. 1999; Johnson and Summerfelt 2000; De Boeck, Alsop et al. 2001; Yamamoto, Konishi et al. 2001; Chaiyapechara, Liu et al. 2003; Simpkins, Hubert et al. 2003; Lellis, Barrows et al. 2004; Liu, Barrows et al. 2004; Simpkins, Hubert et al. 2004; Papoutsoglou, Karakatsouli et al. 2005; Blake, Inglis et al. 2006; Gaylord, Teague et al. 2006; Dumas, de Lange et al. 2007; Eliason, Higgs et al. 2007; Alami-Durante, Wrutniak-Cabello et al. 2010; Lee, Powell et al. 2010) | 3 | (Gunther, Moccia et al. 2005) (Le Francois, Blier et al. 1999; Gunther, Moccia et al. 2007) |
| <i>Oxidative Stress Biomarkers</i> | | | | |
| Liver Retinol (µg/g) | 2 | (Brown, Fisk et al. 2002; Miller, Rasmussen et al. 2009) | 1 | (Miller, Rasmussen et al. 2009) |
| Liver Tocopherol (µg/g) | 2 | (Brown, Fisk et al. 2002; Miller, Rasmussen et al. 2009) | 1 | (Miller, Rasmussen et al. 2009) |
| Liver GSH (µmol/mg protein) | 12 | (Davies, Cook et al. 1994; LindstromSeppa, Roy et al. 1996; Holm 2002; Lange, Ausseil et al. 2002; Ritola, Livingstone et al. 2002; Ait-Aïssa, Ausseil et al. 2003; Schlenk, Zubcov et al. 2003; Leggatt, Scheer et al. 2006; Ferrari, Venturino et al. 2007; Miller, Wang et al. 2007; Miller, Rasmussen et al. 2009) | 1 | (Miller, Rasmussen et al. 2009) |
| Liver Lipid Peroxidation (U/mg protein) | 4 | (Miller, Wang et al. 2007; Miller, Rasmussen et al. 2009; Ramsden, Smith et al. 2009) | 1 | (Miller, Rasmussen et al. 2009) |

| Parameter | Rainbow Trout | | Brook Trout | |
|--------------------------------------|---------------|--|-------------|---|
| | n | References | n | References |
| <i>Physiological Stress Response</i> | | | | |
| Plasma Cortisol (ng/ml) | 59 | (Munoz, Carballo et al. 1991; Johansen, Kennedy et al. 1994; Vijayan, Pereira et al. 1994; Kennedy, Sweeting et al. 1995; Barton and Grosh 1996; Bleau, Daniel et al. 1996; Fuentes, Bury et al. 1996; Hontela, Daniel et al. 1996; Wood, Johnston et al. 1996; Sanchez, Speare et al. 1997; Wagner, Bosakowski et al. 1997; Gregory and Wood 1998; Ricard, Daniel et al. 1998; Gregory and Wood 1999; Procarione, Barry et al. 1999; Barton 2000; Benfey and Biron 2000; Nielsen, Boesgaard et al. 2000; Ruane, Nolan et al. 2000; Ackerman and Iwama 2001; De Boeck, Alsop et al. 2001; Winberg, Overli et al. 2001; Lepage, Tottmar et al. 2002; McGeer, Szebedinszky et al. 2002; Overli, Kotzian et al. 2002; Leonardi, Sandino et al. 2003; Leonardi and Klempau 2003; Lepage, Vilchez et al. 2003; Reddy and Leatherland 2003; Rogers, Richards et al. 2003; Scott, Sloman et al. 2003; Overli, Korzan et al. 2004; Toa, Afonso et al. 2004; Bernier and Craig 2005; Jentoft, Aastveit et al. 2005; Mellina, Hinch et al. 2005; DiBattista, Levesque et al. 2006; Landman, van den Heuvel et al. 2006; Leggatt, Scheer et al. 2006; North, Turnbull et al. 2006; Schjolden, Pulman et al. 2006; Miller, Wang et al. 2007; Salo, Hebert et al. 2007; Auperin and Geslin 2008; Feldhaus, Heppell et al. 2008; Gioacchini, Smith et al. 2008; Person-Le Ruyet, Labbe et al. 2008; Backstrom and Winberg 2009; Miller, Rasmussen et al. 2009; Aluru, Leatherland et al. 2010; Gioacchini, Lombardo et al. 2010) | 12 | (Vijayan, Ballantyne et al. 1991; Audet and Claireaux 1992; Mackett, Tam et al. 1992; McDonald, Goldstein et al. 1993; Biron and Benfey 1994; Sanchez, Speare et al. 1997; Barton 2000; Benfey and Biron 2000; Hiroi and McCormick 2007; Krimmer 2008; Miller, Rasmussen et al. 2009) |

| Parameter | Rainbow Trout | | Brook Trout | |
|---|---------------|--|-------------|---|
| | n | References | n | References |
| Plasma T3 (ng/ml) | 18 | (Ewing, Barratt et al. 1994; Bleau, Daniel et al. 1996; Hontela, Daniel et al. 1996; Burel, Boujard et al. 1998; Ricard, Daniel et al. 1998; Nielsen, Boesgaard et al. 2000; Pereira, Rosa et al. 2002; Reddy and Leatherland 2003; Raine, Cameron et al. 2005; Buckman, Fisk et al. 2007; Miller, Wang et al. 2007; Person-Le Ruyet, Labbe et al. 2008) | 2 | (Audet and Claireaux 1992; Miller, Rasmussen et al. 2009) |
| Plasma T4 (ng/ml) | 17 | (Ewing, Barratt et al. 1994; Bleau, Daniel et al. 1996; Hontela, Daniel et al. 1996; Lanno and Dixon 1996; Burel, Boujard et al. 1998; Ricard, Daniel et al. 1998; Pereira, Rosa et al. 2002; Reddy and Leatherland 2003; Raine, Cameron et al. 2005; Buckman, Fisk et al. 2007; Miller, Wang et al. 2007) | 4 | (Chan and Eales 1976; McCormick and Naiman 1984; Audet and Claireaux 1992; Miller, Rasmussen et al. 2009) |
| Gill Na ⁺ /K ⁺ ATPase (μmol ADP/mg protein) | 15 | (Ewing, Barratt et al. 1994; Galvez and Wood 1999; Shrimpton and McCormick 1999; Galvez and Wood 2002; McGeer, Szebedinszky et al. 2002; Kamunde and Wood 2003; Rogers, Richards et al. 2003; Toa, Afonso et al. 2004; Alves and Wood 2006; Miller, Wang et al. 2007; Poppinga, Kittilson et al. 2007; Miller, Rasmussen et al. 2009; Ramsden, Smith et al. 2009) | 5 | (McCormick and Naiman 1984; Besner and Pelletier 1991; Hiroi and McCormick 2007) |
| <i>Condition & Growth Indices</i> | | | | |
| Hepatosomatic Index | 42 | (Ait-Aïssa, Ausseil et al. 2003; Best, Eddy et al. 2003; Bandarra, Nunes et al. 2006) (Haux and Norberg 1985; Vigano, Arillo et al. 1991; Riley, Higgs et al. 1993; Yang and Dick 1994; Kennedy, Sweeting et al. 1995; Donohoe and Curtis 1996; Lanno and Dixon 1996; Wilson, Wood et al. 1996; Wood, Johnston et al. 1996; Fisk, Yarechewski et al. 1997; Ricard, Daniel et al. 1998; Donohoe, Wang-Buhler et al. 1999; Blom, Andersson et al. 2000; Nielsen, Boesgaard et al. 2000; Mattsson, Tana et al. 2001; Yamamoto, Konishi et al. 2001; Brown, Fisk et al. 2002; Holm 2002; | 3 | (Vijayan, Ballantyne et al. 1991; Rehulka and Minarik 2007; Miller, Rasmussen et al. 2009) |

| Parameter | Rainbow Trout | | Brook Trout | |
|-------------------------------|---------------|--|-----------------|---|
| | n | References | n References | |
| Hepatosomatic Index Continued | | Verslycke, Vandenberg et al. 2002; Kamunde and Wood 2003; Kirchner, Kaushik et al. 2003; Thiessen, Campbell et al. 2003; Nikki, Pirhonen et al. 2004; Ronisz, Finne et al. 2004; Papoutsoglou, Karakatsouli et al. 2005; Raine, Cameron et al. 2005; Gaylord, Teague et al. 2006; Buckman, Fisk et al. 2007; Eliason, Higgs et al. 2007; Gaylord, Barrows et al. 2007; Lefevre, Aubin et al. 2007; Miller, Wang et al. 2007; Gourley and Kennedy 2009; Miller, Rasmussen et al. 2009) | | |
| Condition Factor | 53 | (Gordon and McLeay 1978; Morgan and Iwama 1991; Lohr and West 1992; Riley, Higgs et al. 1993; Ewing, Barratt et al. 1994; Johnsson and Bjornsson 1994; Teskeredzic, Teskeredzic et al. 1995; Lanno and Dixon 1996; Alanara and Brannas 1997; Gregory and Wood 1998; Kieffer, Alsop et al. 1998; Linton, Reid et al. 1998; McDonald, McFarlane et al. 1998; Donohoe, Wang-Buhler et al. 1999; Gregory and Wood 1999; Gregory and Wood 1999; Procarione, Barry et al. 1999; Shrimpton and McCormick 1999; Barton 2000; Johnson and Summerfelt 2000; Nielsen, Boesgaard et al. 2000; Kamunde, Grosell et al. 2001; Mattsson, Tana et al. 2001; Holm 2002; Viant, Werner et al. 2003; Simpkins, Hubert et al. 2004; Toa, Afonso et al. 2004; Papoutsoglou, Karakatsouli et al. 2005; Powell, Speare et al. 2005; Taylor, Migaud et al. 2005; Alves and Wood 2006; Baird, Krueger et al. 2006; Blake, Inglis et al. 2006; Galbreath, Adams et al. 2006; North, Turnbull et al. 2006; Powell, Speare et al. 2006; Schjolden, Pulman et al. 2006; Eliason, Higgs et al. 2007; Lefevre, Aubin et al. 2007; Miller, Wang et al. 2007; Tkatcheva, Franklin et al. 2007; Feldhaus, Heppell et al. 2008; Luchiari and Pirhonen 2008; Fairchild, Feltz et al. 2009; | 24 | (Skyrud, Andersen et al. 1989; Besner and Pelletier 1991; Schofield, Keleher et al. 1991; Audet and Claireaux 1992; Lohr and West 1992; Mackett, Tam et al. 1992; Tang and Boisclair 1995; McDonald, McFarlane et al. 1998; Le Francois, Blier et al. 1999; Barton 2000; Scruton, Ollerhead et al. 2003; Hakala and Johnson 2004; Sweka, Cox et al. 2004; Cox and Hartman 2005; Powell, Speare et al. 2005; Baird, Krueger et al. 2006; Galbreath, Adams et al. 2006; |

| Parameter | Rainbow Trout | | Brook Trout | |
|------------------------------|---------------|---|-------------|--|
| | n | References | n | References |
| Condition Factor Continued | | Kindschi and Barrows 2009; Miller, Rasmussen et al. 2009; Alami-Durante, Wrutniak-Cabello et al. 2010; Rasmussen and Ostenfeld 2010) | | Rajakaruna and Brown 2006; Sotiropoulos, Nislow et al. 2006; Chernoff and Curry 2007; Miller, Rasmussen et al. 2009; Rasmussen and Ostenfeld 2010; Tudorache, O'Keefe et al. 2010) |
| Specific Growth Rate (%/day) | 96 | (Gordon and McLeay 1978; Morgan and Iwama 1991; Wilson and Wood 1992; Davies, Cook et al. 1994; Johansen, Kennedy et al. 1994; Johnsson and Bjornsson 1994; Powell, Speare et al. 1994; Yang and Dick 1994; Dacosta and Curtis 1995; Speare, Macnair et al. 1995; Teskeredzic, Higgs et al. 1995; Teskeredzic, Teskeredzic et al. 1995; Dockray, Reid et al. 1996; Lanno and Dixon 1996; Wilson, Wood et al. 1996; Alanara and Brannas 1997; Alsop and Wood 1997; Fisk, Yarechewski et al. 1997; Burel, Boujard et al. 1998; D'Cruz and Wood 1998; Dockray, Morgan et al. 1998; Gregory and Wood 1998; Ricard, Daniel et al. 1998; Alsop, McGeer et al. 1999; Dabrowska, Fisher et al. 1999; Galvez and Wood 1999; Gregory and Wood 1999; Gregory and Wood 1999; Hollis, McGeer et al. 1999; Morgan, D'Cruz et al. 1999; Procarione, Barry et al. 1999; Vielma, Ruohonen et al. 1999; Burel, Boujard et al. 2000; Hollis, McGeer et al. 2000; Johnson and Summerfelt 2000; Myrick and Cech 2000; Nielsen, Boesgaard et al. 2000; Bailey and Alanara 2001; Galvez, Hogstrand et al. 2001; Kamunde, Grosell et al. 2001; Mattsson, Tana et al. 2001; Yamamoto, Konishi et al. 2001; Brown, Fisk et al. 2002; Campbell, Handy et al. 2002; | 23 | (Sykora, Synak et al. 1972; McCormick and Naiman 1984; Skyrud, Andersen et al. 1989; Audet and Claireaux 1992; Marchand and Boisclair 1998; Harper, Martins et al. 1999; Le Francois, Blier et al. 1999; Tucker and Rasmussen 1999; Morinville and Rasmussen 2003; Gunther, Moccia et al. 2005; Baird, Krueger et al. 2006; Bellgraph, Thompson et al. 2006; Chernoff and Curry 2007; McMahan, Zale et al. 2007; Hartman and Cox 2008) |

| Parameter | Rainbow Trout | | Brook Trout |
|--|---------------|---|-----------------|
| | n | References | n References |
| Specific Growth Rate (%/day) Continued | | Galvez and Wood 2002; Holm 2002; Kamunde, Grosell et al. 2002; Lee, Dabrowski et al. 2002; McGeer, Szebedinszky et al. 2002; Pereira, Rosa et al. 2002; Chaiyapechara, Liu et al. 2003; Ergun, Yigit et al. 2003; Kamunde and Wood 2003; Kirchner, Kaushik et al. 2003; Reddy and Leatherland 2003; Schwertner, Liu et al. 2003; Shima, Yamamoto et al. 2003; Simpkins, Hubert et al. 2003; Thiessen, Campbell et al. 2003; Viant, Werner et al. 2003; Dabrowski, Lee et al. 2004; Lellis, Barrows et al. 2004; Liu, Barrows et al. 2004; Nikki, Pirhonen et al. 2004; Jentoft, Aastveit et al. 2005; Papoutsoglou, Karakatsouli et al. 2005; Raine, Cameron et al. 2005; Taylor, Migaud et al. 2005; Alves and Wood 2006; Baird, Krueger et al. 2006; Bandarra, Nunes et al. 2006; Blake, Inglis et al. 2006; Gaylord, Teague et al. 2006; Heikkinen, Vielma et al. 2006; North, Turnbull et al. 2006; Powell, Speare et al. 2006; Boughton, Gibson et al. 2007; Buckman, Fisk et al. 2007; Eliason, Higgs et al. 2007; Gaylord, Barrows et al. 2007; Luchiari and Pirhonen 2008; Person-Le Ruyet, Labbe et al. 2008; Fairchild, Feltz et al. 2009; Gourley and Kennedy 2009; Peragon, Aranda et al. 2009; Ramsden, Smith et al. 2009; Tiril, Karayucel et al. 2009; Alami-Durante, Wrutniak-Cabello et al. 2010; Gioacchini, Lombardo et al. 2010; Lee, Powell et al. 2010) | |

Table 1.2. The mean, standard error (SE), minimum (Min), and maximum (Max) values of juvenile rainbow trout and brook trout energy reserves, oxidative stress biomarkers, physiological stress parameters and condition indices. ** indicates a highly significant difference between species (t -test; $\alpha = 0.05$) and * indicates a significant difference between species at (t -test; $\alpha = 0.10$).

| Parameter | Rainbow Trout | | | | Brook Trout | | | |
|---|---------------|-------|-------|--------|-------------|-------|-------|--------|
| | Mean | SE | Min | Max | Mean | SE | Min | Max |
| <i>Energy Reserves</i> | | | | | | | | |
| Plasma Glucose (mg/ml) | 1.06 | 0.13 | 0.58 | 4.00 | 0.83 | 0.03 | 0.77 | 0.88 |
| Liver Glycogen (mg/g) | 41.79 | 10.49 | 1.45 | 140.4 | 46.8 | 14.07 | 21.40 | 70.00 |
| Whole-body Lipids (%) | 13.44** | 1.53 | 3.50 | 35.40 | 5.53** | 1.34 | 4.19 | 8.20 |
| <i>Oxidative Stress Biomarkers</i> | | | | | | | | |
| Liver Retinol ($\mu\text{g/g}$) | 3.83 | 1.18 | 2.65 | 5.00 | 5.14 | - | - | - |
| Liver Tocopherol ($\mu\text{g/g}$) | 78.39 | 46.61 | 31.77 | 125.00 | 21.00 | - | - | - |
| Liver GSH ($\mu\text{mol/mg protein}$) | 16.74 | 8.56 | 0.01 | 103.45 | 10.53 | - | - | - |
| Liver Lipid Peroxidation (U/mg protein) | 0.12 | 0.04 | 0.05 | 0.24 | 0.17 | - | - | - |
| <i>Physiological Stress Response</i> | | | | | | | | |
| Plasma Cortisol (ng/ml) | 14.69 | 2.12 | 0.50 | 83.85 | 19.91 | 9.32 | 1.00 | 118.00 |
| Plasma T3 (ng/ml) | 5.73 | 1.16 | 0.90 | 19.44 | 4.59 | 1.51 | 3.08 | 6.10 |
| Plasma T4 (ng/ml) | 10.04* | 1.24 | 2.64 | 23.04 | 4.39* | 2.69 | 0.00 | 11.54 |
| Gill Na^+/K^+ ATPase ($\mu\text{mol ADP/mg protein}$) | 1.55 | 0.23 | 0.54 | 3.50 | 3.60 | 1.38 | 0.42 | 7.90 |
| <i>Condition Indices</i> | | | | | | | | |
| Hepatosomatic Index | 1.24 | 0.07 | 0.63 | 3.45 | 1.18 | 0.33 | 0.60 | 1.73 |
| Condition Factor | 1.24 | 0.03 | 0.66 | 1.87 | 1.19 | 0.08 | 0.87 | 2.96 |
| Specific Growth Rate (%/day) | 4.47 | 0.52 | 0.01 | 25.65 | 4.26 | 1.29 | 0.25 | 24.22 |

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**CHAPTER 2. SPECIES-SPECIFIC SENSITIVITY TO SELENIUM-INDUCED
IMPAIRMENT OF CORTISOL SECRETION IN ADRENOCORTICAL CELLS OF
RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND BROOK TROUT (*SALVELINUS
FONTINALIS*)**

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Abstract

Species differences in physiological and biochemical attributes exist even among closely related species and may underlie species-specific sensitivity to toxicants. Rainbow trout (RT) are more sensitive than brook (BT) to the teratogenic effects of selenium (Se), but it is not known whether all tissues exhibit this pattern of vulnerability. In this study, primary cultures of RT and BT adrenocortical cells were exposed to selenite (Na_2SeO_3) and selenomethionine (Se-Met) to compare cell viability and ACTH-stimulated cortisol secretion in the two fish species. Cortisol, the primary stress hormone in fish, maintains homeostasis when fish are exposed to stressors, such as toxicants. Cell viability was not affected by Se, but selenite impaired cortisol secretion, while Se-Met did not (RT and BT $\text{EC}_{50} > 2000$ mg/L). RT cells were more sensitive ($\text{EC}_{50} = 8.7$ mg/L) to selenite than BT cells ($\text{EC}_{50} = 90.4$ mg/L). To identify the targets where Se disrupts cortisol synthesis, selenite-impaired RT and BT cells were stimulated with ACTH, dbcAMP, OH-

cholesterol, and pregnenolone. Selenite acted at different steps in the cortisol biosynthesis pathway in RT and BT cells, confirming a species-specific toxicity mechanism. To test the hypothesis that oxidative stress mediates Se-induced toxicity, selenite-impaired RT cells were exposed to NAC, BSO and antioxidants (DETCA, ATA, Vit A, Vit E). Inhibition of SOD by DETCA enhanced selenite-induced cortisol impairment, indicating that oxidative stress plays a role in Se toxicity; however, modifying GSH content of the cells did not have an effect. The results of this study, with two closely related salmonid species, demonstrated species-specific differences in sensitivity to Se which should be considered when setting thresholds and water quality guidelines.

Key words

cortisol, adrenocortical cells, selenomethionine, sodium selenite, oxidative stress, trout

Introduction

Fundamental differences in biochemical and physiological attributes exist, even among closely related species. Rainbow trout (*Oncorhynchus mykiss*), a species highly sensitive to environmental stressors and toxicants, digest macronutrients more efficiently than Atlantic salmon (*Salmo salar*) (Krogdahl, Sundby et al. 2004), have higher hepatic glutathione (GSH) reserves than brook trout (*Salvelinus fontinalis*) (Miller, Rasmussen et al. 2009), and have lower phase I biotransformation V_{\max} for sulfoxidation than channel catfish (*Ictalurus punctatus*) or tilapia (*Oreochromis* sp.) (Gonzalez, Shaikh et al. 2009). Such differences in the basic biology of organisms may mediate species-specific sensitivity to toxicants. For example, the greater sensitivity to waterborne cadmium or copper of rainbow trout compared to yellow perch (*Perca*

flavescens) can be explained by differences in the affinity and function of gill binding sites and transport fluxes of the toxicants and of ions such as Ca^{2+} and Na^+ (Taylor, Wood et al. 2003; Niyogi and Wood 2004). Species-specific sensitivity to toxicants in fish have been also documented for pesticides (VanDolah, Maier et al. 1997; Quinn, Rasmussen et al. 2010), pharmaceuticals (Gonzalez, Shaikh et al. 2009), and selenium (Holm, Palace et al. 2005).

Selenium (Se), an essential constituent of glutathione peroxidase, deiodinase and thioredoxin reductase, can be toxic at levels slightly above homeostatic requirement (Janz, DeForest et al. 2010). It occurs at varying levels in the bedrock, with highest concentrations measured in marine shales (Haygarth 1994). Anthropogenic activities such as coal mining and agriculture enhance weathering of seleniferous rock, increasing Se levels in the aquatic environment (Hamilton 2004). Selenium bioaccumulates in the liver and gonads of fish, and uptake occurs primarily through diet, not the water column (Stewart, Grosell et al. 2010). Selenite is the most acutely toxic form of Se to fish, followed by selenate and organic selenomethionine (Se-Met); Se is teratogenic in fish (Coyle, Buckler et al. 1993; Hamilton, Holley et al. 2005; Rigby, Deng et al. 2010) and species-specific sensitivities to Se have been documented. Rainbow trout have higher larval deformity rates than brook trout or cutthroat trout (*Oncorhynchus clarki*) when exposed to elevated Se in the environment (Holm, Palace et al. 2005). It is not known whether processes other than larval development exhibit species-specific sensitivities to Se, and what cellular characteristics and mechanisms underlie these differences.

During the metabolism of both selenite and Se-Met, reactive oxygen species (ROS), leading to increased oxidative damage, are produced (Palace, Spallholz et al. 2004; Misra and Niyogi 2009); however, the metabolic pathways for selenite and Se-Met differ. Selenite is reduced by GSH to hydrogen selenide, which may then react with oxygen to produce ROS (Seko, Saito et al. 1989). This mechanism of oxidative stress only requires the presence of GSH and oxygen. In contrast, the production of ROS by Se-Met is more complex. Selenomethionine must

be first metabolised to methylselenol by methioninase, then methylselenol reacts with GSH to produce the ROS (Palace, Spallholz et al. 2004). Thus GSH, usually acting as an antioxidant protecting cells from damage (Kelly, Havrilla et al. 1998), plays a role in ROS production by selenite and Se-Met. Se-induced oxidative damage in human hepatoma cells was observed in both cells depleted of GSH and those with artificially elevated GSH levels (Shen, Yang et al. 2000), suggesting a dual role for GSH in Se toxicity. Although Palace *et al.* (2004) provided evidence that GSH augments Se toxicity in fish embryos, the universality of oxidative stress and GSH in Se toxicity has not been demonstrated thus far.

The teleost adrenocortical cell model has been used to assess adrenal toxicity of several organic and inorganic toxicants (Hontela and Vijayan 2009), but the adrenotoxicity of Se, either as selenite or Se-Met, has not been investigated. The adrenocortical cells are located in the head kidney, the adrenal tissue of teleost fish (Hontela 2005), a system which has been well characterized *in vitro* (Lacroix and Hontela 2001; Aluru, Renaud et al. 2005; Fuzzen, Van der Kraak et al. 2010). When a fish perceives a stress, adrenocorticotrophic releasing hormone (ACTH) binds a membrane receptor in the steroidogenic adrenocortical cell and activates the cAMP-protein kinase A signalling pathway to stimulate, via the StAR protein, the uptake of cholesterol by the mitochondria (Fig. 2.1). Cholesterol is transformed to pregnenolone, and then a series of cytochrome P450 enzymes in the endoplasmic reticulum and the mitochondria transform pregnenolone to cortisol (Hontela 2005).

The steroidogenic pathway leading to cortisol can be manipulated with steroid precursors and signalling molecules to determine the specific step(s) disrupted by a toxicant (Bisson and Hontela 2002; Hontela and Vijayan 2009). Such mechanism-based data might be extrapolated to other steroidogenic cholesterol-dependent pathways, including synthesis of testosterone and estrogens. It may also be used to compare species-specific sensitivities to toxicants and investigate the mechanisms of toxicity (Lacroix and Hontela 2004). The present study tested the

hypotheses that Se is adrenotoxic, and that adrenocortical cells of rainbow trout (RT) are more sensitive to Se than the adrenocortical cells of brook trout (BT), as has been proposed for the teratogenic effects of Se. The specific objectives were to (1) determine, *in vitro*, the effect of sodium selenite and Se-Met on cortisol secretion by adrenocortical cells in RT and BT, (2) identify the step(s) disrupted by Se in the steroidogenic pathway leading to cortisol secretion, and (3) investigate the role of oxidative stress in Se toxicity.

Materials and methods

Chemicals

Porcine adrenocorticotropin (ACTH I-39), collagenase/dispase, DNAase, minimal essential medium (MEM), bovine serum albumin (BSA), sodium bicarbonate (NaHCO₃), L-buthionine-[S,R]-sulfoximine (BSO), N-acetyl-L-cysteine (NAC), sodium chloride (NaCl), calcium chloride (CaCl₂), potassium chloride (KCl), dextrose, sodium selenite (Na₂SeO₃), selenomethionine (Se-Met), potassium phosphate (KH₂PO₄), ethanol, ethylenediaminetetraacetic acid (EDTA), metaphosphoric acid, 2,6-di-*tert*-butyl-4-methylphenol (BHT), 3-amino-1,2,4-triazole (ATA), sodium diethyldithiocarbamate trihydrate (DETCA), α -tocopherol, trypan blue, retinol, pregnenolone, N⁶,2'-*o*-dibutyryladenine 3',5'-cyclic monophosphate (dbcAMP), and OH-cholesterol were purchased from Sigma-Aldrich (Oakville, Ontario). HEPES was purchased from Fisher Scientific (Ottawa, Ontario) and 3-aminobenzoic acid ethyl ester (MS-222) was purchased from MP Biomedicals (Solon, Ohio).

Fish.

Animal use protocols were approved by the University of Lethbridge Animal Care Committee in accordance with national guidelines. Juvenile rainbow trout, *Oncorhynchus mykiss*, (109.9 ± 4.9 g) and juvenile brook trout, *Salvelinus fontinalis*, (54.8 ± 2.2 g) were obtained from the Allison Creek Brood Trout Station (Blairmore, Alberta). Fish were kept in a 1000 L tank (semi-static system, 25% daily water renewal, 7 mg/L oxygen, 161 mg/L CaCO_3) at 14°C for the duration of experiment. Fish were fed extruded floating steelhead food pellets (Nelson's Silver Cup Fish Feed, Allison Creek Brood Trout Station, Blairmore, Alberta) between 0900 and 1000 hours *ad libitum*. Fish were allowed a minimum of two weeks to acclimate to laboratory conditions before experiments began.

Cell culture

Fish were euthanized with 1 g/L MS-222, bled from the caudal vasculature, and perfused through the heart with 0.7% NaCl. The head kidney was removed and a rough homogenate made (pieces $\sim 1\text{mm}^3$). The tissue was then digested with collagenase/dispase (2 mg/ml) and DNAase (1.2 mg/ml) in MEM (pH = 7.4, supplemented with 5 g/L BSA and 2.2 g/L NaHCO_3) for 1 h at 23°C. The cell suspension was filtered with Nitex monofilament cloth (30 μm) and the cell concentration adjusted to 75×10^6 cells/ml.

Exposure to sodium selenite and selenomethionine

To determine the effective concentration of the toxicant that inhibits 50% of cortisol secretion (EC_{50}), the protocol previously described (Bisson and Hontela 2002) was followed. In summary, the cell suspension (75 – 150 μl) was plated in a 96-well microplate and incubated at 15°C. After 2 h, the microplate was centrifuged (233 x g, 15°C, 3 min) and the MEM replaced

with the appropriate treatment. Adrenocortical cells (individual fish as replicates) were exposed to sodium selenite (0, 2, 4, 8, 16, 30, 100 mg/L) and Se-Met (0, 30, 100, 1000, 2000 mg/L) in Ringer solution (550 M CaCl₂, 9 M NaCl, 370 M KCl, 180 M dextrose, and 40 M hepes; pH = 7.4) for 1 h at 15°C. A Roundup (600 mg/L in Ringer) treatment was included as a positive control, as Roundup has been shown to inhibit cortisol secretion in RT (unpublished data). After 1 h, the microplate was centrifuged (233 x g, 15°C, 3 min) and the supernatant removed and stored at -80°C for determination of lactate dehydrogenase (LDH) release, a measure of cell viability (TOX-7 kit, Sigma-Aldrich, Oakville, Ontario). Cells were rinsed with Ringer, and then cortisol secretion was stimulated with 1 U/ml ACTH in MEM (1 h, 15 °C). The microplate was centrifuged and the supernatant removed and stored at -80°C for later cortisol analysis by a radioimmunoassay kit (#07-221102, Medicorp, Montréal, Canada). Cortisol secretion was expressed as percentage of control (ACTH-stimulated secretion without toxicant, in ng/ml). The cells were re-suspended in MEM for cell viability counts using trypan blue, for comparison to the LDH method in some experiments.

Manipulation of the steroidogenic pathway

Cells were plated and pre-incubated for 2 h as described above. Cells of each fish were then exposed to the sodium selenite EC₅₀ (rainbow trout = 12 mg/L; brook trout = 90 mg/L) for 1 h in Ringer. The microplate was centrifuged (233 x g, 15°C, 3 min) and the supernatant removed and stored at -80°C for LDH determination. Cells were rinsed with Ringer, and then cortisol secretion was stimulated with either 1 U/ml ACTH in MEM (control, ACTH treatment), 2 mM dbcAMP, pregnenolone (rainbow trout, 3.0 µM; brook trout, 1.0 µM), 0.5 mM OH-cholesterol, or 0.5 mM OH-cholesterol + 1 U/ml ACTH for 2 h. Pregnenolone and OH-cholesterol were dissolved in ethanol for a final ethanol concentration of 0.025% and 1% respectively. This

concentration had no effect on cortisol secretion or viability of the adrenocortical cells (Lacroix and Hontela 2001). Microplates were centrifuged, the supernatant removed, and stored at -80°C for later cortisol analyses.

GSH experiment

The cell suspension was plated (100 µl/well), as described in the “Cell culture” section above. The plate was centrifuged (233 x g, 15°C, 3 min) and the media replaced with one of the following pre-exposure treatments: MEM (Control and Se control treatments), 1 mM NAC, 5 mM NAC, 1 mM BSO, or 5 mM BSO in MEM. After 3 h at 15°C, the microplate was centrifuged, the cells washed with MEM, and exposed (15°C, 1h) to Ringer only (Control) or 12 mg/L Na₂SeO₃ (RT EC₅₀) in Ringer (all other treatments). After the exposure, the microplate was centrifuged and the supernatant collected and frozen for later LDH analyses. The cells were then washed with Ringer to remove any toxicant, centrifuged, and cortisol secretion was stimulated with 1 U/ml ACTH in MEM (15°C; 1h). The supernatant was collected and frozen for later cortisol analyses and the cells were washed with 50 mM KH₂PO₄ buffer containing 1 mM EDTA (pH = 7.4) and lysed with a lysis solution (Sigma #TOX-7 kit). Post-exposure reduced glutathione (GSH 400 kit, Bioxytech, Montréal, Québec) was measured (µmol GSH/mg protein) in the supernatant of half the wells for each treatment and post-exposure lipid peroxidation (LPO) was measured (µmol malondialdehyde & 4-hydroxyalkenals/mg protein) in both the pellet and supernatant (LPO 596 kit, Bioxytech, Montréal, Québec) in the other half of the wells, as described previously (Miller, Wang et al. 2007).

Effects of antioxidants

The cell suspension of 75×10^6 cells/ml was plated as described in the “Cell culture” section. The microplate was centrifuged (233 x g, 15°C, 3 min) and the media replaced with one of the following pre-exposure treatments: MEM (Control, Se control), 10 mM ATA (inhibitor of catalase activity), 1 mM DETCA (inhibitor of superoxide dismutase activity), 50 μ M α -tocopherol (Vit E), or 20 μ M retinol (Vit A) in MEM. After 2 h incubation in the dark, cells were centrifuged, washed with MEM, centrifuged again and exposed to 12 mg/l Na_2SeO_3 (~ EC_{50}) in Ringer for 1 h. The control group was exposed to Ringer only. Next, the microplate was centrifuged, and the cells rinsed with Ringer, centrifuged, and stimulated with 1 U/ml ACTH for 1 h. Supernatants were collected for LDH, cortisol, and post-exposure LPO analyses as described in “GSH experiment” section.

Statistical analyses

All statistical analyses were performed using the JMP 7.0.2 software package. Cell viability methods were compared using three-factor analyses of variance (ANOVA) for species, method and treatment nested within experiment. The variability of the cell viability methods was compared by calculating the standard deviation for each treatment in the four EC_{50} experiments, followed by a one-way ANOVA. Positive controls (Round-Up or sodium selenite) for each experiment were compared to the true control (no toxicant) treatment using *t*-tests. EC_{50} s were calculated with regression analyses after probit transformation. Differences in cell viability, cortisol secretion, GSH and LPO were tested with a one-way ANOVA for treatment and a *post hoc* Tukey-Kramer HSD. Species differences in cortisol secretion were determined with a *t*-test or a nested ANOVA (Se exposure nested within treatment). Data was log transformed to respect normality as needed and tests used $\alpha = 0.05$ unless otherwise noted.

Results

Cell viability method

Trypan blue exclusion was a more sensitive measure of cell death than LDH release (3-way ANOVA: $F_1 = 29.24$, $p < 0.0001$; data not shown); however, trypan blue exclusion had significantly higher standard deviations within treatment groups, indicating that it was more variable than the LDH release method (one-way ANOVA: $F_1 = 37.50$, $p < 0.0001$; data not shown).

Effect of sodium selenite on cortisol secretion in vitro (EC_{50})

There was a significant negative relationship between cortisol secretion (probit units) and sodium selenite concentration for both RT ($n = 14$) and BT ($n = 16$) adrenocortical cells (refer to Table 2.1 for regression parameters). The EC_{50} for RT cells was 8.71 mg/L with lower and upper 95% confidence intervals of 4.81 mg/L and 14.05 mg/L, respectively (Fig. 2.2A). The EC_{50} for BT cells was 90.49 mg/L with lower and upper 95% confidence intervals of 40.85 mg/L and 545.65 mg/L (Fig. 2.2B). The EC_{50} for RT cells was significantly lower than the EC_{50} for BT cells, as 95% confidence intervals did not overlap.

Cell viability measured by LDH release was not significantly affected by sodium selenite exposure in RT cells (one-way ANOVA: $F_6 = 0.32$, $p = 0.9244$; data not shown) or BT cells (one-way ANOVA: $F_6 = 1.12$, $p = 0.3593$; data not shown). Adrenocortical cell viability measured by trypan blue exclusion was not significantly influenced by sodium selenite exposure in RT cells (one-way ANOVA: $F_6 = 0.47$, $p = 0.8324$; data not shown). Cell viability measured by trypan

blue exclusion in the BT cells in the 100 mg/L treatment was lower than in the 2, 4, and 8 mg/L treatments (one-way ANOVA: $F_6 = 3.78$, $p = 0.0022$; data not shown).

Effect of selenomethionine on cortisol secretion *in vitro* (EC_{50})

There were no significant relationships between cortisol secretion (probit units) and Se-Met concentrations (Fig. 2.3) for RT ($n = 13$) or BT ($n = 14$) adrenocortical cells ($p > 0.05$). Refer to Table 1 for regression parameters. The EC_{50} s could not be determined in either species, with $EC_{50} > 2000$ mg/L, the highest concentration tested.

Adrenocortical cell viability measured using LDH release was not significantly affected by Se-Met in RT (one-way ANOVA: $F_4 = 0.72$, $p = 0.5811$; data not shown) or BT cells (one-way ANOVA: $F_4 = 0.57$, $p = 0.6838$; data not shown). Similarly, cell viability measured using trypan blue exclusion was not significantly influenced in RT (one-way ANOVA: $F_4 = 2.33$, $p = 0.0691$; data not shown) or BT (one-way ANOVA: $F_4 = 0.81$, $p = 0.5225$; data not shown) adrenocortical cells.

Roundup, a chemical that inhibits cortisol secretion *in vitro* (unpublished data) was used as a positive control in the selenite and Se-Met exposures. Roundup (600 mg/L) significantly decreased cortisol secretion (RT cells = $48.49 \pm 6.32\%$ of control; BT cells = $26.65 \pm 5.54\%$ of control; t -test: $p < 0.05$), but did not alter cell viability (RT cells: $100.17 \pm 0.55\%$; BT cells = $100.06 \pm 0.24\%$; t -test: $p > 0.05$), compared to the un-exposed control.

Manipulation of the steroidogenic pathway

To determine the appropriate concentrations of cortisol precursors required to stimulate cortisol secretion, a range-finding experiment was conducted. In the absence of Se, 1 U/ml ACTH, 2 mM dbcAMP, 0.5 mM OH-cholesterol + 1 U/ml ACTH, and 1, 3 and 4 μ M of pregnenolone stimulated cortisol secretion in RT adrenocortical cells (Table 2.2). Addition of OH-cholesterol (0.5 mM – 2 mM) or 0.5 mM pregnenolone did not stimulate cortisol secretion (Table 2.2). Thus, 1 U/ml ACTH, 2 mM dbcAMP, 0.5 mM cholesterol, 0.5 mM OH-cholesterol + 1 U/ml ACTH, and 3 μ M of pregnenolone were used in the subsequent experiments with RT adrenocortical cells. In the absence of Se, 1 U/ml ACTH, 2 mM dbcAMP, 0.5 mM OH-cholesterol, OH-cholesterol + 1 U/ml ACTH, and 1 μ M of pregnenolone stimulated cortisol secretion in BT adrenocortical cells and were used in the subsequent experiments (Table 2.2). Brook trout adrenocortical cells had significantly higher basal (MEM treatment) and OH-cholesterol stimulated cortisol secretion than RT cells (Table 2.2).

In RT adrenocortical cells ($n = 8$), selenite exposure significantly decreased the ACTH- and pregnenolone-stimulated cortisol secretion, but not dbcAMP-, OH-cholesterol, or OH-cholesterol + ACTH stimulated cortisol secretion (Fig. 2.4A; nested ANOVA: $F_5 = 11.64$, $p < 0.0001$). In BT ($n = 14$) adrenocortical cells, selenite exposure significantly decreased ACTH-, dbcAMP-, OH-cholesterol-, and OH-cholesterol + ACTH stimulated cortisol secretion (Fig. 2.4B; nested ANOVA: $F_5 = 12.73$, $p < 0.0001$). Addition of pregnenolone restored cortisol secretion in presence of selenite (Fig. 2.4B). Treatments did not significantly alter cell viability as measured by LDH release in RT (one-way ANOVA: $F_5 = 1.75$, $p = 0.1213$; data not shown) or BT cells (one-way ANOVA: $F_5 = 0.64$, $p = 0.6677$; data not shown).

GSH experiment

The NAC and BSO treatments used altered intracellular GSH levels in RT cells (Table 2.3, 3 h exposure). One and 5 mM NAC increased intracellular GSH 5.4% and 8.7%, respectively, while 1 and 5 mM BSO significantly decreased intracellular GSH 17.7% and 20.0%, respectively. The NAC and BSO concentrations used in this experiment ($n = 4$) did not alter cortisol secretion (one-way ANOVA: $F_{12} = 0.49$, $p = 0.9075$; data not shown) or cell viability in RT adrenocortical cells (one-way ANOVA: $F_{12} = 0.74$, $p = 0.7055$; data not shown).

Exposure to 12 mg/L selenite (EC_{50} for RT cells) decreased cortisol secretion (Fig. 2.5A) as expected; however, treatment with NAC or BSO at concentrations that altered the GSH content of the cells after 3 h, did not significantly affect cortisol secretion in adrenocortical cells after exposure to selenite and stimulation with ACTH ($n = 9$; Fig. 2.5A). The GSH and LPO content of the cells at the end of the experiment (5 h) was not significantly influenced by selenite exposure, NAC, or BSO exposure (Table 2.3). Adrenocortical cell viability, as measured by LDH release, was also not significantly altered by treatment (ANOVA: $F_5 = 0.29$, $p = 0.9168$; data not shown).

Antioxidant experiment

Treatment with ATA (inhibitor of catalase), DETCA (inhibitor of SOD), Vit E or Vit A ($n = 3$) in absence of selenite did not influence LPO (ANOVA: $F_4 = 0.43$, $p = 0.7830$; data not shown), cell viability (ANOVA: $F_4 = 1.07$, $p = 0.4223$; data not shown), or cortisol secretion (ANOVA: $F_4 = 0.64$, $p = 0.6365$; data not shown).

Exposure to the selenite ($EC_{50} = 12$ mg/L) significantly decreased cortisol secretion in RT adrenocortical cells, as also shown in the previous experiments (Fig. 2.5B). Exposure to DETCA

significantly decreased cortisol secretion compared to exposure to sodium selenite alone (n=8; Fig. 2.5B); however, exposure to ATA, Vit A, and Vit E did not alter the sodium selenite-induced impairment of cortisol secretion (Fig. 2.5B). LPO levels in the adrenocortical cells did not significantly vary with ATA, DETCA, Vit, A or Vit E treatments (Table 2.3). Similarly, cell viability (measured by LDH release) was not significantly influenced by treatment (ANOVA: $F_5 = 0.31$, $p = 0.9072$; data not shown).

Discussion

Species-specific sensitivities to the teratogenic effects of Se have been documented in fish, with higher rates of larval deformities detected in RT than in the closely related BT (Holm, Palace et al. 2005). The aim of this study was to compare the effects of selenite and Se-Met on the adrenocortical cells and cortisol secretion of RT and BT, identify the steps disrupted by Se in cortisol's biosynthetic pathway, and investigate the role of oxidative stress in Se toxicity. Exposure of RT and BT adrenocortical cells to selenite identified a species-specific sensitivity to Se. Sodium selenite impaired cortisol secretion in both trout species, but the EC_{50} in cells of RT was substantially lower than the EC_{50} in BT cells. This sensitivity pattern mirrors the teratogenicity of Se observed in field studies where RT embryos from Se-contaminated streams showed a greater rate of teratogenesis than BT embryos from the same stream (Holm, Palace et al. 2005). Species-specific toxicodynamics of Se have been proposed as a mechanism for these species-specific teratogenesis rates. The uptake of Se may be lower in BT eggs than in RT eggs, as RT eggs had higher loads of Se than BT eggs, for similar exposure levels and muscle Se accumulation (Holm, Palace et al. 2005). However, differences in exposure do not explain the results observed in the present study with the adrenocortical cells, as the cells from the two species were exposed to the same Se concentrations *in vitro*. Cellular uptake rates of Se in RT and

BT cells, or other model systems, have not been characterized, and a higher uptake of Se by the RT cells cannot be excluded at present.

The present study compared the sensitivity of the adrenocortical cells in two fish species, and also provided new data on comparative effects of two forms of Se. Sodium selenite was more toxic than Se-Met to RT and BT adrenocortical cells. Se-Met did not impair cortisol secretion, even at concentrations as high as 2000 mg/L. Similarly, selenite was more toxic than Se-Met to human HepG2 cells (Weiller, Latta et al. 2004) and larvae of *Chironomus*, an aquatic invertebrate (Maier and Knight 1993). In the aquatic environment, all animals are exposed to Se-Met through their diet (Janz, DeForest et al. 2010) while selenite exposure is primarily waterborne through the gills (Hodson, Hilton et al. 1986); however, the form of Se that acts on the target cells and organs depends on seleno-metabolism of the organism. In human K-562 cells, selenite is taken up faster than Se-Met (Frisk, Yaqob et al. 2000). Selenite may be reduced to selenide by thiols, before it is absorbed by cells (Ganyc and Self 2008; Olm, Fernandes et al. 2009), but Se-Met uptake is controlled by the same amino acid transporters as methionine (Bakke, Tashjian et al. 2010). The different uptake mechanisms and cellular uptake rates may drive the different toxicities of selenite and Se-Met observed in the present study.

Both selenite (Misra and Niyogi 2009) and Se-Met (Spallholz, Palace et al. 2004) generate ROS and oxidative damage when reduced by GSH; however, selenite is reduced directly (Tarze, Dauplais et al. 2007; Gabel-Jensen and Gammelgaard 2010) and Se-Met must be first metabolised to methylselenol (Palace, Spallholz et al. 2004). Although there is some evidence to suggest that methioninase is present in fish embryos (Palace, Spallholz et al. 2004), and its activity has been detected in RT hepatocytes (Misra, Peak et al. 2010), it has not been directly measured in fish adrenocortical cells. If methioninase is absent or present at very low levels in trout adrenocortical cells, Se-Met should not impair cortisol secretion in RT or BT cells, as has

been observed in the present study. Future studies should investigate the biotransformation of Se and Se-induced generation of ROS in fish adrenocortical cells.

The impairment of cortisol secretion by selenite in the present study was not due to cell death, as LDH release was low in all experiments. Similarly, exposure for 1 h to 100 mg/L (587 μ M) sodium selenite did not significantly alter cell viability of RT hepatocytes (Misra and Niyogi 2009) and 19,000 mg/L Se-Met did not decrease human HepG2 cell viability (Weiller, Latta et al. 2004). Instead, the impairment of cortisol secretion in RT and BT adrenocortical cells appears to be caused by a disruption of the cortisol biosynthetic pathway. The biosynthesis and secretion of cortisol in fish has been well studied, and the key steps and intermediates identified (Hontela and Vijayan 2009; Fuzzen, Van der Kraak et al. 2010). In the present study, cortisol secretion by the steroidogenic adrenocortical cells could be stimulated *in vitro* by ACTH, dbcAMP, or pregnenolone in both RT and BT cells. Some species differences in the basal, ACTH-, and OH-cholesterol stimulated cortisol secretion were detected, with higher secretion in BT than RT cells. OH-cholesterol did not stimulate cortisol secretion to the same extent as the other compounds used, but this may be due to lower conversion rates of the artificial substrate (Lacroix and Hontela 2001). Similar to other studies designed to identify the target(s) of specific toxicants in the steroidogenic pathway, including cadmium (Laskey and Phelps 1991; Mathias, Mgbonyebi et al. 1998) and some pesticides (Bisson and Hontela 2002; Dorval, Leblond et al. 2003), restoration of cortisol synthesis following substitution of specific precursors or signalling molecules was used to identify the step where selenite exerted its action.

In BT cells, selenite-induced disruption of cortisol secretion was restored by pregnenolone, but not by ACTH, dbcAMP or OH-cholesterol, the intermediates or signalling molecules acting upstream of pregnenolone in the biosynthetic pathway. This suggests that in BT cells, selenite may be disrupting one specific step in the pathway, the synthesis of pregnenolone. Normal synthesis of pregnenolone requires transport of cholesterol to the mitochondria by the

StAR protein (Stocco 2001) and activity of several steroidogenic enzymes, including $cP450_{sc}$, that transforms cholesterol to pregnenolone, and then to cortisol. Although StAR protein has been identified as a target of several toxicants with endocrine disrupting activity, including Dimethoate (Walsh, Webster et al. 2000) and some pharmaceuticals (Gravel and Vijayan 2006), the role of StAR protein in selenite-induced disruption of cortisol synthesis has not been investigated.

The present study provided evidence that species differences exist in the mode of action of selenite-induced cortisol disruption. While selenite disrupted one specific step in steroidogenesis of BT adrenocortical cells, it impaired cortisol secretion at multiple sites in the RT cells. Stimulation of cortisol synthesis by dbcAMP restored selenite-impaired cortisol secretion, indicating that selenite acted early in the biosynthesis pathway; however, pregnenolone also failed to restore secretion. Selenite may interfere with ACTH binding to the melanocortin 2 receptor, affect production of cAMP by adenylyl cyclase, and, since cortisol secretion was not restored by pregnenolone, selenite must also disrupt steps downstream of cholesterol transport into mitochondria. Chemicals damaging multiple cellular sites may exert their toxicity by a non-specific mechanism. Oxidative stress, the proposed mechanism of Se toxicity in teratogenesis (Palace, Spallholz et al. 2004), generates oxidative radicals which will indiscriminately attack proteins, DNA or lipids in their vicinity (Kelly, Havrilla et al. 1998) in a non-specific manner.

To investigate the role of GSH and oxidative stress in selenite toxicity to RT adrenocortical cells, cells were treated with NAC to boost GSH production and with BSO to lower GSH levels. Studies with human hepatoma cells reported that GSH can both protect and facilitate Se-induced oxidative stress (Shen, Yang et al. 2000). In the present study with RT cells, GSH levels were substantially lowered with BSO pre-treatment, but NAC only slightly increased intracellular GSH. Despite the alterations of GSH content of the RT cells detected before the start of the selenite exposure, no effects on selenite-induced impairment of cortisol secretion,

lipid peroxidation, or GSH levels at the end of the experimental period were observed. Our results suggest that GSH does not play a significant role in selenite-induced cortisol impairment in RT adrenocortical cells, in contrast to human hepatoma cells. Biochemical differences between human hepatoma cells and fish cells may underlie this difference; however, it is also possible that the selenite concentration used in this experiment (12 mg/L) may not have been high enough to observe GSH-mediated effects. In RT hepatocytes, GSH induction then depletion was not observed until 17 mg/L and 35 mg/L selenite, respectively (Misra and Niyogi 2009).

To further investigate the role of oxidative stress in selenite-induced cortisol disruption in RT adrenocortical cells, the concentrations of antioxidants were manipulated. Addition of α -tocopherol (vitamin E), a lipid soluble scavenger of the superoxide anion radical (Kelly, Havrilla et al. 1998), retinol (vitamin A), and ATA (catalase inhibitor) did not alter the sensitivity of the RT cells to selenite; however, exposure to DETCA, an inhibitor of superoxide dismutase (SOD), increased the toxicity of selenite to adrenocortical cells. Superoxide dismutase removes the superoxide anion radical to form oxygen and hydrogen peroxide (Kelly, Havrilla et al. 1998). Thus, production of the superoxide anion radical may be one of the toxicity mechanisms of selenite and SOD may play an important role in the defence against selenite toxicity in fish cells, as decreasing the cell's ability to scavenge the superoxide anion when exposed to selenite further inhibited cortisol secretion. It has been previously shown that SOD decreases Se-induced ROS production *in vitro* in a chemiluminescent assay (Spallholz, Palace et al. 2004) and is induced by selenite in RT hepatocytes (Misra and Niyogi 2009). Superoxide dismutase has isoforms found in the cytosol and mitochondria, as well as the cellular membrane (Kelly, Havrilla et al. 1998). Intracellular site-specific activities of antioxidant enzymes such as SOD may be related to site-specific toxicity of selenite in the steroidogenic pathways, and underlie species differences in vulnerability.

In conclusion selenite, but not Se-Met, impaired cortisol secretion of RT and BT adrenocortical cells. Rainbow trout cells were more sensitive to selenite-induced impairment of cortisol secretion than BT cells, a species-specific sensitivity hierarchy also observed for Se-induced teratogenesis (Holm, Palace et al. 2005). Selenite appeared to act at different points in the cortisol steroidogenic pathway in the two species, further highlighting the different responses of closely related species to Se exposure. While the present study did not provide evidence for a role of GSH in Se-induced cortisol impairment, a protective role for SOD was demonstrated in RT cells. The different sensitivities to Se of the two salmonid species suggest that extrapolation of water quality guidelines and thresholds values across species should consider species-specific responses. Steroidogenic pathways in wild fish may provide sensitive endpoints for environmental assessment and monitoring, in addition to data useful for the protection of biodiversity. Future work will investigate the effect of Se and the role of oxidative stress in adrenocortical cells of other salmonids potentially impacted by Se and coal mining, including brook trout, cutthroat trout, and bull trout.

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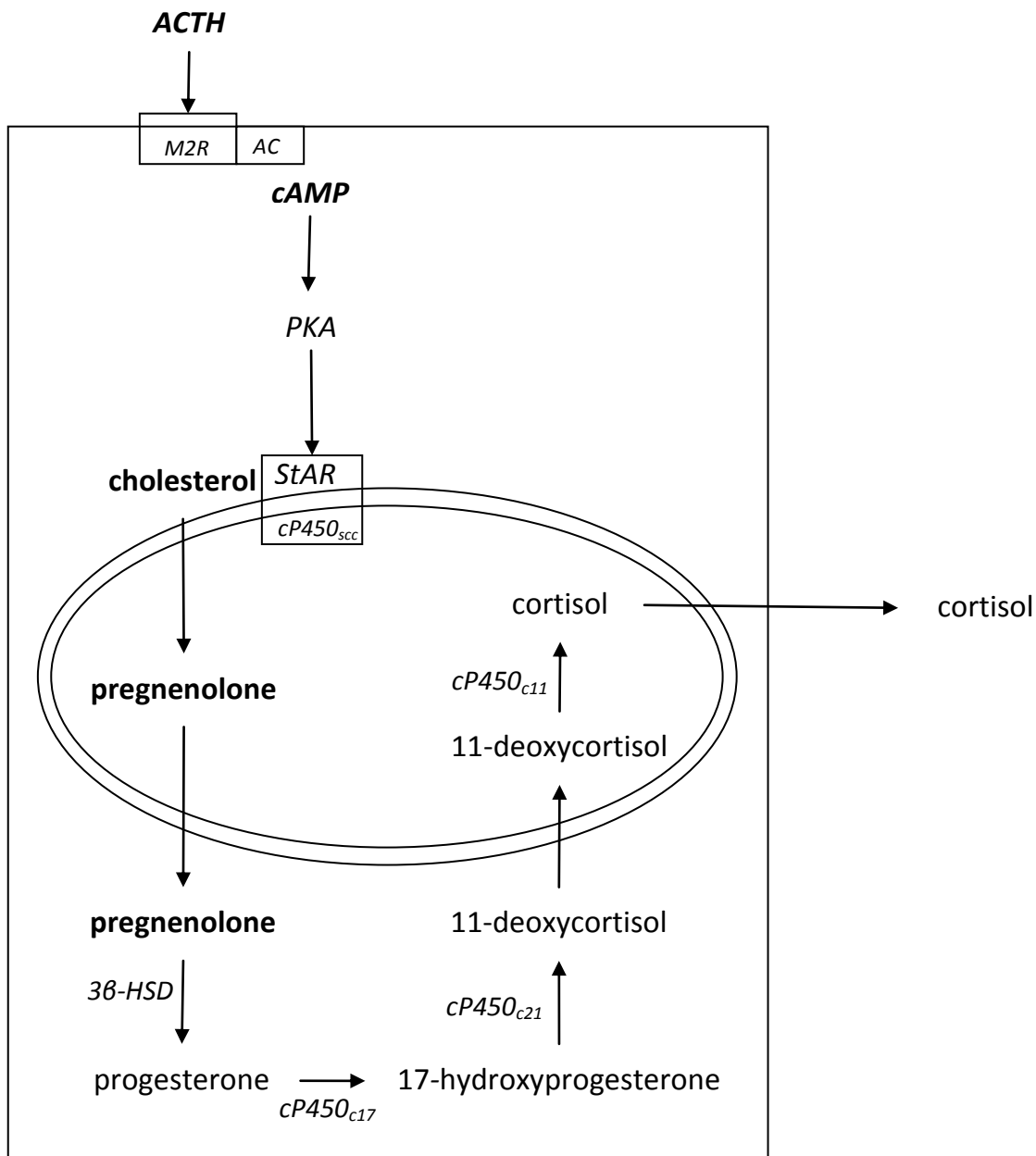


Figure 2.1. Cortisol biosynthesis pathway in a adrenocortical steroidogenic cell of the teleost fish. ACTH = adrenocorticotrophic hormone, M2R = melanocortin 2 receptor, AC = adenylyl cyclase, cAMP = cyclic adenosine monophosphate, PKA = protein kinase A, StAR = steroidogenic acute regulatory protein, cP450_{sc} = cytochrome P450 side chain cleavage, 3β-HSD = 3β-hydroxysteroid-Δ⁵-steroid dehydrogenase, cP450_{c17} = cytochrome P450 17α-hydroxylase-17,20 lyase, cP450_{c21} = cytochrome P450 21-hydroxylase, and cP450_{c11} = cytochrome P450 11β-hydroxylase. Bolded compounds were used in the pathway experiment. Adapted from (Hontela 2005; Hontela and Vijayan 2009).

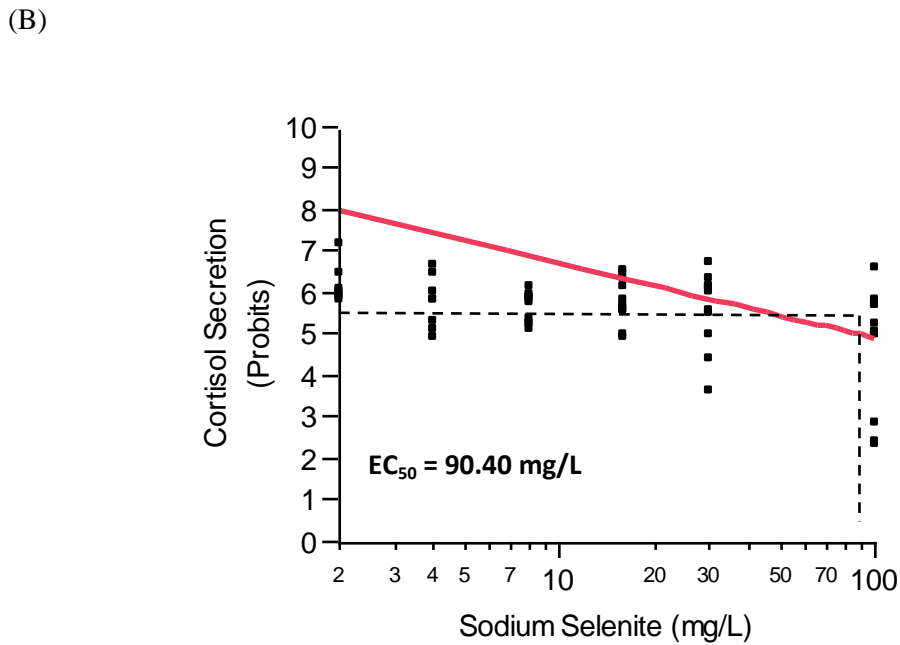
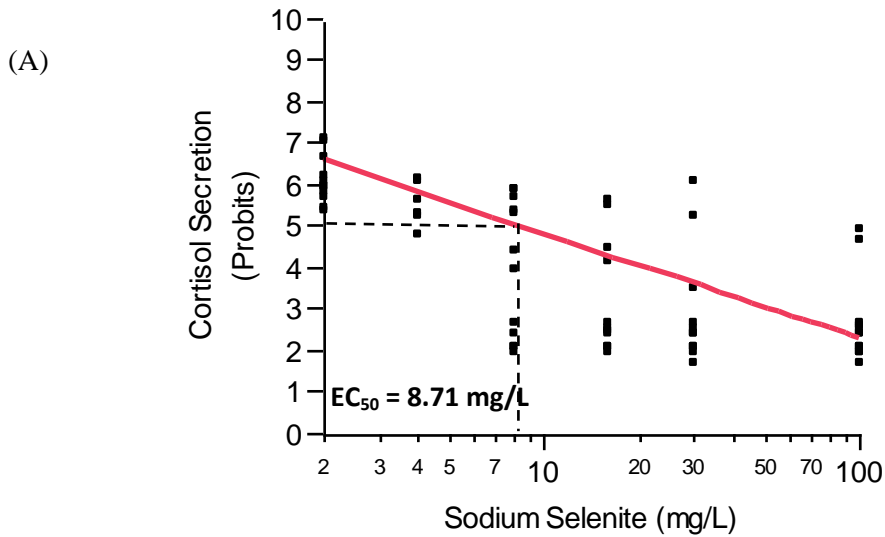
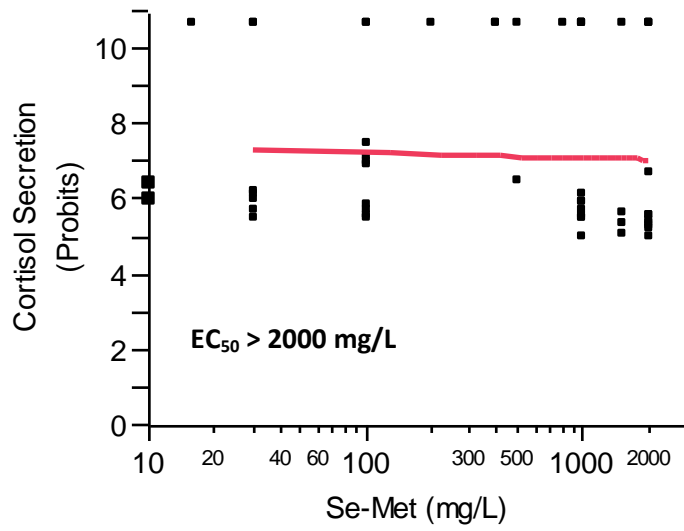


Figure 2.2. The effect of sodium selenite (Na_2SeO_3) on cortisol secretion of (A) rainbow trout ($n = 14$) and (B) brook trout ($n = 16$) adrenocortical cells. EC_{50} = concentration of toxicant that impairs 50% of ACTH-stimulated cortisol secretion. Refer to Table 2.2 for regression parameters. Exposure to positive control (Roundup) significantly decreased cortisol secretion, but did not alter cell viability (t -test, data not shown).

(A)



(B)

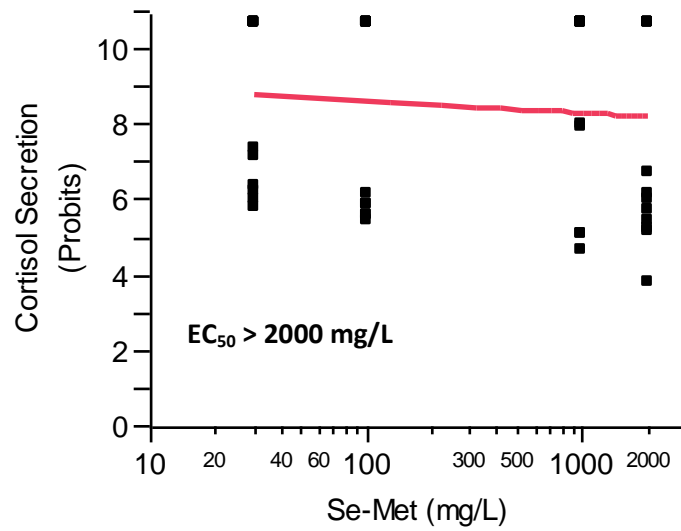
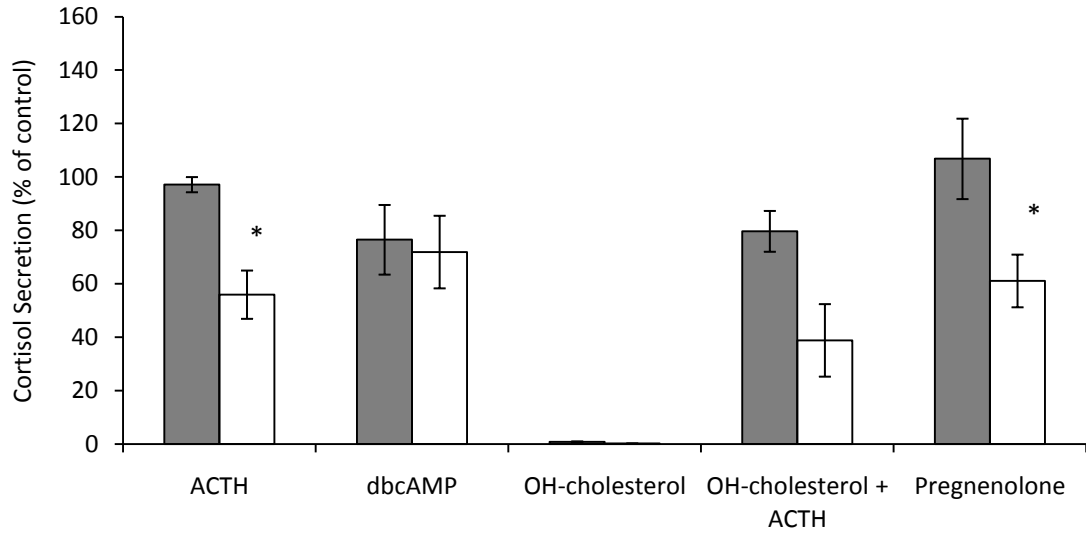


Figure 2.3. The effect of selenomethionine (Se-Met) on cortisol secretion of (A) rainbow trout (n = 13) and (B) brook trout (n = 14) adrenocortical cells. See Fig. 2.2 for details.

(A)



(B)

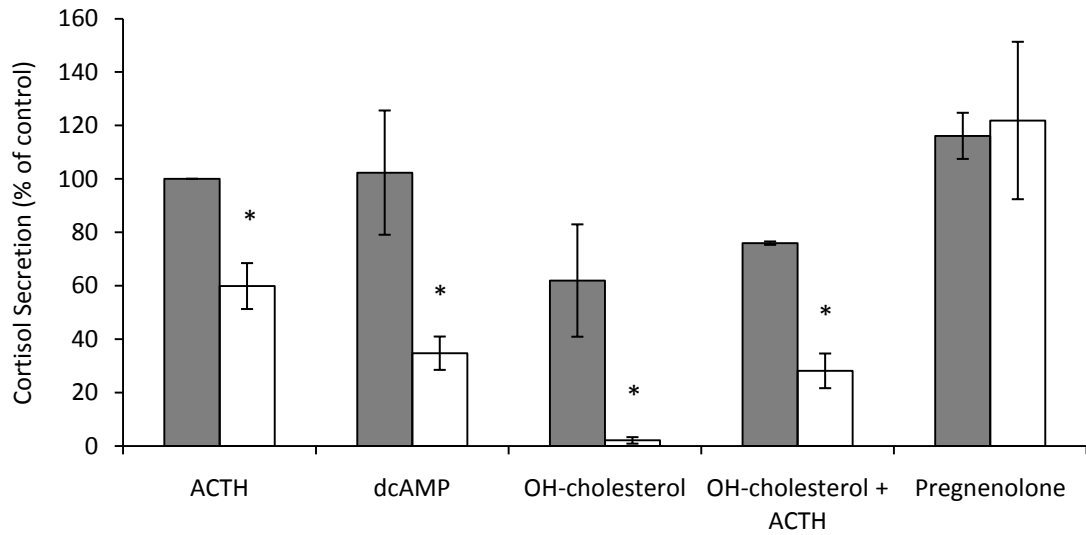
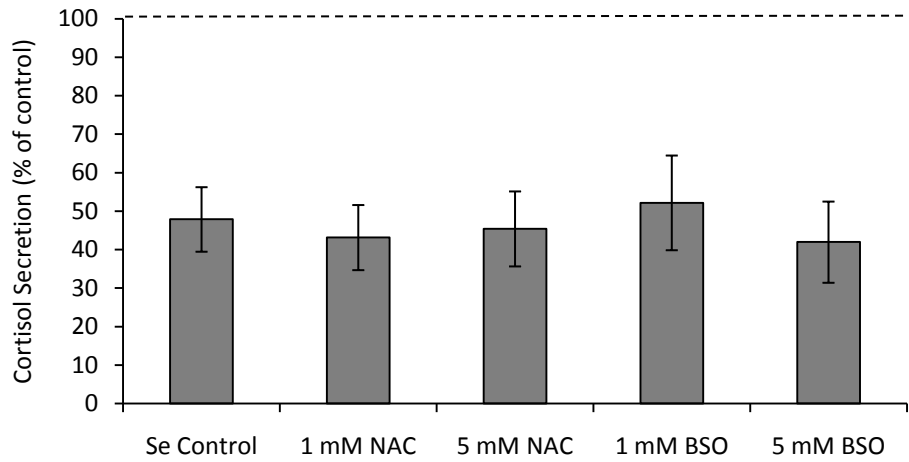


Figure 2.4. Cortisol secretion (mean % of ACTH stimulated control \pm SE) of (A) rainbow trout (n = 8), and (B) brook trout (n = 14) adrenocortical cells exposed to sodium selenite (clear bars) at EC_{50} (12 mg/L for RT cells, 90 mg/L for BT cells) or control (shaded bars, no toxicant) and stimulated with ACTH, dbcAMP, OH-cholesterol, OH-cholesterol and ACTH, and pregnenolone.

* indicates treatments in which the control and Se groups were significantly different (nested ANOVA).

(A)



(B)

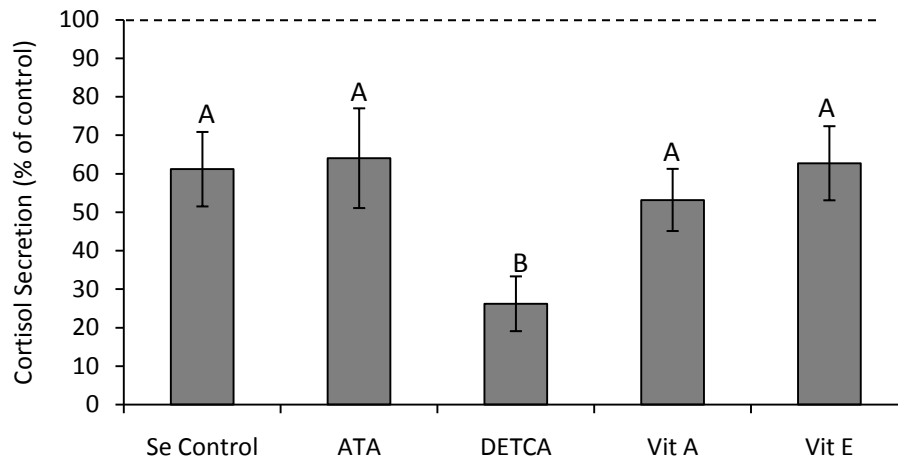


Figure 2.5. Cortisol secretion (mean % of control \pm SE) in adrenocortical cells of rainbow trout exposed to 12 mg/L sodium selenite (EC_{50} Na_2SeO_3) treated with (A) NAC or BSO ($n = 9$), and (B) ATA, DETCA, Vit A or Vit E ($n = 8$). The dashed line indicates cortisol secretion (100%) of control cells not exposed to Se. Different letters indicate a significant difference between treatment groups exposed to selenite in (A) one-way ANOVA: $F_5 = 5.97$, $p = 0.0002$; and (B) one-way ANOVA: $F_4 = 0.17$, $p = 0.9545$. NAC = N-acetyl-L-cysteine; BSO = L-buthionine-[S,R]-sulfoximine; ATA = 3-amino-1,2,4-triazole; DETCA = diethyldithiocarbamate trihydrate; Vit A = retinol; Vit E = tocopherol.

Table 2.1. Regression parameters used for the calculations of the EC₅₀ of selenite (Na₂SeO₃) and selenomethionine (Se-Met) in rainbow trout (RT) and brook trout (BT) adrenocortical cells.

| Experiment | Species | R ² | R ² | Intercept | Slope | p-value |
|---|---------|----------------|----------------|-----------|-------|-----------|
| | | | Adjusted | | | |
| Na ₂ SeO ₃ EC ₅₀ | RT | 0.3014 | 0.2911 | 7.38 | -1.10 | < 0.0001* |
| Na ₂ SeO ₃ EC ₅₀ | BT | 0.2159 | 0.2054 | 8.52 | -0.78 | < 0.0001* |
| Se-Met EC ₅₀ | RT | 0.0016 | -0.0247 | 7.47 | -0.05 | 0.8084 |
| Se-Met EC ₅₀ | BT | 0.0103 | -0.0080 | 9.32 | -0.15 | 0.4557 |

* indicates a significant dose-response relationship between cortisol secretion (probit units) and log Se concentration.

Table 2.2. Cortisol secretion (ng/ml) of rainbow trout (RT) and brook trout (BT) adrenocortical cells stimulated by ACTH, dbcAMP or cortisol precursors.

| Treatment | RT cells | BT cells |
|---|----------------------------|---------------|
| MEM | 0.20 ± 0.16 ^b | 6.64 ± 0.14* |
| ACTH (1 U/ml) | 23.39 ± 4.77 ^a | 26.13 ± 11.07 |
| dbcAMP (2 mM) | 19.14 ± 1.48 ^a | 29.33 ± 17.41 |
| OH-cholesterol (0.5 mM) | 0.15 ± 0.08 ^b | 12.50* |
| OH-cholesterol (1 mM) | 0.33 ± 0.28 ^b | - |
| OH-cholesterol (2 mM) | 0.11 ± 0.06 ^b | - |
| OH-cholesterol (0.5 mM) + ACTH (1 U/ml) | 18.01 ± 4.28 ^a | 11.34 |
| Pregnenolone (1 µM) | 12.98 ± 5.45 ^a | 31.07 ± 11.15 |
| Pregnenolone (3 µM) | 24.49 ± 6.81 ^a | - |
| Pregnenolone (4 µM) | 37.00 ± 13.35 ^a | - |

Different letters indicate a significant difference (*post hoc* Tukey Kramer HSD) in cortisol secretion (one-way ANOVA: RT cells: $F_9 = 20.97$, $p < 0.0001$; BT cells: $F_5 = 1.41$; $p = 0.3805$). * indicates a significant difference between species (*t*-test, $p < 0.05$).

Table 2.3. GSH and LPO levels (% of control) in rainbow trout adrenocortical cells exposed to 12 mg/L selenite and NAC, BSO, Vit E, Vit A, ATA or DETCA.

| GSH Experiment | | | | Antioxidant Experiment | |
|----------------|----------------------------------|---|--|------------------------|--|
| Treatment | Pre-Exposure GSH ^a | Post Exposure & Stimulation GSH ^b | Post Exposure & Stimulation LPO ^c | Treatment | Post Exposure & Stimulation LPO ^d |
| Control | 100.00 ± 2.54 ^A | 100 ± 0.00 | 100.00 ± 0.00 | Control | 100 ± 0.00 |
| Se 12 mg/L | - | 118.19 ± 24.63 | 83.49 ± 8.22 | Se 12 mg/L | 101.52 ± 7.85 |
| 1 mM NAC | 105.41 ± 2.25 ^A | 122.44 ± 27.39 | 66.97 ± 11.55 | ATA | 98.75 ± 9.93 |
| 5 mM NAC | 108.76 ± 2.99 ^A | 115.352 ± 62.02 | 79.52 ± 7.91 | DETCA | 76.35 ± 6.62 |
| 1 mM BSO | 82.28 ± 4.67 ^B | 113.04 ± 34.03 | 70.59 ± 7.18 | Vit E | 110.19 ± 13.21 |
| 5 mM BSO | 79.93 ± 1.95 ^B | 96.09 ± 41.48 | 88.50 ± 17.42 | Vit A | 94.69 ± 13.08 |

Different letters indicate a significant difference: ^a one-way ANOVA for treatment: $F_4 = 19.52$, $p < 0.0001$; $n = 4$; ^b one-way ANOVA for treatment: $F_5 = 0.09$, $p = 0.9929$; $n = 6$; ^c one-way ANOVA for treatment: $F_5 = 1.41$, $p = 0.2546$; $n = 5$; ^d one-way ANOVA for treatment: $F_5 = 1.39$, $p = 0.2415$; $n = 10$.

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**CHAPTER 3. ACCUMULATION AND EFFECTS OF CHRONIC SELENIUM
EXPOSURE IN RAINBOW TROUT AND BROOK TROUT EXPERIMENTALLY
STOCKED INTO PIT LAKES ON RECLAIMED COAL MINES**

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Abstract

Selenium (Se), an essential element, is toxic at concentrations slightly above those required for homeostasis and species-specific vulnerability to teratogenic effects of Se has been reported. To test the null hypothesis that rainbow trout (more vulnerable to Se toxicity) and brook trout (less vulnerable to Se toxicity) accumulate Se at similar rates, hatchery-reared juvenile rainbow trout and brook trout were stocked into reference and Se-contaminated pit lakes. Water quality, food availability, Se accumulation in fish and invertebrates, fish condition, and growth rates were measured at 0, 5, 12, 17 and 24 months after stocking. Selenium accumulation patterns in invertebrates and fish followed water Se levels, with lower Se levels in the reference

pit lakes (water Se: < 2.5 µg/L Se) than the Se-contaminated pit lakes (water Se: 15 – 35 µg/L Se); however, no consistent species-specific Se-accumulation pattern was observed. After 24 months of exposure, fish exceeded the proposed whole-body criterion in all the pit lakes, and lower growth and condition was observed in fish from Se-contaminated lakes with poor-food availability. Thus, duration of exposure and site-specific factors, such as food availability, are important aspects of lentic Se risk assessments. Additionally, for the first time, whole-body: muscle Se regressions were developed for rainbow trout and brook trout (no species difference) allowing future use of muscle biopsies in non-lethal Se exposure monitoring.

Key words

selenium; accumulation; trout; growth; tissue Se

Introduction

The creation of pit lakes is a common reclamation strategy for open pit coal and metal mines as they provide both aquatic habitat and recreational opportunities. The number of open pit mines is increasing (Castro and Moore 2000), yet there is limited information on the suitability of pit lakes for aquatic biota. Pit lakes are formed when a mined-out pit is filled up with surface runoff or groundwater. These lakes have a greater maximum depth to width ratio than natural lakes, have a relatively smaller littoral zone, and often remain stratified all year (Castro and Moore 2000). Pit lakes are often acidic and may have high sulphate and metal concentrations (Eary 1999; Castro and Moore 2000; Dessouki, Hudson et al. 2005); however, if the water is in contact with rock containing high levels of carbonate (e.g. limestone and dolomite), the acid produced by pyrite oxidation is neutralized (Castro and Moore 2000). In these pit lakes, selenium (Se) and arsenic may be the contaminants of concern (Eary 1999).

Selenium is often elevated in the water column of lakes with high Se content in the parent rock (e.g. cretaceous shales) (Kharaka, Kakouros et al. 2001). It bioaccumulates through the food chain, but the greatest trophic transfer occurs during uptake by primary producers (Stewart, Grosell et al. 2010). Primary producers concentrate Se from the water column $10^2 - 10^6$ times, while invertebrates and fish have trophic transfer factors of only 0.6 – 23 and 1 – 3, respectively (Stewart, Grosell et al. 2010). Diet is the most important exposure pathway for secondary and tertiary consumers (Stewart, Grosell et al. 2010), but for some invertebrates exposure through water may be also important (Wiramanaden, Forster et al. 2010).

Selenium is an essential element, but it is toxic at levels only slightly greater than those required for homeostasis (Janz, DeForest et al. 2010). The major toxic effects are teratogenic deformities observed in oviparous vertebrates exposed to Se (Janz, DeForest et al. 2010), but recently similar deformities have been observed in mayfly *Centroptilum triangulifer* (Conley, Funk et al. 2009). Amphipods are the most sensitive invertebrate taxa to acute Se exposure and Hirudinea the least (Debruyne and Chapman 2007). Sub-lethal effects on growth may occur in aquatic invertebrates at body-burdens of 1 to 30 $\mu\text{g/g dw}$; chironomid larvae appear to be highly sensitive to these effects (Debruyne and Chapman 2007). In addition to different sensitivities to Se, different invertebrate species may also accumulate different forms of Se at different rates. The species of Se present in invertebrates appears linked to trophic position. A Se speciation study with stream insects reported a higher proportion of selenite in primary consumers than invertebrates further up the food chain, which contained more organic selenides (Andrahnadi, Wayland et al. 2007). However, the amount of Se accumulated by an invertebrate species was highly variable, and could not be linked to taxonomic or trophic position (Casey and Siwik 2000). Thus, the current understanding of Se accumulation in invertebrates is limited, yet these important prey species may influence the Se exposure of fish and birds, as organisms higher in the food chain are exposed primarily through diet.

Species-specific sensitivity to Se has been documented, with reproductive effect thresholds ranging from 16 to 40 $\mu\text{g/g}$ dw Se in the egg or ovary (Janz, DeForest et al. 2010). Rainbow trout, *Oncorhynchus mykiss*, appears to be a more sensitive fish species to the teratogenic effects of Se ($\text{EC}_{50} = 21.1 \mu\text{g/g}$ dw egg Se) than cutthroat trout, *Salvelinus clarkii*, ($\text{NOEC} > 21.2 \mu\text{g/g}$ dw egg Se) (Janz, DeForest et al. 2010), while the NOEC for brook trout, *Salvelinus fontinalis*, has been reported as $>20.5 \mu\text{g/g}$ dw egg Se (Janz, DeForest et al. 2010). Thresholds for non-reproductive effects, including survival and growth, range from 7 to 51 $\mu\text{g/g}$ dw whole-body in fish (Janz, DeForest et al. 2010). Recent work has shown that relative differences in Se-induced teratogenesis may also extend to other aspects of physiology. Rainbow trout head kidney cells were more sensitive to Se-induced impairment of cortisol secretion ($\text{EC}_{50} = 8.71 \text{ mg/L}$ sodium selenite) than brook trout ($\text{EC}_{50} = 90.40 \text{ mg/L}$ sodium selenite) head kidney cells *in vitro* (Miller and Hontela 2011).

The wide range of thresholds, both for the reproductive and non-reproductive effects of Se in fish may be due to differences in exposure duration, accumulation rates, tissue partitioning, or metabolic pathways between species. Rainbow trout, a species more sensitive to Se-induced teratogenesis than brook trout, load more Se into their eggs than brook trout with similar muscle Se concentrations (Holm, Palace et al. 2005); however, the species-specific relationships between tissue (egg and muscle) Se concentrations and Se exposures still remain unclear. Fish may also move in and out of the contaminated streams while developing their gonads (Palace, Halden et al. 2007), partially accounting for species differences in tissue-specific accumulation and effects observed in previous studies. While field studies (Kennedy, McDonald et al. 2000; Holm, Palace et al. 2005; Rudolph, Andreller et al. 2008; Miller, Rasmussen et al. 2009; Muscatello and Janz 2009) represent environmentally relevant exposures, they may be limited by fish movement, lack of information on the organism's exposure history, and other confounding factors such as age of the fish or temperature fluctuations. In contrast, laboratory studies provide controlled exposures,

but usually are short-term and their environmental relevance may be limited by their experimental design.

The present study, designed to compare Se accumulation and effects in rainbow trout and brook trout exposed to environmentally relevant concentrations of Se over two years, used methodological approaches of both the field and the laboratory. Two pit lakes with low water Se concentrations and two pit lakes with elevated Se concentrations were selected from a reclaimed coal mining area and stocked with hatchery-reared juvenile rainbow trout and brook trout of the same age. The pit lakes and the introduced fish populations were sampled over two years, to test the null hypothesis that the two fish species accumulate Se at similar rates and have similar sensitivities to Se toxicity. The specific objectives of this study were to characterize the exposure to Se through water and diet in the pit lakes, and to compare the Se accumulation patterns, growth, and condition in rainbow trout and brook trout. To our knowledge this is the first time pit lakes have been used as “large mesocosms” in a chronic aquatic toxicity experiment.

Materials and methods

Study sites

Pit lakes formed by open pit coal mining were chosen based on Se concentration, access, and size. Reference lakes (Lake 24 and Lake 44) were located on an open pit coal mine in the Rocky Mountain foothills (53.1 °N, 117.5 °W) near Edson, Alberta, Canada. The Se-contaminated lakes (Luscar Lake and Lake C4) were located on open pit coal mines on the north eastern slopes of the Rocky Mountains (53.0 N, 116.7 °W) near Hinton, Alberta, Canada (Figure 3.1). All pit lakes had no outflow or inflow, and were closed to recreational fisheries. Morphometric characteristics of these lakes are described in Table 3.1.

Juvenile rainbow trout (weight = 10.64 ± 2.25 g) were obtained from the Allison Creek Brood Trout station (Blairmore, Alberta, Canada). Juvenile brook trout (weight = 3.13 ± 0.41 g) were obtained from the Sam Livingston Fish Hatchery (Calgary, Alberta, Canada). At the hatcheries, fish were raised in water containing 0.47 ± 0.11 $\mu\text{g/L}$ Se and fed a diet containing 1.86 ± 0.66 $\mu\text{g/g dw}$ Se. Both juvenile rainbow trout and brook trout were stocked into the four pit lakes in June of 2007 and 2008 at a rate of 100 fish of each species per hectare.

Water quality

Water temperature, oxygen, and conductivity profiles to a depth of 10 m were measured (YSI, model 85), along with Secchi disk transparency and surface pH (VWR, model SP21) at each site during all sampling periods. Surface water samples were also collected during each sampling period for total Se and Se-speciation analyses. Total Se (detection limit = 0.1 $\mu\text{g/L}$) was measured by inductively coupled plasma-mass spectrometry (ICP-MS) as previously described (Miller, Rasmussen et al. 2009). Selenate (detection limit = 0.36 $\mu\text{g/L}$) and selenite (detection limit = 0.22 $\mu\text{g/L}$) were measured with a non-metallic liquid chromatograph interfaced with an ICP-MS as previously described (Hu, Wang et al. 2009). Additional surface water samples were collected during the 24 month sampling period for analyses of nutrient and trace metal concentrations (49 parameters; Tables A.A.1 and A.A.2) using standard methods (Eaton, Cllesceri et al. 2005).

Invertebrates

Pelagic invertebrate samples were collected using a plankton tow net. At each site and sampling period, three 25 m surface tows and three 10 m vertical tows were completed, and samples were frozen for later identification to order and Se analyses. During the 24 month sampling period, kick net samples were also used to collect littoral invertebrate samples from

each pit lake. Invertebrates were sorted, identified to order (Clifford 1991), and freeze dried for later total Se analyses. Invertebrate diversity was defined as the order richness (number of orders) in littoral and pelagic invertebrate samples.

Fish

Animal-use protocols were approved by the Animal Care Committee of the University of Lethbridge in accordance with national guidelines. Rainbow trout and brook trout were collected at 5, 12, 17, and 24 months after the initial stocking in June 2007. Fish were captured using one hour gill net sets (22 to 62 mm mesh), euthanized with clove oil (160 ppm, emulsified in ethanol). Fish were kept on ice during transportation to the field laboratory. Dissections began 2.5 – 3.0 hours after the last net was retrieved to standardize the travel times from all the field sites. At the field laboratory, fork lengths and weights were collected and stomach contents (for diet composition and Se), otoliths (age), and a muscle sample (moisture and Se) were removed and frozen for later analyses. Condition factor, $K = (\text{weight} \times 100) / \text{length}^3$, and specific growth rates, $\text{SGR} = 100 \times (\log_e \text{final weight} - \log_e \text{initial weight}) / \text{days after stocking}$, were calculated. During each sampling time, one fish of each species from each pit lake was used for whole-body Se analyses. A muscle sample and the gonads were removed from this fish and frozen, with the carcass, for later Se analyses.

All stomach contents were sorted and identified to order (Clifford 1991). For each fish, the total volume (mL) of the stomach content and the volume (mL) of each invertebrate order were determined using a 10 mL graduated cylinder. For each site and sampling period, all of the samples from each order were pooled, freeze dried and weighed. For later Se analyses, invertebrate samples were pooled into the following functional groups: predators (Hydrachnidia, Odonata, Hemiptera, Hirudinea, and Nematoda), detritivores (Ephemeroptera and Amphipoda), filter feeders and herbivores (Cladocera, Copepoda, Gastropoda, and Pelecypoda), miscellaneous

(eggs, rocks, plants), terrestrial (Hymenoptera, Neuroptera, Orthoptera, and Lepidoptera), and omnivores (Trichoptera, Coleoptera, and Diptera).

Tissue Se analyses & accumulation indices

Total Se was measured in frozen fish muscle tissue, and freeze dried whole-body samples, stomach contents, gonads, and invertebrates by hydride generation-atomic absorption (detection limit = 0.05 µg/g), as previously described (Miller, Rasmussen et al. 2009). Selenium bioaccumulation factors ([Se] in tissue/[Se] in water) and Se biomagnification factors ([Se] in fish muscle/[Se] in diet) were calculated for rainbow trout and brook trout stocked into the pit lakes and sampled after 5, 12, 17, and 24 months.

Statistical analyses

All statistical analyses were performed using the JMP 7.0.2 software package. Length distributions were used to distinguish age classes of fish during the 18 and 24 month sampling periods. These were confirmed by ages determined from the otoliths. Surface water quality data, diversity, invertebrate Se levels, diet Se levels, and muscle Se levels were compared using a one-way analyses of variance (ANOVA) for site and a post hoc Tukey-Kramer HSD. Analyses of covariance (ANCOVAs) were used to investigate the effect of species and months after stocking (continuous variable) on muscle Se levels. Two-way ANOVAs were used to investigate the effect of species and site on condition factor and specific growth rate. ANCOVA and regression analyses were used to investigate the relationships between whole-body Se concentrations, and gonad and muscle Se concentrations in rainbow trout and brook trout. All analyses used $\alpha = 0.05$. Refer Table A.A.3 for details of all statistical tests (F -statistics, p -values, degrees of freedom).

Results

Water quality

Water Se levels were elevated above 1 µg/L (CCME Canadian Water Quality Guideline) in all the pit lakes except Lake 24, and above 5 µg/L (USEPA's Aquatic Life Criteria) in Lake C4 and Luscar Lake; however, total Se concentrations were lower in Lake 24 and Lake 44 than in Lake C4 and Luscar Lake (Table 3.1). Lake 24 and Lake 44 were classified as "reference" pit lakes, and Lake C4 and Luscar Lake as "Se-contaminated" pit lakes. Selenate was the most abundant species of Se present in water column of Lake 44, Lake C4, and Luscar Lake, but selenite accounted for 100, 8.85, 4.62 %, of the Se present in Lake 24, Lake C4, and Luscar Lake respectively (Table 3.1).

Surface oxygen levels, pH, and water temperature were similar between all four pit lakes (Table 3.1). Surface conductivity was lower in the Lake 24 and 44 than in Luscar Lake, and Secchi disk depth was highest in Lake 24 (Table 3.1). Refer to Table A.A.4 for oxygen, conductivity, and water temperature profiles, and to Table A.A.5 for surface pH and Secchi disk transparency for each sampling time. Water hardness and sulfates were greater in Lake C4 and Luscar Lake than Lake 24 and Lake 44 during June 2009 (Table 3.1). Nitrites were below detection limits at all sites, and nitrates were only detectable in Lake C4 and Luscar Lake. Phosphorus levels were low in Lake 44 and Lake C4, and slightly higher in Lake 24 and Luscar Lake in June 2009. The cation regime was $\text{Na}^+ > \text{Ca}^+ > \text{Mg}^+ > \text{K}^+$ for all of the lakes and the anion regime was $\text{HCO}_3^- > \text{SO}_4^- > \text{Cl}^-$ for all of the lakes (Table A.A.1). With the exception of Se, none of the trace elements measured were above the CCME Canadian Water Quality Guidelines for the protection of aquatic life or the USEPA's Aquatic Life Criteria (Table A.A.2).

Invertebrate community

Lake 24 and Luscar Lake both had higher total invertebrate biomass in the water column than Lake 44 and C4 (Figure 3.2A); thus, Lake 24 and Luscar Lake were classified as “food-rich” lakes, and Lake 44 and Lake C4 as “food-poor” lakes. There was a significant positive linear relationship between invertebrate biomass and phosphorus levels in the pit lakes ($R^2 = 0.9838$; R^2 adjusted = 0.9757; Biomass = $10.55 \times (\text{phosphorus}) - 16.37$). Invertebrate tow samples also identified slightly different pelagic community assemblages at each pit lake (Figure 3.2A). Pelagic diversity was greatest in Lake 24 (7 ± 1.2) and Luscar Lake (7 ± 1.3), followed by Lake C4 (4 ± 1.4) and Lake 44 (3 ± 0.3). Kick samples collected during June 2009 also showed different littoral community assemblages present in each of the pit lakes (Figure 3.2B). Littoral diversity was 11 in Lakes 24 and 44, 9 in Luscar Lake and 8 in Lake C4.

Fish stomach contents

Although omnivores were a large part of all fish diets, slight differences in invertebrate prey in stomach contents (% volume of stomach contents; Table 3.2) of rainbow trout and brook trout were observed. Rainbow trout consumed significantly less detritivores and more miscellaneous food items than brook trout in Lake 44, but no other species differences were observed (Table 3.2).

Se accumulation

Selenium content of the benthic invertebrates (Table A.A.6) followed the Se water concentrations. Benthic invertebrates from Luscar Lake ($13.18 \pm 2.67 \mu\text{g/g dw}$) had significantly higher levels of Se than those collected from Lake 24 ($4.51 \pm 0.91 \mu\text{g/g dw}$). Benthic invertebrates collected from Lake 44 ($6.55 \pm 2.67 \mu\text{g/g dw}$) and Lake C4 ($8.54 \pm 2.72 \mu\text{g/g dw}$) had intermediate Se levels, not statistically different from any of the other pit lakes.

Similarly, Se levels of stomach contents followed water Se concentrations (Table 3.2). Detritivores and miscellaneous items from the stomachs of fish caught in Luscar Lake had significantly higher Se content than those from the reference lakes (Lake 24 and Lake 44). A similar trend was noted for omnivores and predators' Se content, but it was not statistically significant. There was no obvious trend of Se levels in terrestrial invertebrates or herbivores eaten by the fish in the pit lakes.

Fish muscle Se levels (Figure 3.3) were significantly different between sites. Muscle Se levels in the hatchery rainbow trout ($1.25 \pm 0.62 \mu\text{g/g dw}$) and brook trout ($0.60 \pm 0.07 \mu\text{g/g dw}$) on Day 0 were similar, and were significantly lower than those stocked into the pit lakes. Fish from Lake 24 (mean of all sampling times = $6.87 \pm 0.37 \mu\text{g/g dw}$) and Lake 44 ($7.42 \pm 0.43 \mu\text{g/g dw}$) had significantly lower muscle Se levels than those from in Lake C4 ($20.25 \pm 0.60 \mu\text{g/g dw}$) and Luscar Lake ($27.12 \pm 0.87 \mu\text{g/g dw}$). Fish from Luscar Lake had significantly higher muscle Se levels than fish caught from all of the other pit lakes. Refer to Table A.A.3 for statistical details.

In Lake 24 (reference, food-rich), brook trout muscle Se levels increased with exposure duration. No rainbow trout were caught in Lake 24. In Lake 44 (reference, food-poor), brook trout accumulated more Se in their muscle than rainbow trout, but this difference was not apparent until 24 months of Se exposure (Figure 3.3). In Lake C4 (Se-contaminated, food-poor), there was no significant difference in the muscle Se levels between the two species; however, muscle Se levels increased with time (Figure 3.3). In Luscar Lake (Se-contaminated, food-rich), brook trout muscle Se levels were greater than rainbow trout levels after 5 months of exposure; however, this difference was not evident after 12 months of Se exposure (Figure 3.3).

There was no significant difference between rainbow trout and brook trout Se bioaccumulation factors; however, fish from Lake 24 (17.43 ± 3.30) had significantly greater Se bioaccumulation factors than fish from Lake 44 (3.80 ± 0.66), Lake C4 (1.23 ± 0.19), or Luscar

Lake (0.57 ± 0.10). There was no significant effect of species or site on the Se biomagnification factors (mean of all fish = 2.11 ± 0.20).

Fish tissue Se relationships

There was a significant positive relationship between muscle Se levels and whole-body Se levels in both rainbow trout and brook trout stocked into the pit lakes and sampled over a 24 month period (Figure 3.4A); and there was no significant difference in this relationship between the two species. There was a significant positive relationship between gonad Se levels in immature rainbow trout and whole-body Se levels (Table 3.3; Figure 3.4B); however, while there was a positive trend linking immature rainbow trout gonad Se levels to muscle Se levels, this relationship was not statistically significant (Table 3.3; Figure 3.4C). There was also a significant positive relationship between female brook trout gonad and whole-body Se levels; however, the relationship for male brook trout was not statistically significant (Table 3.3; Figure 3.4B). Similarly, there was a significant positive relationship between brook trout female gonad and muscle Se levels, but this relationship was not statistically significant for the males (Table 3.4; Figure 3.4C).

Condition and growth

Twenty-four months after stocking, rainbow trout from Lake C4 (Se, food-poor) had significantly lower condition factors than brook trout (Figure 3.5A). Brook trout condition factor was the highest in fish from Lake 24 (food-rich; reference), and brook trout from Lake C4 (food-poor; Se-contaminated) had significantly lower condition factors than fish from Luscar Lake (food-rich; Se-contaminated; Figure 3.5A). The condition factor of rainbow trout was similar in all of the pit lakes (Figure 3.5A) where rainbow trout were sampled. In contrast to condition factor, the specific growth rate of rainbow trout was significantly lower than brook trout in both

Se-contaminated lakes. Both rainbow trout and brook trout had the lowest specific growth rate in Lake C4 (low-food; Se-contaminated; Figure 3.5B). Brook trout from the food-rich lakes had the highest growth rate (Figure 3.5B).

Discussion

This study demonstrates how well characterized pit lakes may be used to investigate species-specific accumulation and effects of toxicants in fish and invertebrates. Experiments using pit lakes have several advantages over conventional field studies and mesocosms. Pit lakes may mimic natural conditions of the area, allow replication as they may be a common feature on a reclaimed landscape, contain natural food sources appropriate for the area, and allow investigations of chronic truly environmentally relevant exposures. Additionally, many conventional field studies in lotic environments are confounded by fish movement (e.g. (Palace, Halden et al. 2007)); pit lakes eliminate this factor by limiting the movement of fish out of the exposure area. In this study, pit lakes were used to investigate Se accumulation and effects in rainbow trout and brook trout by stocking unexposed juvenile fish into four pit lakes with different Se levels, and sampling over a period of 24 months.

The water quality of the pit lakes used in this study was similar to the water quality of natural lakes in west-central Alberta, but with a few key differences. Pit lakes were permanently stratified, had the same anionic composition ($\text{HCO}_3^- > \text{SO}_4^- > \text{Cl}^-$) as natural lakes ((Stemo 2005), Alberta Surface Water Quality Data: <http://environment.alberta.ca/01288.html>), were slightly alkaline, and their pH supported aquatic life (CCME Guidelines: <http://mst.ccme.ca>). However, there were differences in conductivity, cationic composition, and sulphate levels between natural lakes and some of the pit lakes used in this study. Conductivity was higher in the Se-contaminated pit lakes than the reference pit lakes and natural lakes in west-central Alberta (Alberta Surface Water Quality Data: <http://environment.alberta.ca/01288.html>), possibly caused

by different contributions of groundwater, which has higher conductance than rainfall or runoff (McNeely, Neimanis et al. 1979). The investigated pit lakes were seepage lakes with no stream inflow or outflow; thus, water came from precipitation, surface runoff, or groundwater inflow. The conductance values suggest a higher proportion of the water in Lake C4 and Luscar Lake came from groundwater than the water in Lake 24, Lake 44, and natural lakes in the area. Similarly, the difference in cationic composition between the pit lakes ($\text{Na}^+ > \text{Ca}^+ > \text{Mg}^+ > \text{K}^+$) and natural lakes ($\text{Ca}^+ > \text{Na}^+ > \text{Mg}^+ > \text{K}^+$) may also be due to the prevalence of groundwater in pit lakes (McNeely, Neimanis et al. 1979). Typical of other pit lakes, sulphate concentrations in the pit lakes were higher than the natural lakes (Castro and Moore 2000), probably due to the leaching from the surrounding rock (McNeely, Neimanis et al. 1979). Shale, the dominant rock type at Lake C4 and Luscar Lake, contains high concentrations of sulphates explaining the elevated levels in these two lakes.

Many other pit lakes formed by mining activities are acidic (Castro and Moore 2000), but pit lakes in areas with high carbonate content in the underlying rock, neutralize the acid released by pyrite oxidation (Castro and Moore 2000). These pit lakes often have elevated levels of arsenic and Se (Eary 1999). In the pit lakes used in this experiment, arsenic was below detection limits in all of the lakes; however Se was elevated in Lake C4 and Luscar Lake. The Se present in the water column was primarily selenate, with a small amount of selenite. The parent rock at these lakes consists predominantly of cretaceous shale (Langenburg, MacDonald et al. 1989), a rock that releases selenate and small amounts of selenite when oxidized (Kharaka, Kakouros et al. 2001). Conversely, the parent rock at Lakes 24 and 44 originates from the tertiary period and has a much lower Se content (MacDonald, Langenburg et al. 1989). The difference in Se contents among the pit lakes used in this study created an excellent system to experimentally study the chronic accumulation of Se and its effects in fish.

The water Se levels in Lake 44, Luscar Lake and Lake C4 exceeded the Canadian Water Quality Guideline for the Protection of Aquatic Life ($1 \mu\text{g/L}$); however, no other trace element or

water quality parameter exceeded these guidelines. The presence of other trace elements, including As or Hg, can alter the toxicity of Se (Janz, DeForest et al. 2010), but in this study, most of these elements were below detection limits. Copper and zinc levels were slightly elevated in Lake C4, but levels were well below water quality guidelines (CCME Guidelines: <http://mst.ccme.ca>). Sulphur may lower Se bioaccumulation in fish and invertebrates (Janz, DeForest et al. 2010), and sulphates, while below the water quality guidelines, were elevated in the Se-contaminated pit lakes. Thus, Se accumulation rates may be even higher in systems with lower sulphate levels.

The lakes in this study were oligotrophic to ultra-oligotrophic, with low phosphorus concentrations. The pelagic invertebrate biomass was greater in the pit lakes with more phosphorus (Lake 24 and Luscar Lake), irrespective of Se contamination. Pelagic diversity also increased with productivity; however, littoral diversity did not follow this pattern. Instead, littoral diversity was highest in the reference pit lakes and lowest in the Se-contaminated pit lakes. Low biomass of benthic invertebrates has also been documented in streams contaminated by Se-laden coal ash (Hopkins, Staub et al. 2004). The pit lakes studied had similar shoreline development ratios indicating they had similar littoral zone availability; thus, declines in littoral invertebrate diversity may be due to the close link between benthic invertebrates and sediments, as elevated Se levels in the sediment have been implicated in the elevation of Se in food chains, even after water concentrations declined (Bowie, Sanders et al. 1996).

Sensitivity of aquatic invertebrates to Se follows a general taxonomic pattern. Organism sensitivity (based on lethal response to acute aqueous Se exposure), from the most sensitive to the least is as follows: amphipods > dipterans > gastropods > Hirudinea (Debruyn and Chapman 2007). However, sensitivity to dietary Se levels or internal Se burdens does not appear to follow any clear taxonomic pattern (Debruyn and Chapman 2007). In the pit lakes used in this study, invertebrate community composition did not follow the sensitivity pattern described above. Instead, community composition appeared to be driven by succession and littoral zone

availability. Lakes 44 and C4, the ultra-oligotrophic lakes, are younger than the other two pit lakes and may still be in the early stages of succession. These pit lakes had more abundant diptera populations than the older oligotrophic pit lakes, regardless of Se concentration. Changes from a chironomid (Diptera) dominated community to a more natural and diverse community has also been documented in another pit lake as it aged (Wolanski 1999). Selenium levels were generally greatest in invertebrates collected from the Se-contaminated pit lakes; however, there were exceptions. Diptera collected from Lake 24 (oligotrophic, reference pit lake) and Hydrachnidia collected from Lake 44 (ultra-oligotrophic, reference lake) had higher Se concentrations than those collected from the Se-contaminated lakes. Diptera are very diverse and encompass a wide range of feeding strategies (Clifford 1991). Even within families and feeding strategies, different species may accumulate different amounts of Se (Andrahennadi, Wayland et al. 2007); thus, the observed invertebrate Se concentrations may reflect species-specific accumulation patterns. Studies that investigate Se levels in invertebrates from pit lakes at finer taxonomic detail are needed to test this hypothesis and may reveal patterns that have not been previously documented in the field.

Lemly suggested a dietary threshold concentration of $\leq 3 \mu\text{g/g dw}$ (Lemly 1993) to protect fish populations from the toxic effects of Se. In all the pit lakes studied, invertebrate body burdens and stomach contents of fish sampled exceed that dietary threshold, suggesting Se-induced effects on fish may occur. However, insects from reference streams in west-central Alberta had Se burdens of 4-7 $\mu\text{g/g dw}$ (Wayland and Crosley 2006; Andrahennadi, Wayland et al. 2007), also exceeding Lemly's proposed threshold to protect fish populations. Fish collected from these same streams did not exhibit high rates of teratogenesis (Holm, Palace et al. 2005); thus, the Se body burdens of aquatic invertebrates from the reference pit lakes appear to be within the normal range for the area and Lemly's dietary threshold may be too conservative.

The diet of rainbow trout and brook trout varied with species and lake. Fish stomach contents did not match kick or tow sample composition suggesting that, while trout are

opportunistic predators, some prey selection occurred. Generally, rainbow trout consumed more pelagic organisms and brook trout consumed more benthic organisms. Similar observations have been made at a natural lake in west-central Alberta where brook trout diets were dominated by Tricoptera, a benthic organism, and rainbow trout diets by cladocerans (Shelast and Luoma 2004). Prey selection may influence Se exposure, as different species of invertebrates accumulate different levels of Se (Andrahennadi, Wayland et al. 2007). No clear patterns linking stomach content composition and muscle Se levels in rainbow trout or brook trout were evident in the present study; however, the reliance of brook trout on benthic organisms, which may accumulate high Se loads from the sediment (Muscatello and Janz 2009; Stewart, Grosell et al. 2010), suggests feeding strategies may explain species-specific Se accumulation patterns.

Similar to the invertebrate Se burdens, fish muscle Se levels were higher in the Se-contaminated lakes than in the reference lakes. In Lakes 24 and C4, fish muscle Se levels increased with exposure duration. Similar but non-significant trends were observed in fish from Lake 44, suggesting that even after a two year exposure, Se accumulation in trout from the pit lakes may not have reached equilibrium. Likewise, cutthroat trout exposed to dietary Se for 44 weeks also accumulated slightly more Se than fish exposed to the same levels for a shorter amount of time (Hardy, Oram et al. 2010). Equilibrium periods longer than 24 months may impact the applicability of tissue-based criteria. To be effective, the criterion should be applied to fish in equilibrium with their environment and, thus, accurately reflecting the exposure.

The USEPA has proposed a whole-body criterion of 7.91 $\mu\text{g/g dw}$ for long-term reproductive effects in fish (USEPA 2004). Using the whole-body : muscle regression equations developed in this study, the muscle threshold for long-term effects corresponding to this proposed criterion is 7.09 $\mu\text{g/g dw}$ for rainbow trout and 6.34 $\mu\text{g/g dw}$ for brook trout. All of the fish from the Se-contaminated lakes exceeded this criterion. Fish from the reference pit lakes also exceeded this criterion after two years of exposure. This suggests long-term reproductive effects could occur in all the pit lakes; however, such effects were outside the scope of this experiment

since our fish were stocked as very young individuals and no spawning habitat was available. Conversely, rainbow trout (2.34 - 2.91 $\mu\text{g/g}$ dw muscle Se) and brook trout (1.45 – 2.73 $\mu\text{g/g}$ dw muscle Se) collected from reference streams in west-central Alberta (Holm, Palace et al. 2005; Miller, Rasmussen et al. 2009) did not exceed the proposed whole-body threshold and reproductive effects were not observed (Holm, Palace et al. 2005). This difference may be due to higher Se accumulation rates in fish from lentic areas than those from lotic areas (Orr, Guiguer et al. 2006) or the ability of the fish in the streams to move in and out of the Se-contaminated reaches (Palace, Halden et al. 2007). The duration of exposure is important and should be considered, with exposure level, during risk assessments of lentic areas, even if the water levels are below water quality guidelines, as Se equilibrium times in lentic systems may be longer than 24 months.

Regressions between whole-body Se levels and muscle Se levels for rainbow trout and brook trout were developed using fish collected over the course of this experiment. The whole-body : muscle relationship was similar for the two species and can be used to estimate whole-body Se levels from muscle Se levels. Estimated whole-body Se levels can then be compared to the whole-body tissue criterion of 7.91 $\mu\text{g/g}$ dw (USEPA 2004) as demonstrated above or used to estimate egg Se concentrations if whole-body : egg relationships have also been derived. The development of a muscle : whole-body regression is very important, as it creates an opportunity for non-lethal monitoring activities, through use of muscle biopsies (e.g. (Palace, Baron et al. 2004)). In west-central Alberta, native rainbow trout populations in streams impacted by coal mines are declining, while the introduced brook trout inhabiting the same streams are increasing (Rasmussen and Taylor 2007). The regression equations developed here will allow fisheries managers to predict whole-body concentrations (and thus potential effects) from muscle levels, monitoring the Se levels without further negatively impacting native rainbow trout populations.

The only other fish species for which muscle : whole-body Se regressions have been developed is bluegill sunfish, a warm-water species (deBruyn, Hodaly et al. 2008). The

relationship between muscle and whole-body Se levels for bluegill has a slightly shallower slope than the relationships observed for rainbow trout and brook trout in the present study; thus, for similar whole-body Se concentrations bluegill have lower muscle Se concentrations than brook trout and rainbow trout. This supports earlier suggestions by deBruyn et al. (deBruyn, Hodaly et al. 2008) that regressions used to estimate tissue concentrations should be species specific or conservatively derived using the most sensitive species if species-specific regressions are not available.

Selenium criteria and thresholds focus on preventing teratogenic deformities, which can lead to population declines (USEPA 2004). The rate of teratogenesis is positively correlated with egg Se burdens in cutthroat trout, bluegill sunfish, rainbow trout, and northern pike (deBruyn, Hodaly et al. 2008); thus the ability to estimate egg burdens from whole-body or muscle Se concentrations is valuable. The gonad: muscle relationship for brook trout observed in the present study is similar to those observed previously for this species (slopes = 1.8) (Holm, Palace et al. 2005), suggesting that species relationships are fairly constant across sites. This relationship is different from those that have been previously observed in rainbow trout; however, fish movement in and out of contaminated areas may account for this difference. Rainbow trout have higher ovary Se burdens for similar muscle concentrations than brook trout (Holm, Palace et al. 2005; deBruyn, Hodaly et al. 2008). In fact, rainbow trout appear to be the fish species with the highest egg Se burden for a given Se exposure (deBruyn, Hodaly et al. 2008). They also appear to be the most sensitive species to Se-induced teratogenesis (DeForest 2008). The rainbow trout captured from the pit lakes in this study were not mature; thus, egg Se regressions are not available; however, there was a significant positive relationship between whole-body Se levels and gonad Se burdens in immature rainbow trout (mean GSI = 0.03 ± 0.01). The slope of this regression was lower than that observed for female brook trout (mean GSI = 0.62 ± 0.11) which deposit two times as much Se into their eggs for a given whole-body burden than immature rainbow trout. As expected, no significant relationship between male brook trout (mean GSI =

2.79 ± 0.73) gonad Se burdens and whole-body or muscle Se levels was observed. This data supports the use of female fish in Se assessment activities as whole-body Se and ovary Se were significantly correlated.

The exposure differences in the pit lakes used in the present study provided a system to investigate the effects of chronic Se exposure in hatchery-reared juvenile rainbow trout and brook trout. Moreover, the interaction between Se exposure and food availability could also be investigated as there was a food-rich and food-poor pit lake within each exposure category. Such interactions were observed, as fish from Lake 24 (reference, food-rich) had the highest condition and growth, and fish from Lake C4 (Se, food-poor) had the lowest condition and growth, while fish from the other lakes were intermediate. Moreover, the specific growth rate of rainbow trout in Lake C4 was lower than in brook trout, providing additional evidence for greater sensitivity of rainbow trout than brook trout. There is substantial evidence for lower condition and growth rates of fish in contaminated systems (Laflamme, Couillard et al. 2000; Goto and Wallace 2010). Fish can also increase their energy intake, if enough food is available, to compensate for the elevated energy demand of polluted sites (Goto and Wallace 2010). The results of our study suggest that chronic Se exposure has an energetic cost for trout; however, the adverse effects of Se on growth may be mitigated by abundant food supplies. Whether food availability influences the effects of Se on reproductive endpoints including teratogenic effects, could not be assessed within the 24 months of this study.

This project validated the use of pit lakes as large `mesocosms` in experiments investigating accumulation and effects of toxicants. Pit lakes, created during the reclamation process, provide realistic environmental exposure regimes, and opportunity for replication. As demonstrated, unexposed hatchery-reared fish can be stocked into these systems, and the populations studied over a number of years. In the present study, even though all the pit lakes were located on reclaimed mining sites, a significant difference in Se levels in water, invertebrates and fish were observed between the two sets of lakes, providing a system for

comparisons. Whole-body Se : muscle Se relationships were developed for rainbow trout and brook trout, to facilitate non-lethal monitoring of Se exposure and comparison to whole-body tissue guidelines. Fish from the Se-contaminated pit lakes accumulated Se above the proposed USEPA criterion for effects. A similar pattern was observed in fish from the reference pit lakes after two years, suggesting duration of exposure is important in lentic systems. The present study also provided data on the effects of environmental exposure to Se, indicating that chronic exposure to Se, interacting with food availability, may act as an energetic stressor, decreasing condition and growth of rainbow trout and brook trout.

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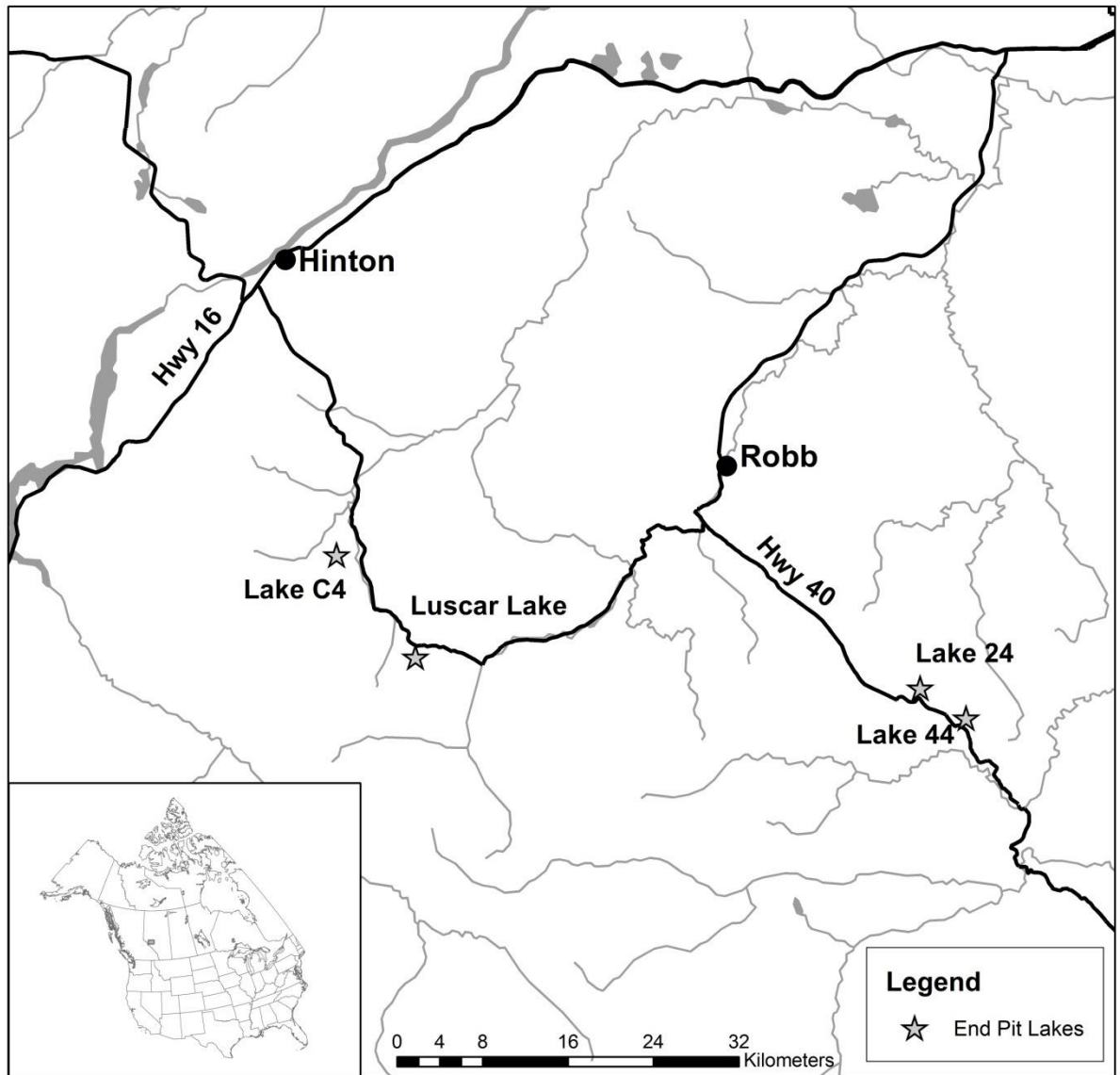
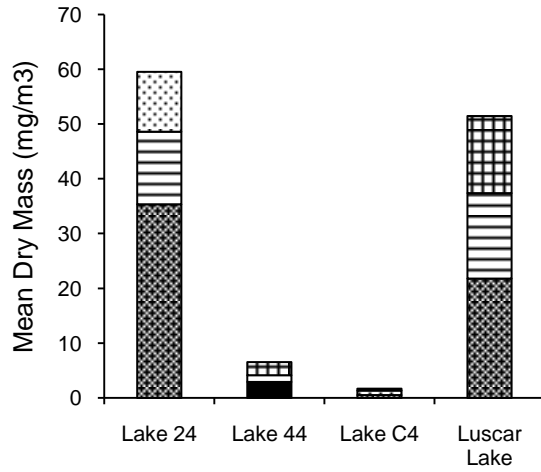


Figure 3.1. Locations of pit lakes experimentally stocked with rainbow trout and brook trout in coal mining region near Hinton, Alberta, Canada. (Reference lakes, Lake 24 and Lake 44; Se-contaminated lakes, Lake C4, Luscar Lake).

(A)



(B)

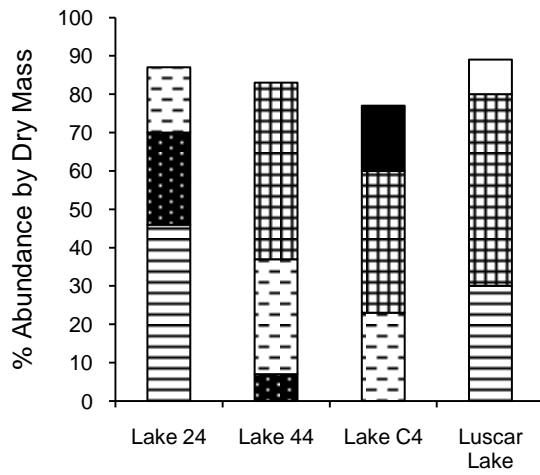


Figure 3.2. (A) Invertebrate biomass (mg/m^3) collected during pelagic invertebrate tows and (B) benthic invertebrate community composition (% of dw; kick samples) in June 2009. Bar shading identifies the three most abundant orders at each site. Data shown is an average of all sampling times. Cladocera (▨), Gastropoda (▤), Copepoda (▧), Diptera (■), Pelecypoda, (□), Ephemeroptera (▩), Odonata (▥), and Trichoptera (▦).

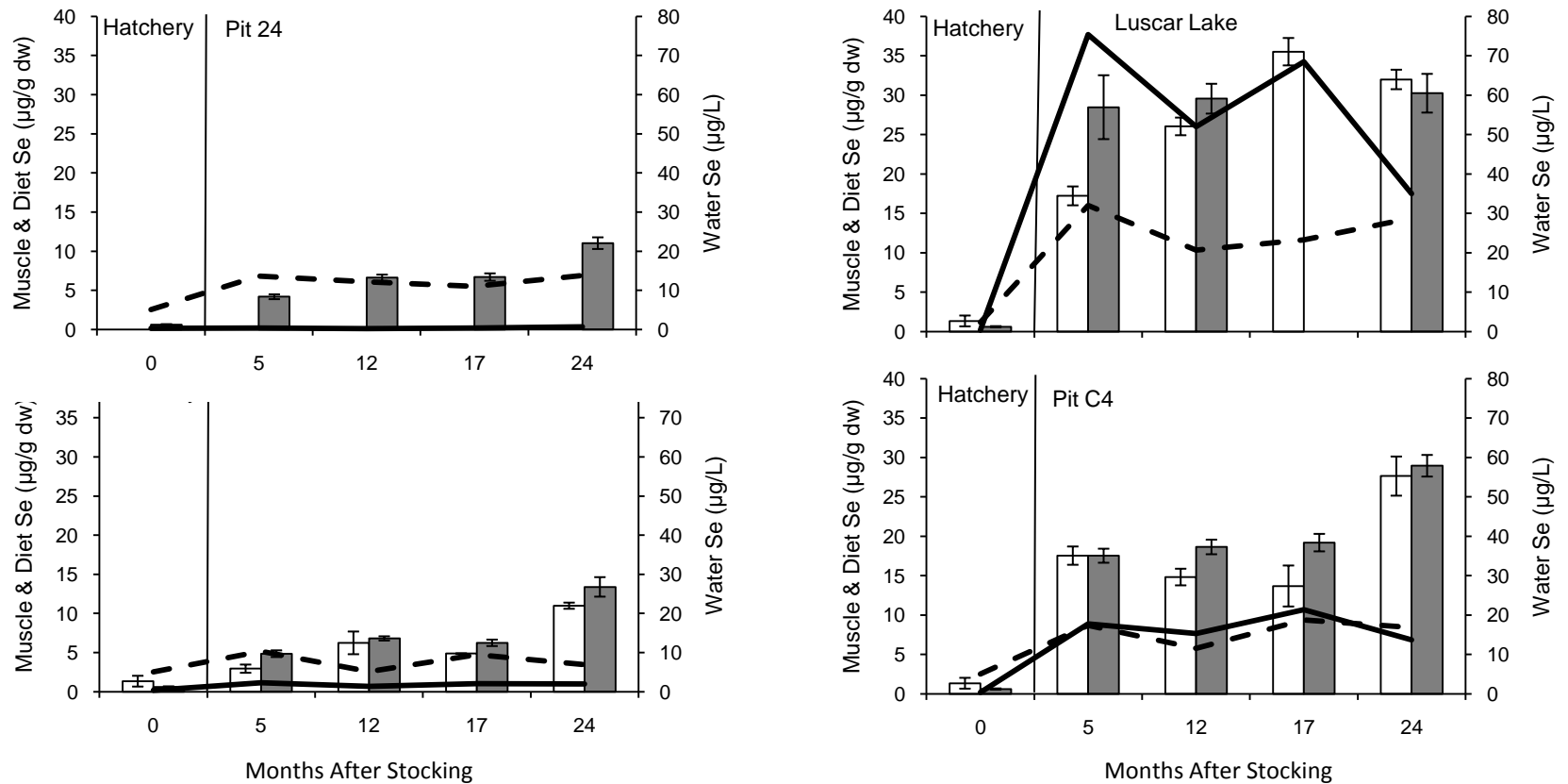


Figure 3.3. Selenium levels of water (solid line), stomach contents (dashed line), rainbow trout (mean \pm SE) muscle (open bars) and brook trout muscle (shaded bars) from the pit lakes at 0, 5, 12, 17, and 24 months after stocking. ANCOVAs for each lake were significant ($R^2 = 0.46, 0.39, 0.21, 0.28$ for Lakes 24, 44, C4 and Luscar respectively; $p < 0.0001$ for each). The months after stocking term was significant for each lake ($p < 0.0001$). The species term was significant for Lake 44 ($p = 0.0386$) and Luscar Lake ($p = 0.0162$), but not Lake C4. ($p = 0.0845$). There was a significant interaction between species and months after stocking for Lake 44 ($p = 0.0495$) and Luscar Lake ($p = 0.0020$), but not Lake C4 ($p = 0.3367$). Muscle moisture was 88.66 ± 0.17 and 78.44 ± 0.11 % for rainbow trout and brook trout, respectively.

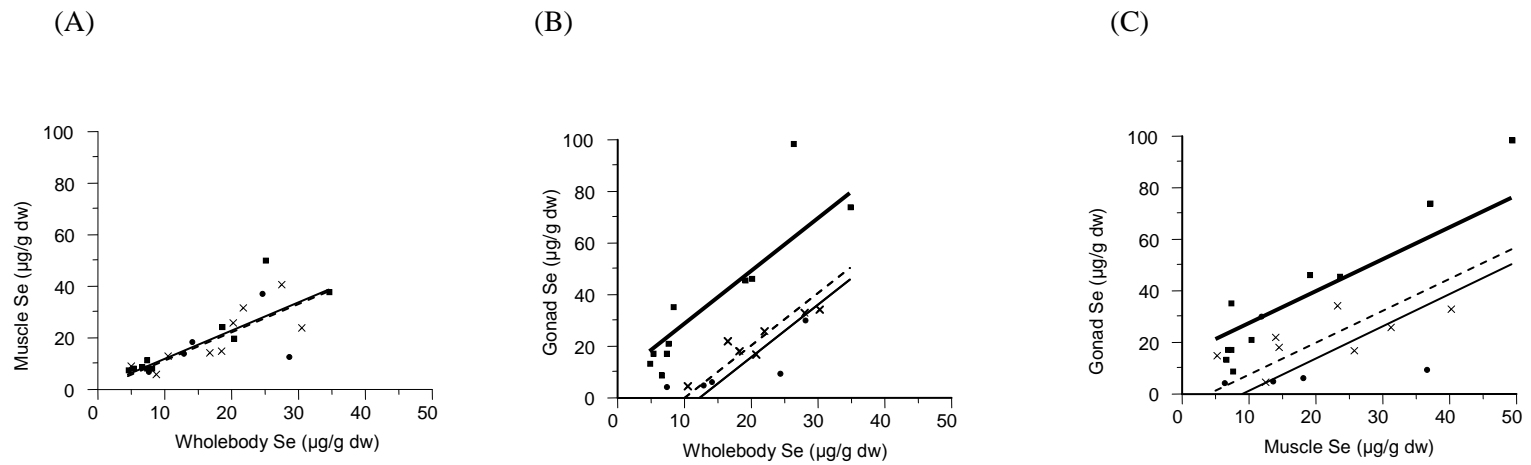
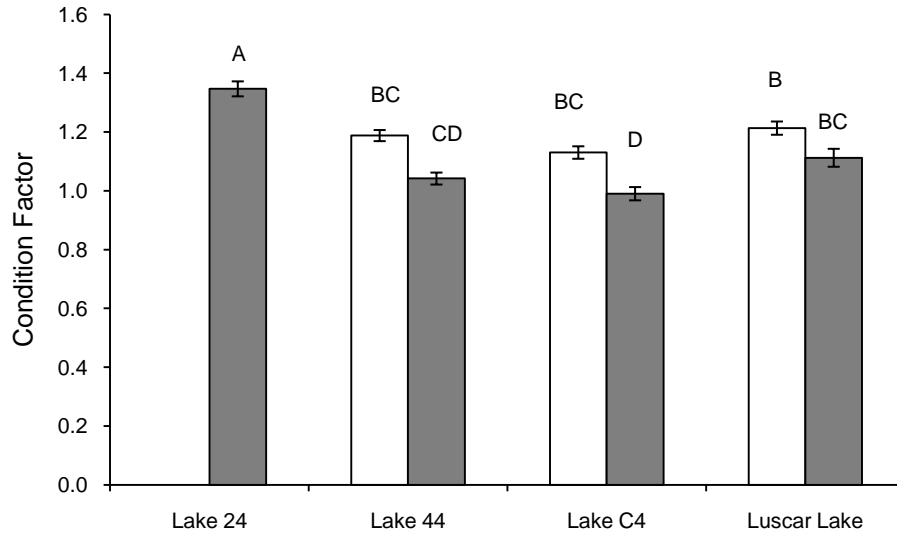


Figure 3.4. Relationship between whole-body, muscle, and gonad Se levels for rainbow trout (dashed line and X = immature) and brook trout (bold line and ■ = female; solid line and ● = male) captured throughout the experiment. Refer to Table 3.3 for individual regression parameters.

(A)



(B)

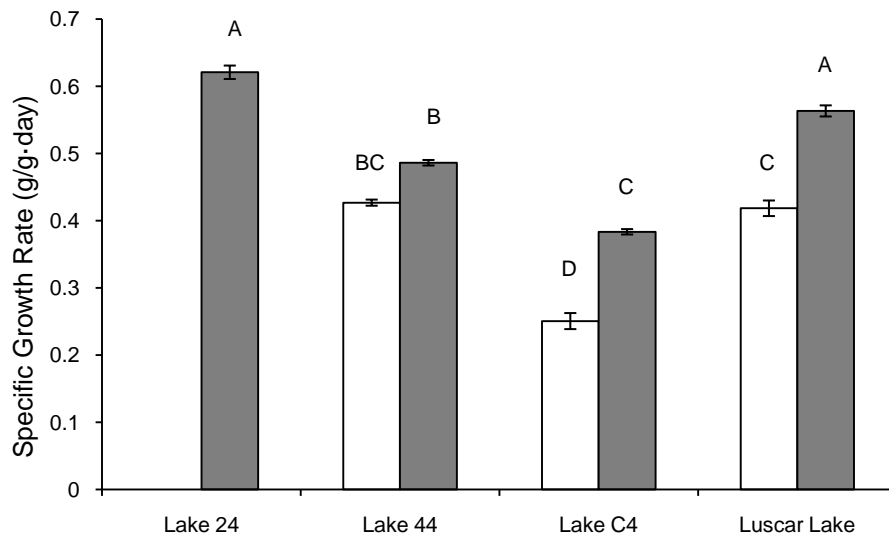


Figure 3.5. (A) Condition factor and (B) specific growth rate of rainbow trout (open bars) and brook trout (shaded bars) from pit lakes 24 months after stocking. Different letters indicate a significant difference between groups (2-way ANOVA: condition factor $p < 0.0001$; SGR $p < 0.0001$, refer to Table A.A.3 for further statistical details).

Table 3.1. Surface water quality data (mean \pm SE) and morphometric characteristics of the pit lakes stocked with rainbow trout and brook trout in north-eastern Alberta, Canada. Morphometric data sources: (Stemo 2005) (Elk Valley Coal 2006).

| | Lake 24 | Lake 44 | Lake C4 | Luscar Lake |
|---|---|---|---|--------------------------------|
| Total Se ($\mu\text{g/L}$) | 0.44 \pm 0.08 ^A | 2.00 \pm 0.17 ^A | 16.72 \pm 1.33 ^A | 50.47 \pm 10.07 ^B |
| Selenite (% of total) | 100 ^A | <DL ^A | 8.85 \pm 3.04 ^B | 4.62 \pm 1.24 ^{AB} |
| Selenate (% of total) | <DL ^A | 100 \pm 0.00 ^B | 89.68 \pm 5.53 ^B | 92.68 \pm 3.64 ^B |
| Oxygen (mg/L) | 9.0 \pm 0.4 | 8.7 \pm 0.6 | 9.0 \pm 0.2 | 8.8 \pm 0.8 |
| Conductivity (μS) | 292 \pm 15 ^A | 363 \pm 17 ^{AB} | 666 \pm 87 ^{BC} | 803 \pm 146 ^C |
| Water Temperature ($^{\circ}\text{C}$) ^a | F = 6.3 \pm 0.6 S = 13.8 \pm 1.4 | F = 6.1 \pm 1.4 S = 12.1 \pm 1.1 | F = 3.8 \pm 1.7 S = 12.8 \pm 1.5 | F = 4.4 S = 12.2 \pm 1.0 |
| pH | 7.86 \pm 0.24 | 8.00 \pm 0.20 | 7.97 \pm 0.22 | 7.68 \pm 0.28 |
| Secchi Disk Depth (m) | 6.3 \pm 0.8 ^A | 2.7 \pm 0.6 ^B | 2.2 \pm 0.3 ^B | 1.8 \pm 0.4 ^B |
| Water Hardness (mg/L CaCO ₃) ^b | 66 | 72 | 175 | 112 |
| Nitrates (mg/L N) ^b | < 0.02 | < 0.02 | 5.1 | 4.3 |
| Nitrites (mg/L N) ^b | < 0.02 | < 0.02 | < 0.02 | < 0.02 |
| Sulfates (mg/L) ^b | 44 | 67 | 202 | 211 |
| Phosphorus ($\mu\text{g/L}$) ^b | 7.5 | 2.0 | 2.0 | 6.0 |
| Area (Ha) | 5.00 | 8.06 | 5.41 | 10.80 |
| Max. Length (m) | 492 | 688 | 450 | 560 |
| Max. Width (m) | 141 | 132 | 153 | 340 |
| Max. Depth (m) | | 24 | 37 | 7 |
| Shoreline Length (m) | 1111 | 1700 | 1143 | 1568 |
| Shoreline Development ^c | 1.4 | 1.7 | 1.4 | 1.3 |
| % area < 3 m | | 25.3 | 7.0 | 65.0 |
| Elevation (m) | 1371 | 1336 | 1559.8 | 1696 |

^a F indicates the fall water temperature and S the summer water temperature. DL (detection limits) were 0.1, 0.22, 0.36 $\mu\text{g/L}$ for total Se, selenite, and selenate respectively. Different letters indicate a significant difference between sites (1-way ANOVA: selenite $p = 0.0136$, selenate $p < 0.0001$, total Se $p < 0.0001$, O₂ $p = 0.9501$, pH $p = 0.3699$, fall temperature $p = 0.5407$, spring temperature $p = 0.7849$, conductivity $p = 0.0008$, Secchi depth $p < 0.0001$; refer to Table A.A.3 for additional statistical results); ^b only collected during June 2009 (24 month Se exposure); ^c shoreline development = ratio of shore line length to the circumference of a circle with an area equal to that of the lake.

Table 3.2. Selenium^a levels ($\mu\text{g/g dw}$) and percent composition^b of stomach contents of rainbow trout and brook trout collected from the pit lakes.

| Functional Group | Lake 24 | | Lake 44 | | Lake C4 | | Luscar Lake | |
|-----------------------------|---------------------------|----------------------------------|---------------------------|------------------------------------|---------------------------|------------------------------------|---------------------------|-----------------------------------|
| | Se ($\mu\text{g/g dw}$) | Composition (%) | Se ($\mu\text{g/g dw}$) | Composition (%) | Se ($\mu\text{g/g dw}$) | Composition (%) | Se ($\mu\text{g/g dw}$) | Composition (%) |
| Detritivores | 4.84 \pm | - | 5.31 \pm | RT: 6.30 \pm 1.60 ^{A*} | 11.68 \pm | RT: 6.59 \pm 1.35 ^A | 17.92 \pm | RT: 18.26 \pm 2.46 ^A |
| | 0.79 ^A | BT: 35.2 \pm 4.56 ^A | 0.85 ^A | BT: 24.70 \pm 3.51 ^A | 0.91 ^{AB} | BT: 5.71 \pm 1.84 ^A | 4.20 ^B | BT: 27.60 \pm 4.45 ^A |
| Herbivores & Filter Feeders | 9.16 \pm | - | 6.06 \pm 1.09 | RT: 14.79 \pm 4.23 | 2.72 | RT: 7.82 \pm 2.40 | 9.20 \pm 1.86 | RT: 16.77 \pm 3.50 |
| | 4.83 | BT: 24.50 \pm 4.15 | | BT: 17.99 \pm 3.99 | | BT: 6.80 \pm 2.16 | | BT: 4.63 \pm 2.07 |
| Miscellaneous | 35.87 | - | 1.45 \pm | RT: 26.93 \pm 3.88 ^{A*} | 3.80 \pm | RT: 16.77 \pm 3.01 ^A | 11.47 \pm | RT: 12.58 \pm 2.49 ^A |
| | | BT: 2.63 \pm 0.76 ^A | 0.31 ^A | BT: 8.41 \pm 1.50 ^B | 0.76 ^{AB} | BT: 14.22 \pm 2.52 ^{AB} | 3.22 ^B | BT: 5.12 \pm 2.54 ^{AB} |
| Omnivores | 5.74 \pm | - | 4.51 \pm 0.97 | RT: 28.48 \pm 3.33 | 13.08 \pm | RT: 50.97 \pm 4.00 | 17.13 \pm | RT: 41.62 \pm 3.75 |
| | 1.18 | BT: 25.90 \pm 3.83 | | BT: 31.97 \pm 3.67 | 1.76 | BT: 50.84 \pm 3.86 | 8.38 | BT: 45.20 \pm 5.77 |
| Predators | 4.90 \pm | - | 1.24 \pm 0.84 | RT: 4.55 \pm 2.81 ^A | 18.83 \pm | RT: 3.38 \pm 0.85 ^A | 11.63 \pm | RT: 2.69 \pm 1.59 ^A |
| | 0.84 | BT: 8.47 \pm 2.36 ^A | | BT: 6.57 \pm 1.72 ^A | 14.56 | BT: 11.12 \pm 2.43 ^A | 0.29 | BT: 5.27 \pm 2.21 ^A |
| Terrestrial | - | - | - | RT: 0.61 \pm 0.37 | 2.71 \pm 0.85 | RT: 3.31 \pm 1.07 | 2.47 | RT: 0.63 \pm 0.53 |
| | | BT: 1.70 \pm 0.69 | BT: 1.71 \pm 1.21 | BT: 5.42 \pm 1.88 | | BT: 0.09 \pm 0.09 | | |

^a Stomach contents of both species were pooled into functional groups before Se analyses. Different letters indicate a significant difference between sites for each functional group (1-way ANOVA: predators $p = 0.7230$, detritivores $p = 0.0043$, filter feeders & herbivores $p = 0.5437$, terrestrial $p = 0.8971$, omnivores $p = 0.1557$, miscellaneous $p < 0.0001$; refer to Table A.A.3 for statistical details).

^b Different letters indicate a significant difference between sites for a species; * indicates a significant species difference at a site for a functional group (nested ANOVA: predators $p = 0.0234$, detritivores $p = 0.0067$, filter feeders & herbivores $p = 0.1299$, terrestrial $p = 0.5274$, omnivores $p = 0.9567$, miscellaneous $p = 0.0002$ refer to Table A.A.3 for statistical details).

Table 3.3. Regression parameters for the relationships between muscle, gonad, and whole-body Se levels in rainbow trout and brook trout from the pit lakes.

| | R ² | R ² Adjusted | ANOVA <i>p</i> -value | Y- intercept | Slope |
|------------------------------------|----------------|----------------------------|--------------------------|-----------------|--------|
| Brook trout | | | | | |
| Muscle vs. whole-body (both sexes) | 0.6129 | 0.5831 | 0.0006 | 0.9648 | 1.0950 |
| Male Gonad vs. whole-body | 0.6767 | 0.5689 | 0.0873 | -7.7110 | 1.0273 |
| Female Gonad vs. whole-body | 0.7848 | 0.7579 | 0.0006 | 2.2907 | 2.5222 |
| Female Gonad vs. muscle | 0.9409 | 0.9336 | 0.0001 | 3.8514 | 1.8898 |
| Male Gonad vs. muscle | 0.0070 | - | 0.8938 | - | - |
| Rainbow trout | | | | | |
| Muscle vs. whole-body (immature) | 0.6636 | 0.6156 | 0.0075 | 0.1573 | 1.0939 |
| Gonad vs. whole-body (immature) | 0.8010 | 0.7679 | 0.0027 | -1.4238 | 1.1506 |
| Gonad vs. muscle (immature) | 0.4675 | 0.3788 | 0.0615 | 8.5300 | 0.5802 |

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**CHAPTER 4. EFFECT OF CHRONIC SELENIUM EXPOSURE ON ENERGY
RESERVES AND GROWTH OF RAINBOW TROUT AND BROOK TROUT
EXPERIMENTALLY STOCKED INTO PIT LAKES ON RECLAIMED COAL MINES**

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Abstract

The objective of this experiment was to investigate species-specific effects of chronic selenium (Se) exposure on energy storage and growth in two salmonid species, rainbow trout and brook trout. Hatchery-reared juvenile rainbow trout and brook trout were stocked into two reference (water Se ≤ 2 $\mu\text{g/L}$; one food-rich and one food-poor lake) and two Se-contaminated (water Se > 15 $\mu\text{g/L}$; food-rich and food-poor) pit lakes on reclaimed coal mines. Over two years, fish were sampled in the spring and fall, and their energy storage (liver glycogen, muscle triglycerides, hepatosomatic index) and energy use (condition factor, specific growth rate,

gonadal somatic index) were investigated to compare energy allocation strategies in the two species. Chronic Se exposure had an associated energetic cost (lower glycogen in rainbow trout, lower condition, and growth in both species), but this cost was greater for rainbow trout than brook trout, and most apparent in the spring. The effect of Se was mitigated by high food availability and there was no evidence for winter stress syndrome. The results of this study indicate that site-specific risk assessments for Se are needed, as food availability can alter the effect of Se on growth and energy storage in fish.

Key words

rainbow trout, brook trout, selenium, glycogen, growth, condition, triglycerides

Introduction

Energy acquired through feeding is allocated to maintenance, growth, reproduction, or storage as fats, protein and carbohydrates (Congdon, Dunham et al. 2001). Maintenance activities comprise 80% of an organism's energy budget and include maintenance costs and the cost of assimilating energy (Congdon, Dunham et al. 2001). Even small changes to the amount of energy required for maintenance has a large impact on the energy available for growth, reproduction, or storage (Congdon, Dunham et al. 2001). Exposure to stressors such as predation (Lippert, Gunn et al. 2007), habitat degradation (Feldhaus, Heppell et al. 2010) or contaminants (van den Heuvel, Landman et al. 2008) may increase maintenance energy requirements, thus, decreasing energy available for other activities.

In temperate climates, where food supplies fluctuate seasonally, the effect of contaminants may be compounded by natural cycles in food availability and energy requirements. In a pristine temperate environment, fish store energy in the form of lipids, protein or glycogen

during periods of abundance (e.g. summer) to meet the energy demands during periods of scarcity (e.g. winter) (Morgan, McCarthy et al. 2002). When they are simultaneously exposed to contaminants and winter conditions, winter stress syndrome may develop. Winter stress syndrome is defined as the decrease in over-winter survival, growth, or energy reserves when an organism is exposed to a metabolic stressor (e.g. contaminants) and responds to cold water temperatures by decreasing feeding and activity levels (Lemly 1996). Winter stress syndrome has been documented in bluegill (*Lepomis macrochirus*) exposed to selenium (Lemly 1993), slimy sculpin (*Cottus cognatus*) (Bennett and Janz 2007) and fathead minnow (*Pimephales promelas*) (Driedger, Weber et al. 2010) exposed to mining effluent, but not in northern pike (*Esox lucius*) and burbot (*Lota lota*) (Bennett and Janz 2007).

Selenium (Se), an essential element that can be toxic at concentrations slightly above those required for homeostasis (Hamilton 2004), may be present in aquatic systems due to anthropogenic disturbances of Se-rich marine shales (Maher, Roach et al. 2010). It bioaccumulates rapidly in primary producers (Stewart, Grosell et al. 2010) and fish are exposed primarily to organo-selenium compounds through their diet (Janz, DeForest et al. 2010). The most pronounced toxic effect of Se is the increased rate of teratogenesis due to the maternal transfer to the embryo (Janz, DeForest et al. 2010). Selenium also acts as a physiological stressor at acute exposures, causes skin lesions, cataracts, edema, tissue necrosis, and decreased white blood cell counts (Sorensen 1986; Lohner, Reash et al. 2001c; Lemly 2002; Miller, Wang et al. 2007). Similar to winter stress syndrome, species-specific teratogenic vulnerabilities to Se have been observed. Rainbow trout (RT; *Oncorhynchus mykiss*) exhibit a higher rate of Se-induced teratogenesis (Holm, Palace et al. 2005) and are more sensitive to Se-induced cortisol impairment (Miller and Hontela 2011) than brook trout (BT; *Salvelinus fontinalis*). Juvenile bluegill (Lemly 1993), slimy sculpin (Bennett and Janz 2007), and fathead minnows (Driedger, Weber et al. 2010) exhibit symptoms of winter stress syndrome when exposed to Se and winter

conditions, but northern pike and burbot do not (Bennett and Janz 2007). However, it is not known if the RT and BT are also impacted by winter stress syndrome when exposed to elevated Se levels in temperate environments.

This experiment was designed to investigate species-specific differences in seasonal energy storage (glycogen and triglyceride reserves) and energy allocation strategies (condition, growth, gonadal somatic index) in RT and BT exposed to chronic environmental levels of Se for 24 months. Hatchery reared young-of-the-year RT and BT were experimentally stocked into two reference and two Se-contaminated lakes formed by open pit coal mining to test the hypotheses that Se alters seasonal energy storage or allocation patterns in RT and BT, and RT are more vulnerable to Se than BT.

Materials and methods

Study sites and fish stocking

Animal-use protocols were approved by the Animal Care Committee of University of Lethbridge in accordance with national guidelines. Juvenile rainbow trout (weight = 10.64 ± 2.25 g) obtained from Allison Creek Brood Trout Station (Blairmore, Alberta, Canada) and brook trout (weight = 3.13 ± 0.41 g) from Sam Livingstone Hatchery (Calgary, Alberta, Canada) were each stocked at a rate of 100 fish/ha into two reference and two Se-contaminated pit lakes in west-central Alberta, Canada. An initial sample was collected at the hatcheries before the fish were stocked into the pit lakes (water [Se] = 0.47 ± 0.11 $\mu\text{g/L}$; diet [Se] = 1.86 ± 0.66 $\mu\text{g/L}$). A complete characterization of the pit lakes can be found in (Miller, Rasmussen et al. submitted) a summary is presented in Table 4.1.

Fish sampling

Fish were sampled at 5 (fall), 12 (spring), 17 (fall), and 24 (spring) months after stocking. Fish (n = 5-29) were captured with one hour gill net sets (22 to 62 mm mesh), euthanized with clove oil (160 ppm, emulsified in ethanol), bled, and kept on ice during transportation to the field laboratory. Dissections began 2.5 – 3.0 hours after the last net was pulled to standardize travel from field sites. Fork length, weight, sex, liver weight, and gonad weight were recorded. Livers were removed and frozen in liquid nitrogen for later glycogen analysis and a muscle sample was collected for triglyceride and Se analysis, as described in (Miller, Rasmussen et al. submitted). Stomach contents were removed, sorted, and freeze dried for Se analyses as described in Miller, Rasmussen et al. submitted).

Livers were digested in potassium hydroxide (1 N) and acetic acid (1.5 N) before glycogen determination. Glycogen was digested with amyloglucosidase (1 N acetate buffer; 30 min; 37°C) and the liberated glucose concentration measured (GOD-PAP reagent, 60 min, 23°C, 510 nm) with a 96-well microplate reader as previously described (Bleau, Daniel et al. 1996; Miller, Rasmussen et al. 2009). Results were expressed as mg glycogen per g wet liver weight. Muscle triglycerides were measured with a modification of Sigma-Aldrich clinical kit as previously described (Weber, Higgins et al. 2003; Bennett and Janz 2007). Briefly, muscle was homogenized in sodium citrate (0.2 M) and frozen at -80°C until analysis the next day. In a 96-well microplate, samples and standards (diluted in isopropanol) were incubated with free glycerol reagent (5 minutes, 37°C) and absorbance read at 540 nm (free glycerols). Triglyceride reagent which hydrolyses triglycerides to glycerol and free fatty acids, was added to each well, the plate was incubated (20 minutes, 37°C), and the absorbance read at 540 nm (total glycerols). Total triglycerides were calculated (total triglycerides = total glycerols – free glycerols) and results

expressed as mg triglycerides per mg wet muscle weight. Refer to Supplemental Information for a list of chemicals used and QA/QC parameters (Table A.B.1).

Condition factor ($K = (\text{weight} \times 100) / \text{length}^3$), hepatosomatic index ($\text{HSI} = (\text{liver weight} / \text{body weight}) \times 100$), and gonadal somatic index ($\text{GSI} = (\text{gonad weight} / \text{body weight}) \times 100$) were calculated for each fish. Specific growth rates ($\text{SGR} = 100 \times (\log_e \text{final weight} - \log_e \text{initial weight}) / (\text{number of days after stocking})$) were also calculated.

Statistical analyses

All statistical analyses used the JMP 7.0.2. software package and data was log transformed to respect normality. A one-way analyses of variance (ANOVA) was used to investigate the effect of site on water Se, invertebrate Se, and water pH. A two-way ANOVA was used to investigate the effects of site and species on stomach content Se, muscle Se, liver glycogen, muscle triglycerides, K, HSI, and SGR in the spring and in the fall. *Post hoc* Tukey-Kramer HSD tests were used to further investigate significant ANOVAs. Refer to Table A.B.2 for details of all statistical tests.

Results

Lake 44 and Lake C4 are ultraoligotrophic lakes with low food availability, while Lake 24 and Luscar Lake are oligotrophic with high food availability (Miller, Rasmussen et al. submitted). Water hardness, concentrations of sulphates and nitrogen compounds were greater in the Se-contaminated pit lakes than the reference pit lakes, but pH was similar (Table 4.1). Selenium was the only trace element that exceeded Canadian Water Quality Guidelines (Miller, Rasmussen et al. submitted).

Water Se levels were lowest in Lake 24 followed by Lake 44, Lake C4, then Luscar Lake (Table 4.1). Selenium levels of the stomach content were the lowest in fish from Lake 44, followed by Lake 24 and Lake C4, and then Luscar Lake (Table 4.1). Muscle Se levels were similar in fish from the reference lakes (Lakes 24 and 44), where no species differences were observed (Table 4.1). Muscle Se levels in fish from the Se-contaminated lakes were higher than those from the reference lakes, and BT from Luscar Lake had significantly higher muscle Se levels than other fish from the contaminated lakes (Table 4.1).

Energy storage

In the spring, RT had lower liver glycogen reserves than BT in Lake C4 (Se; food-poor), but in the other pit lakes the glycogen levels were similar (Figure 4.1A). In the fall, RT had lower liver glycogen levels than BT at all sites (Figure 4.1B). In the spring, BT from the food-poor lakes (Lakes 44 and C4) had lower glycogen levels than those from Lake 24, while BT from Luscar Lake (Se; food-rich) were intermediate (Figure 4.1A); however, no site differences were observed in the fall (Figure 4.1B). Rainbow trout from Lake C4 had lower liver glycogen levels than those from Lake 44 and Luscar Lake (Figure 4.1A) in the spring, but in the fall, no effect of site was observed (Figure 4.1B).

No species differences were observed in muscle triglyceride levels from fish collected in the spring or the fall (Table 4.2). In the spring, muscle triglyceride levels were lower in fish from the food-poor lakes (Lakes 44 and C4) than the food-rich lakes (Lake 24 and Luscar Lake); although, fish from Luscar Lake (Se; food-rich) were similar to fish from Lake 44 (ref.; food-poor; Table 4.2). Conversely, in the fall, a food-dependant pattern was only observed in the Se-contaminated lakes (Lake C4 and Luscar Lake; Table 4.2), as fish from the food-rich lake had higher triglyceride levels than fish from the food-poor lake.

No species differences were observed in the HSI of fish collected in the spring (Table 4.2). The HSIs of both RT and BT were lower in the food-poor lakes (Lakes 44 and C4) than the food-rich lakes (Lake 24 and Luscar Lake; Table 4.2). In the fall, BT from the food-poor lakes had higher HSIs than RT from the same lakes (Table 4.2). Both fish species from the food-poor lakes had the lowest HSIs in the fall, but HSIs of BT from Luscar Lake (Se; food-rich) were intermediate (Table 4.2).

Energy use

In both the spring and fall, RT had higher K than BT in Lake 44 (Figure 4.2A). Rainbow trout from Lake C4 in the spring and from Luscar Lake in the fall also had higher K than BT (Figure 4.2B). Condition factor of fish was lower in lakes with poor-food availability and contaminated with Se. In the spring, RT from Lake C4 (Se; food-poor) had lower K than those from the other pit lakes (Figure 4.2A). Similarly, BT from the food-poor lakes had lower K than those from the food-rich pit lakes, and BT from the Se-contaminated food-rich lake (Luscar Lake) had lower K than BT from the reference food-rich lake (Pit 24; Figure 4.2A). A similar pattern was seen in the fall, as RT from Lake C4 (Se, food-poor) had the lowest K and BT from Lake 24 (ref., food-rich) had highest K (Figure 4.2B).

In the spring, SGR was lower in RT than BT from the Se-contaminated lakes, but this pattern was not observed in the reference lakes (Figure 4.3A). In the fall, no significant species differences in SGR were observed (Figure 4.3B). In both seasons, SGR of RT and BT was the lowest in Lake C4 (Se; food-poor) and similar in Lake 44 (ref.; food-poor) and Luscar Lake (Se; food-rich; Figure 4.3A).

In the spring, there was no effect of site or species on the GSI of fish from the pit lakes (Table 4.2); however, in the fall, BT had significantly greater GSI than RT, but no differences among pit lakes were observed (Table 4.2).

Discussion

Hatchery-reared juvenile RT and BT were experimentally stocked into reference (water Se ≤ 2 $\mu\text{g/L}$) and Se-contaminated pit lakes (water Se > 15 $\mu\text{g/L}$) with different food availabilities, to investigate species-specific effects of Se on energy storage and allocation. In this exposure system, RT and BT were exposed to and accumulated Se in the muscle, at low levels in the reference pit lakes and higher levels in the Se-contaminated pit lakes. Muscle Se levels measured in fish in this experiment were greater than those observed in RT collected from streams with water Se at 25 – 32 $\mu\text{g/L}$ Se (Holm, Palace et al. 2005; Miller, Rasmussen et al. 2009); however, this may be explained by the different Se accumulation patterns of lentic and lotic habitats (Orr, Guiguer et al. 2006) and the ability of fish to move out of Se-contaminated streams (Palace, Halden et al. 2007), but not out of the pit lakes.

Glycogen and triglyceride levels were used in this long term study as indicators of energetic status of fish. Glycogen is the storage form of glucose in animals and building up liver glycogen reserves is an important over-winter and anoxia survival strategy for fish (Hemre, Mommsen et al. 2002). Normally, fish from temperate regions build up their glycogen stores over the summer when food is abundant, and use glycogen stores over the winter when food may be scarce (Hemre, Mommsen et al. 2002). This pattern was observed in BT from the pit lakes as liver glycogen levels were elevated in the fall. Conversely, RT did not appear to elevate liver glycogen levels over the summer, as similar levels were observed in the fall and the spring. Species differences in seasonal liver glycogen cycles may be due to differences in energy

assimilation ability or energy allocation strategies. Moreover, rainbow trout spawn in the spring while BT spawn in fall; however, only BT in this experiment reached sexual maturity. Both RT and BT feed throughout the winter months (Riehle and Griffith 1993; Utz and Hartman 2007), although feeding rates may decrease during the winter (Sweka and Hartman 2001; Utz and Hartman 2007). Laboratory experiments have shown that BT have a competitive advantage over RT at low water temperatures (Cunjak and Green 1986), but direct competitive interactions may not be occurring in the pit lakes. Instead, RT and BT may be partitioning the resources, RT exploiting the pelagic zone and BT the littoral zone, as suggested by stomach content analyses and catch data (Miller, Rasmussen et al. submitted).

Toxicants may interfere with the accumulation, storage, and utilization of energy reserves. Yellow perch from reference lakes had greater liver glycogen and triglyceride levels in the summer than the fall, but this pattern was not present in fish from the metal contaminated lakes (Levesque, Moon et al. 2002). In the pit lake experiment, Se exposure, in conjunction with food availability alters liver glycogen levels. In the spring, RT liver glycogen levels were lower in fish from lakes with poor food availability, but were the lowest in the pit lake that also had elevated Se levels (Lake C4). However, RT from Luscar Lake, a food-rich Se-contaminated lake, had the highest liver glycogen levels suggesting that high food availability can modulate the effects of Se exposure. Brook trout liver glycogen levels exhibited a food-dependant pattern in spring, as BT from the two food-poor lakes had lower liver glycogen levels than those from the food-rich lakes. These patterns were not evident in RT and BT collected in the fall. This suggests that both Se exposure and low food availability may have an associated energetic cost for RT, but food availability was more important for BT than Se exposure over the winter. Lower liver glycogen levels in fish exposed to toxicants may be caused by an impairment of glycogen storage pathways (Levesque, Moon et al. 2002) or increased energy demands associated with the exposure to the toxicant (McGeer, Szebedinszky et al. 2000; Campbell, Handy et al. 2002; Goto

and Wallace 2010). There is no evidence thus far to indicate that Se impairs liver glycogen synthesis or accumulation. In other Se-contaminated systems with sufficient food availability, liver glycogen levels of juvenile northern pike (Kelly and Janz 2008) and sunfish (Lohner, Reash et al. 2001) were higher in Se-contaminated systems; however, Se exposure may still have an energetic cost. The results from the present study suggest that the interaction between low food availability and Se exposure results in low liver glycogen reserves, since RT from Lake C4 in the spring had the lowest glycogen reserves, and they were subjected to low potential supply of energy (poor food availability) and a greater energetic demand (Se exposure). Fish with low glycogen reserves would be expected to also exhibit lower growth, condition, and gonadal growth (Levesque, Moon et al. 2002).

Triglyceride reserves are another form of energy storage in animals. They may be used for over winter survival, migration, and reproduction in fish (Morgan, McCarthy et al. 2002). In both RT and BT, muscle triglyceride levels were lower in fish from Lake C4 than Luscar Lake in the spring and the fall. This suggests that food availability, not Se exposure, may underlie the differences observed in triglyceride levels as both of these lakes are Se-contaminated, but with different food availabilities. Furthermore, the BT from food-rich lakes had similar triglyceride levels regardless of Se status. The effect of contaminants on triglyceride levels in fish is variable. In burbot (Bennett and Janz 2007), fathead minnows (Driedger, Weber et al. 2009), and northern pike (Kelly and Janz 2008) exposed to mining effluent containing Se in the field, triglyceride levels were elevated; however, in sculpin exposed in the field (Bennett and Janz 2007) and fathead minnows exposed in the laboratory (Driedger, Weber et al. 2010) triglyceride levels decreased or did not change when exposed to mining effluent containing Se. The different responses observed in these studies appear to also depend on food availability. If food availability was low (e.g. over winter, or in a system with low productivity), then triglycerides decreased or did not change (Driedger, Weber et al. 2010); however, if food availability was high

(e.g. due to nutrient enrichment of the system), then the triglyceride levels increased with exposure to the mining effluent (Kelly and Janz 2008; Driedger, Weber et al. 2009). The present experiment did not observe significant Se-induced alteration of triglycerides levels, regardless of food availability.

Unlike liver glycogen levels, seasonal patterns or species differences in muscle triglyceride levels were not observed. This suggests that RT and BT allocate similar amounts of energy to triglyceride storage; however, the trout from this study appear to allocate more energy reserves to muscle triglycerides (8.6 – 22.4 mg/g) than northern pike from Se-contaminated systems (1.5 – 2.0 mg/g; (Kelly and Janz 2008)). Additionally, the difference observed between the brook trout liver glycogen and muscle triglyceride seasonal patterns may indicate that muscle triglycerides are less sensitive to changes in net energy deficits than liver glycogen. Previous research has shown that fish use glycogen reserves first, followed by fat and then protein as energy sources during starvation (Collins and Anderson 1995). Rainbow trout and BT may use energy stores in a similar pattern during the winter months, as liver glycogen reserves were more sensitive to Se exposure than muscle triglycerides.

Together with energy reserves, hepatosomatic index (HSI; ratio of liver weight to body weight) is widely used as an indicator of fish health, and is one of the indicators used in Canada's Environmental Effects Monitoring program (Barrett, Lowell et al. 2010). Enlarged livers (elevated HSI) can indicate large glycogen deposits and a positive energetic status (Hemre, Mommsen et al. 2002), or alternatively, disease and inability to mobilize the stored energy as in steatosis (Rehulka and Minarik 2007). In this experiment, HSI was more affected by food availability than Se exposure. Fish from the pit lakes with high food availability had higher HSIs than those from lakes with the low food availability, independently of Se exposure. This suggests that the elevated HSI is linked to glycogen deposits, not toxicant exposure. Similarly, Se exposure did not alter the HSI of juvenile northern pike (Kelly and Janz 2008) or juvenile RT

(Miller, Wang et al. 2007), but decreased HSI have been documented in fasted fish (Gourley and Kennedy 2009).

No species differences were observed in muscle triglyceride levels, and species differences in liver glycogen may be related to species-specific vulnerability to Se, as Se appears to have a metabolic cost for RT, but not BT. A review of scientific literature reporting juvenile RT and BT liver glycogen levels also found no species differences in liver glycogen reserves in fish from reference systems (Chapter 1). Energy accumulated by an organism (in the form of glycogen, lipid or protein) may be stored, as discussed above, or used for maintenance, growth, or reproduction activities (Congdon, Dunham et al. 2001). Maintenance activities account for the largest proportion of an individual's energy budget (Congdon, Dunham et al. 2001) and are difficult to directly measure; however, energy is not available for allocation to growth and reproductive activities until after these maintenance requirements have been met (Congdon, Dunham et al. 2001).

Condition factor, a ratio of weight to length, has been used as an indicator of health and nutritional status of fish (Willis and Hobday 2008; Cox and Heintz 2009). Selenium exposure and food availability appear to interact to impact the condition factor of fish stocked into the pit lakes. In the spring both RT and BT from Lake C4 (Se, food-poor) had the lowest condition, and fish from a lake that was either Se-contaminated or food-poor were intermediate. Similar to liver glycogen reserves, the differences in condition suggest that contaminants, such as Se, have an associated energetic cost over the winter. To survive with an increased energy demand due to toxicant exposure, a fish must increase its energy intake (Goto and Wallace 2010), use up its energy stores (Teh, Deng et al. 2005; Becker, Moraes et al. 2009), or re-allocate energy from reproduction and growth to maintenance (Congdon, Dunham et al. 2001; Teh, Deng et al. 2005). For example, chronic copper exposure increased oxygen consumption (a measure of metabolic energy requirement) of RT and the fish increased their feeding rates to meet this increased energy

demand (McGeer, Szebedinszky et al. 2000). If the fish are unable to increase their feeding rate due to limited food availability, a decrease in energy reserves or the indices of condition is expected. Both lower energy reserves (liver glycogen) and condition were observed in fish from Lake C4, suggesting chronic Se exposure has an energetic cost.

Food availability and Se exposure also appear to interact to lower specific growth rates in RT and BT. Similar to liver glycogen reserves and condition, fish from Lake C4 (Se-contaminated; low food) had the lowest growth rates as they had limited energy available and most likely, higher maintenance costs. When exposed to Se in a food rich environment (Luscar Lake), fish were able to compensate and have growth rates similar to those from the reference lakes. Rainbow trout (McGeer, Szebedinszky et al. 2000) and mummichug (Goto and Wallace 2010) have also been shown to increase food consumption to compensate for the energy demand of a metabolic stressor.

While energy storage patterns were similar between RT and BT, energy allocations may differ slightly. RT from the food-poor lakes were generally in better condition than BT from the same lakes. Conversely, a review of juvenile RT and BT literature revealed no species-specific differences in average condition factor (Miller, unpublished results). Maturation rates may explain species differences in condition observed in the pit lakes. Rainbow trout mature more slowly than BT (Scott and Crossman 1973) and some of the BT reached sexual maturity in the 24 months of the experiment; thus BT but not RT, allocated some of their assimilated energy to reproduction. The energetic trade-off between maturity and survival has been well documented. For example, non-reproductive BT had greater survival and used less lipid reserves over winter than the more mature fish of the same age (Hutchings, Pickle et al. 1999). Thus, RT may be in better condition simply because they have not yet reached sexual maturity, as shown by the low percentage of fish with GSI greater than 1.0 % in this experiment. In contrast to the condition indices, specific growth rates of RT were lower than those of BT from the Se-contaminated lakes

in the spring. When exposed to Se, BT appear to allocate more of their available energy to somatic growth than RT or, due to the species-specific vulnerability to Se, RT may have less energy available for allocation to growth than BT after the winter.

Winter stress syndrome is the mortality or decrease in energy reserves and condition over winter in the presence of a chemical stressor (Lemly 1996). Selenium caused winter stress syndrome in laboratory experiments with bluegill (Lemly 1993) and slimy sculpin exposed to Se-contaminated uranium mine effluent (Bennett and Janz 2007); but field studies with other coldwater fish species, such as northern pike and burbot have not reported decreased energy reserves over winter (Bennett and Janz 2007). The current experiment did not explicitly test the winter stress syndrome in RT and BT; but it does not appear to support it. For winter stress syndrome to occur three conditions must be met: a significant metabolic stressor must be present, water temperatures must be less than 10°C, and the fish must have lower feeding rates or energy intake (Lemly 1996). In this experiment, the first two conditions were met. Chronic selenium exposure is a potential metabolic stressor (Lemly 1993) and water temperatures were below 10°C in the winter; however, trout do feed throughout the winter (Sweka and Hartman 2001), and fish from the lakes with high food availability should not experience a reduced energy intake throughout the winter. Winter stress syndrome may be occurring in fish from Lake C4 as liver glycogen, muscle triglycerides, condition indices and growth rate were the lowest; however the presence or absence of winter stress syndrome in trout appears to depend more on food availability than the presence of Se as a metabolic stressor.

Chronic Se exposure has an energetic cost for RT and BT, as indicated by decreased condition and growth; although, this cost appears to be greater for RT than BT, with lower glycogen reserves and growth in RT, but not BT. This species-specific pattern of vulnerability to Se has been also observed for teratogenesis rates (Holm, Palace et al. 2005) and cortisol production (Miller and Hontela submitted). The energetic cost of Se may be mitigated if fish

have the ability to increase their energy intake, as is possible in lakes with high food availability. The results of this chronic Se exposure study provide evidence in support of site-specific risk assessments for Se, as the effect of chronic Se exposure can be affected by site-specific factors such as food availability.

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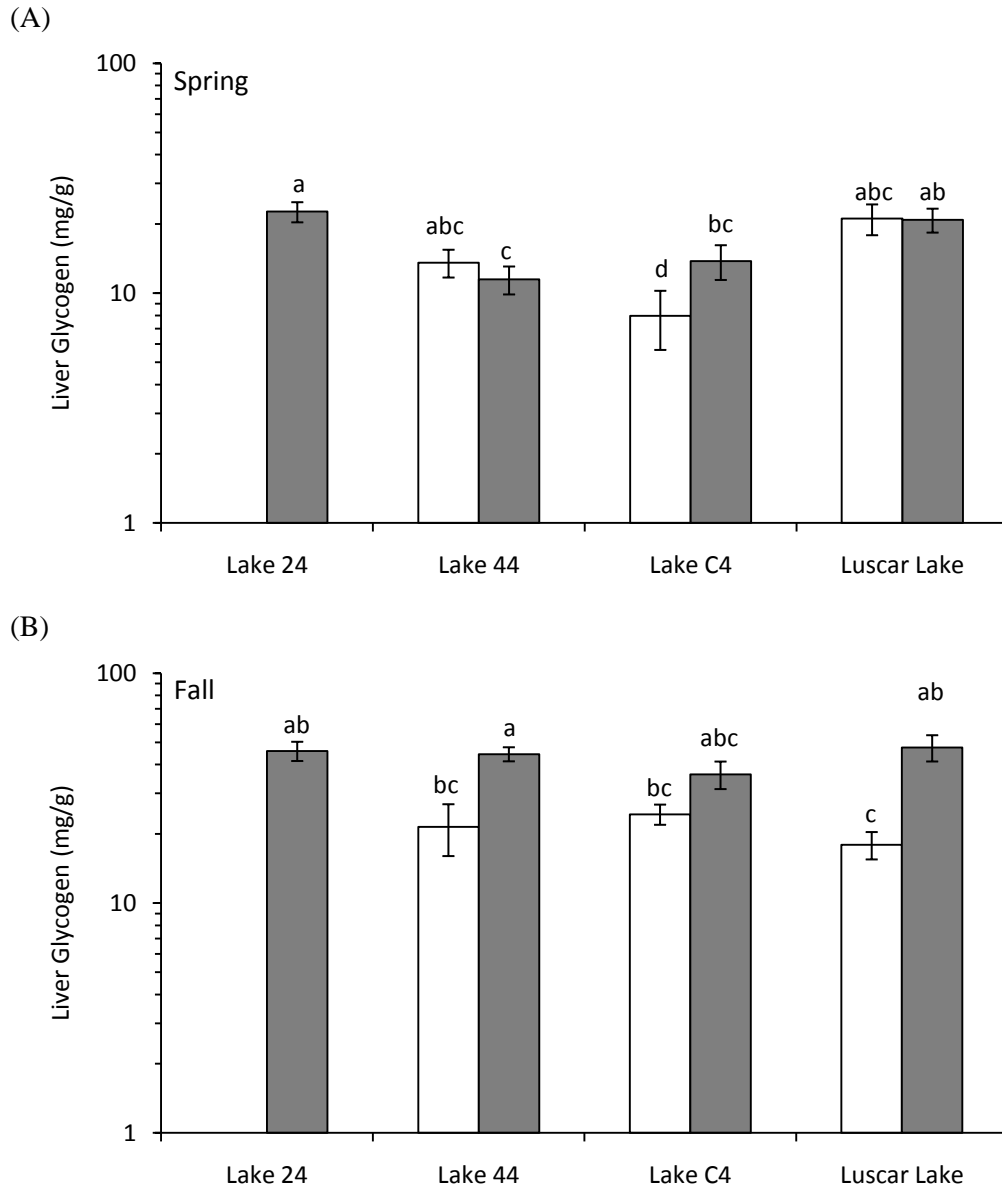


Figure 4.1. Liver glycogen (mean \pm SE) of rainbow trout (open bars) and brook trout (shaded bars) in (A) spring (12 and 24 months after stocking) and (B) fall (5 and 17 months after stocking). Different letters indicate a significant difference (2-way ANOVA: site x season; log transformed to respect normality; refer to Table A.B.2 for details).

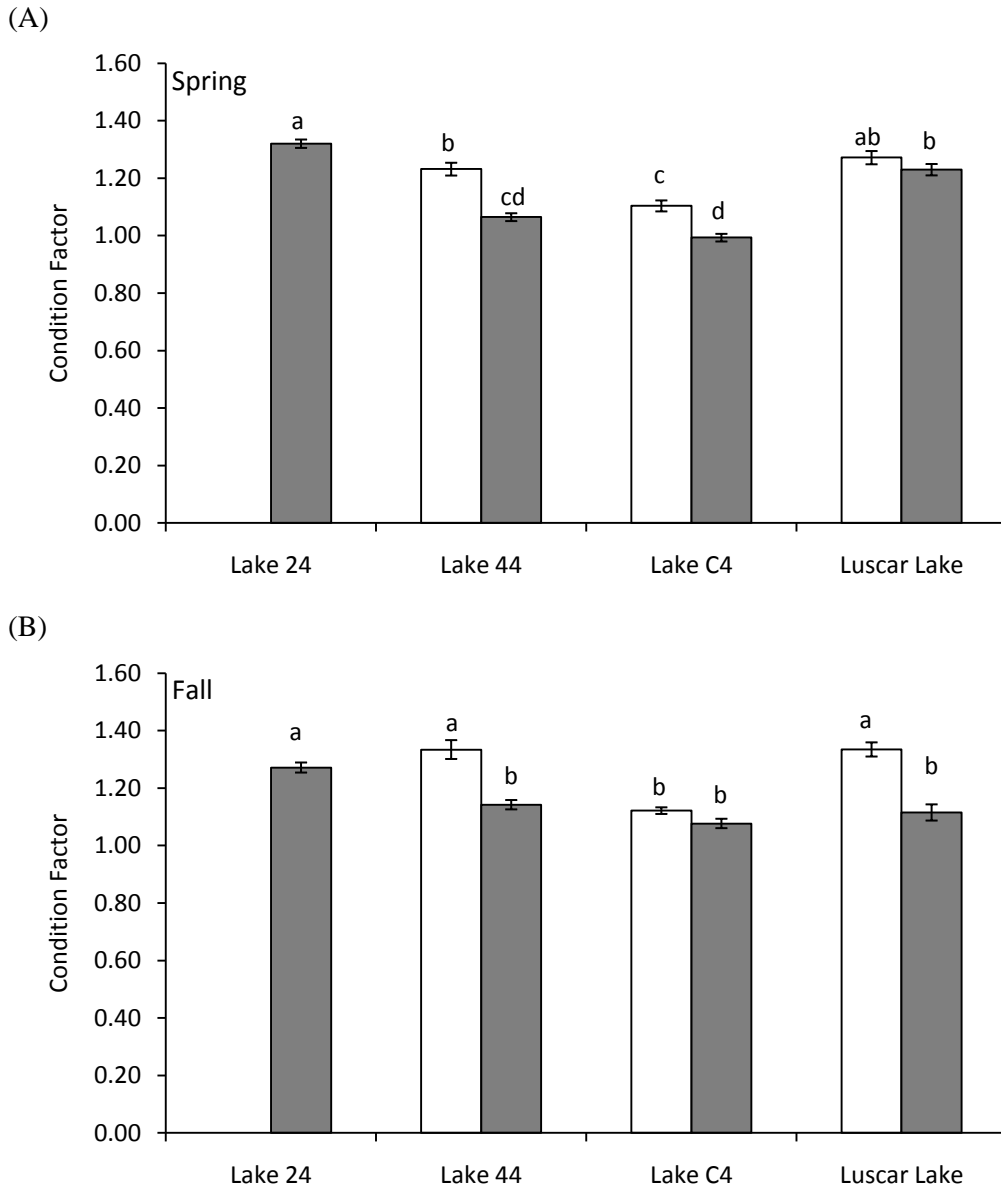


Figure 4.2. Condition factor of rainbow trout (open bars) and brook trout (shaded bars) in (A) spring (12 and 24 months after stocking) and (B) fall (5 and 17 months after stocking). Different letters indicate a significant difference (2-way ANOVA: site x season; refer to Table A.B.2 for details).

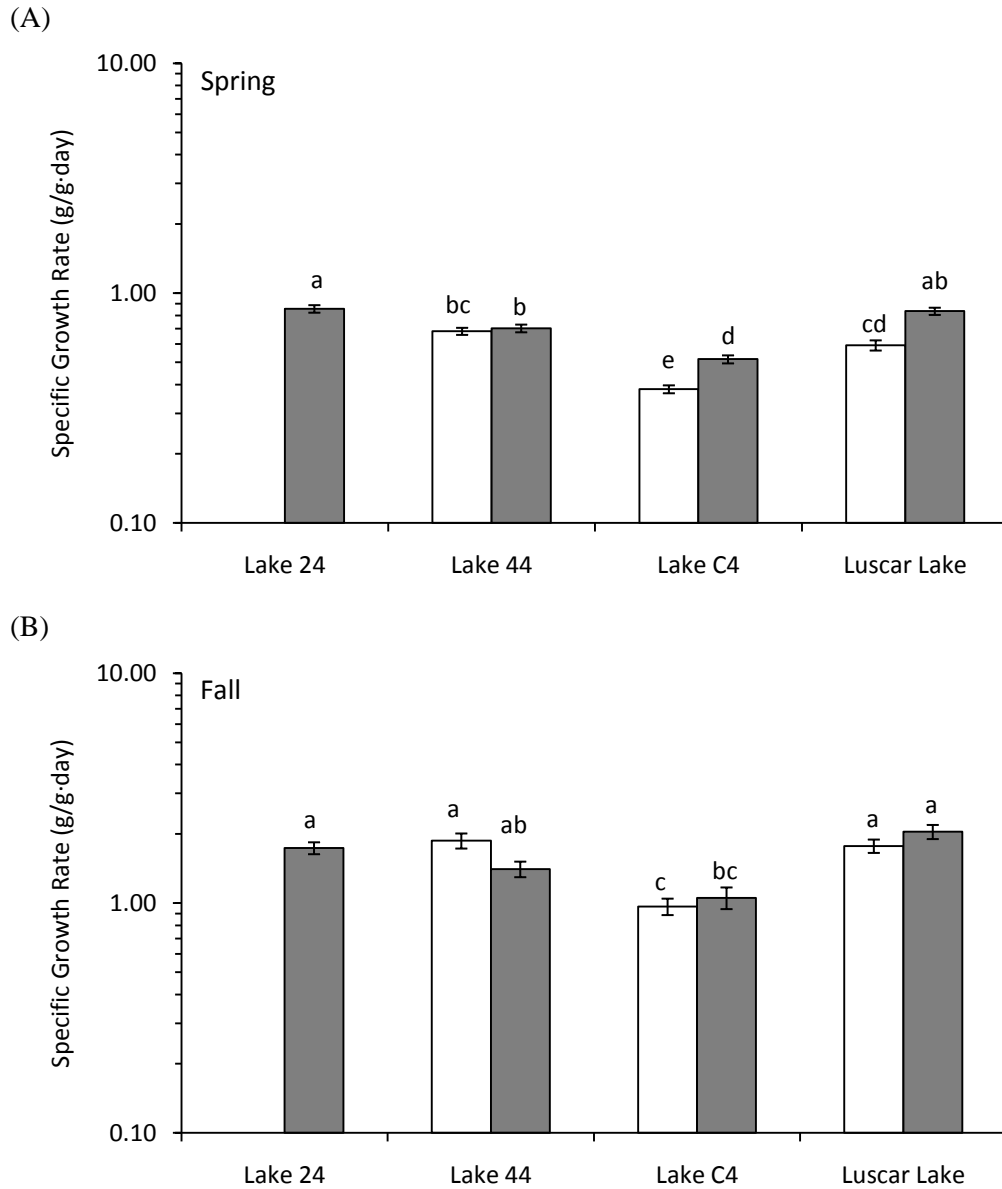


Figure 4.3. Specific growth rate of rainbow trout (open bars) and brook trout (shaded bars) in (A) spring (12 and 24 months after stocking) and (B) fall (5 and 17 months after stocking). Different letters indicate a significant difference (2-way ANOVA: site x season; log transformed to respect normality; refer to Table A.B.2 for statistical details).

Table 4.1. Selenium exposure and water quality parameters in the four pit lakes stocked with rainbow trout and brook trout.

| | Lake 24 | Lake 44 | Lake C4 | Luscar Lake |
|---|-------------------|----------------------|----------------------|---------------------|
| Lake Type | Reference | Reference | Se | Se |
| Water Total Se ($\mu\text{g/L}$) ² | 0.44 ± 0.08^a | 2.00 ± 0.17^b | 16.72 ± 1.33^c | 50.43 ± 10.07^d |
| Benthic Invertebrate Se ($\mu\text{g/g dw}$) ^{3,5} | 4.51 ± 0.9^b | 6.55 ± 1.34^{ab} | 8.54 ± 2.72^{ab} | 13.18 ± 2.67^a |
| Stomach Content Se ($\mu\text{g/g dw}$) ^{2,6} | 6.37 ± 0.33^b | 3.58 ± 0.20^a | 8.85 ± 0.37^b | 13.46 ± 0.38^c |
| Muscle Se ($\mu\text{g/g dw}$): RT ² | - | 6.35 ± 0.78^A | 18.77 ± 0.79^B | 25.44 ± 1.41^C |
| Muscle Se ($\mu\text{g/g dw}$): BT ² | 6.87 ± 0.37^A | 7.96 ± 0.50^A | 21.63 ± 0.79^B | 29.41 ± 1.06^D |
| pH ² | 7.86 ± 0.24 | 8.00 ± 0.20 | 7.97 ± 0.22 | 7.68 ± 0.28 |
| Water Temperature ($^{\circ}\text{C}$): Spring ² | 6.3 ± 0.6 | 6.1 ± 1.4 | 3.8 ± 1.7 | 4.4 |
| Water Temperature ($^{\circ}\text{C}$): Fall ² | 13.8 ± 1.4 | 12.1 ± 1.1 | 12.8 ± 1.5 | 12.2 ± 1.0 |
| Surface Dissolved Oxygen (mg/L) ² | 9.0 ± 0.4 | 8.7 ± 0.6 | 9.0 ± 0.2 | 8.8 ± 0.8 |
| Trophic Status ³ | oligotrophic | ultraoligotrophic | ultraoligotrophic | oligotrophic |
| Food Availability ⁴ | rich | poor | poor | rich |
| Hardness (as mg/L CaCO_3) ³ | 66 | 72 | 175 | 112 |
| Phosphorus ($\mu\text{g/L}$) ³ | 7.5 | 2.0 | 2.0 | 6.0 |
| Sulphates (mg/L) ³ | 44 | 67 | 202 | 211 |
| Nitrates + Nitrites as N (mg/L) ³ | < 0.02 | < 0.02 | 5.1 | 4.3 |
| Aluminum ($\mu\text{g/L}$) ³ | 10.8 | 29.1 | 12.0 | 63.2 |
| Arsenic ($\mu\text{g/L}$) ³ | < 0.04 | < 0.04 | < 0.04 | < 0.04 |
| Cadmium ($\mu\text{g/L}$) ³ | < 0.05 | < 0.05 | < 0.05 | < 0.05 |
| Copper ($\mu\text{g/L}$) ³ | 0.5 | 0.4 | 3.5 | 0.8 |
| Lead ($\mu\text{g/L}$) ³ | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Mercury ($\mu\text{g/L}$) ³ | < 0.05 | < 0.05 | < 0.05 | < 0.05 |
| Silver ($\mu\text{g/L}$) ³ | < 0.04 | < 0.04 | < 0.04 | < 0.04 |

¹ refer to (Miller, Rasmussen et al. submitted) for more details about water quality and Se exposure.

² mean \pm SE of: all 5 sampling times (pH, dissolved oxygen, water Se), spring or fall sampling times (water temperature), or 4 sampling times (fish muscle and stomach content Se); different letters indicate a significant difference (lowercase: 1-way ANOVA for site; uppercase: 2-way ANOVA for site x species; refer to Table A.B.2 for statistical details).

³ data from June 2009, the 24 month spring sampling period.

⁴ based on plankton tow biomass: food-rich lakes had $> 50 \text{ mg/m}^3 \text{ dw}$ and food-poor lakes had $< 10 \text{ mg/m}^3 \text{ dw}$ (Miller, Rasmussen et al. submitted).

⁵ different letters indicate a significant difference (1-way ANOVA for site); mean of all invertebrate groups collected by kick sample (Miller, Rasmussen et al. submitted).

⁶ contents of RT and BT were pooled for Se analyses at each site.

Table 4.2. Muscle triglycerides (mg/g), hepatosomatic index (HSI), gonadal somatic index (GSI), and sample size of rainbow trout (RT) and brook trout (BT) collected in the spring (12 and 24 months after stocking) and the fall (5 and 17 months after stocking) from pit lakes on reclaimed coal mines.

| | | Lake 24 | | Lake 44 | | Lake C4 | | Luscar Lake | |
|--------|----------------------------|---------|----------------------------|-----------------------------|-----------------------------|---------------------------|---------------------------|----------------------------|----------------------------|
| | | RT | BT | RT | BT | RT | BT | RT | BT |
| Spring | Triglycerides ¹ | - | 22.41 ± 3.07 ^a | 10.23 ± 0.54 ^{cd} | 12.41 ± 0.84 ^{bcd} | 9.15 ± 0.67 ^d | 8.65 ± 0.55 ^d | 13.55 ± 1.16 ^{bc} | 20.00 ± 3.09 ^{ab} |
| | HSI ¹ | - | 1.35 ± 0.03 ^a | 0.83 ± 0.03 ^c | 0.75 ± 0.03 ^c | 0.93 ± 0.07 ^c | 0.85 ± 0.03 ^c | 1.12 ± 0.05 ^b | 1.10 ± 1.26 ^{ab} |
| | GSI | - | 0.31 ± 0.05 | 0.21 ± 0.03 | 0.43 ± 0.04 | 0.18 ± 0.07 | 0.39 ± 0.05 | 0.07 ± 0.15 | 0.30 ± 0.04 |
| | Sample Size ² | 0 | 39 | 11 | 41 | 38 | 40 | 41 | 41 |
| Fall | Triglycerides ¹ | - | 12.67 ± 0.99 ^{ab} | 12.72 ± 1.96 ^{abc} | 10.58 ± 0.94 ^{abc} | 12.00 ± 4.14 ^c | 8.94 ± 0.89 ^{bc} | 18.07 ± 1.64 ^a | 19.05 ± 1.43 ^a |
| | HSI ¹ | - | 1.65 ± 0.08 ^a | 0.93 ± 0.02 ^b | 1.04 ± 0.05 ^b | 0.80 ± 0.02 ^c | 1.06 ± 0.09 ^b | 1.20 ± 0.05 ^b | 1.30 ± 0.06 ^{ab} |
| | GSI | - | 4.04 ± 0.80 ^a | 0.30 ± 0.23 ^{bc} | 3.33 ± 0.61 ^a | 0.43 ± 0.17 ^{bc} | 3.81 ± 0.66 ^a | 0.45 ± 0.27 ^c | 2.91 ± 1.05 ^{ab} |
| | Sample Size ² | 0 | 40 | 29 | 37 | 31 | 32 | 24 | 10 |

¹ different letters indicate a significant difference (2-way ANOVA: site x species) for each season. Refer to Table A.B.2 for statistical details.

²stocked RT were not captured in this Lake 24.

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CHAPTER 5. EFFECT OF CHRONIC SELENIUM EXPOSURE ON ENDOCRINE AND OXIDATIVE STRESS BIOMARKERS IN RAINBOW TROUT AND BROOK TROUT

Abstract

Hatchery-reared juvenile rainbow trout and brook trout were experimentally stocked into reference and selenium-contaminated pit lakes to investigate the species-specific effect of selenium on endocrine and oxidative stress biomarkers. Selenium exposure decreased plasma T3 and T4 levels, but did not alter cortisol secretion, testosterone, estradiol, GSH, LPO, vitamin A or vitamin E levels. Species differences in T3, T3:T4, and vitamin A levels were also observed.

Key words

brook trout; endocrine disruption; oxidative stress; rainbow trout; selenium

Introduction

Many different classes of chemicals, including pesticides (Iwanowicz, Blazer et al. 2009), personal care products (Kidd, Blanchfield et al. 2007), metals (Lizardo-Daudt, Bains et al. 2007), and industrial effluents (Lister, Nero et al. 2008), contaminating aquatic systems have been linked to endocrine disruption. While the effects of endocrine disruptors on the reproductive system of fish (Flores-Valverde, Horwood et al. 2010) have been investigated in many studies, effects on thyroid (Iwanowicz, Blazer et al. 2009), stress (Lizardo-Daudt, Bains et al. 2007), and growth hormones (McCormick, O'Dea et al. 2005) also occur. Endocrine disrupting compounds may bind to the hormone receptors mimicking or blocking the endogenous hormones, alter the number of hormone receptors present, interfere with protein synthesis, directly interact with the hormones

to alter their synthesis and clearance, or generate reactive oxygen species that cause oxidative damage (Choi and Lee 2004). Oxidative stress is the damage to lipids, proteins, and DNA caused by reactive oxygen species (ROS) or other radicals. The cell has a variety of antioxidants that remove ROS, but if ROS production occurs faster than the antioxidants' scavenging rate, damage such as lipid and protein peroxidation will occur. Reactive oxygen species are a natural by-product of cellular respiration (Kelly, Havrilla et al. 1998), but can also be produced by toxicants, such as selenium (Spallholz, Palace et al. 2004).

Selenium (Se) is an essential metalloid that may be toxic at concentrations only slightly greater than those required for homeostasis (Janz, DeForest et al. 2010). The major toxic effect in fish is teratogenesis from maternally transferred Se (Janz, DeForest et al. 2010); however, Se can also inhibit cortisol secretion *in vitro* (Miller and Hontela 2011), activate the stress response in juvenile rainbow trout (Miller, Wang et al. 2007), and inhibit androgen receptors and estrogen receptors in cancer cells (Shah, Kaul et al. 2005). The effects of Se appear to be species-specific. Rainbow trout (RT), *Oncorhynchus mykiss* (Holm, Palace et al. 2005) have greater rates of Se-induced teratogenesis than brook trout (BT), *Salvelinus fontinalis* (Holm, Palace et al. 2005), dolly varden, *Salvelinus malma malma* (McDonald, DeBruyn et al. 2010), and cutthroat trout, *Oncorhynchus clarki* (Hardy, Oram et al. 2010). Similar patterns have been noted in endocrine disruption, as RT are also more vulnerable to Se-induced cortisol impairment than BT (Miller and Hontela 2011). Oxidative stress has been proposed as the major mechanism behind Se toxicity (Janz, DeForest et al. 2010). Excess Se, both as selenite and selenomethionine, can produce ROS in the presence of reduced glutathione (GSH). Selenite, key form of Se in waterborne exposures in the wild, can be reduced to elemental Se by GSH producing ROS (Misra and Niyogi 2009). Conversely, selenomethionine, important in dietary and maternal transfer exposures, is transformed to methylselenol by methioninase, and then methylselenol reacts with GSH to produce ROS (Palace, Spallholz et al. 2004).

The objective of this paper was to investigate species-specific effects of chronic Se exposure on endocrine and oxidative stress biomarkers in juvenile RT and BT. Hatchery-reared fish were experimentally stocked into two reference pit lakes and two Se-contaminated pit lakes formed by open pit coal mines. Fish were sampled over two years and the following hypotheses were tested: (1) Se alters endocrine and oxidative stress biomarkers, and (2) species differences between RT and BT endocrine and oxidative stress biomarkers exist.

Materials and methods

Chemicals

EDTA, imidazole-HCl, NaCl, KCl, ouabain, Na₂ATP, MgCl₂·6H₂O, (NH₄)₆Mo₇O₂₄·4H₂O, FeSO₄·7H₂O, H₂SO₄, potassium phosphate (KH₂PO₄), metaphosphoric acid, butylated hydroxytoluene (BHT), porcine ACTH I-39, minimal essential medium, bovine serum albumin, NaHCO₃, Bradford reagent, HPLC grade dichloromethane, ethyl acetate, hexane, acetonitrile, and methanol, as well as the authentic standards tocopherol, tocopherol acetate, retinol, and retinol palmitate were purchased from Sigma–Aldrich (Oakville, Ontario). Didehydroretinol was a gift from Hoffman LaRoche (Basel, Switzerland). Retinyl esters were synthesized as previously described (Palace, Hill et al. 1999). Clove oil was purchased from Nutter’s Bulk and Natural Foods (Lethbridge, Alberta, Canada).

Fish

Juvenile RT (average weight = 10.64 ± 2.25 g; muscle Se = 1.25 ± 0.62 µg/g dw) and BT (average weight = 3.13 ± 0.41 g; muscle Se = 0.60 ± 0.07 µg/g dw) were obtained from Alberta fish hatcheries (water Se = 0.47 ± 0.11 µg/L; diet Se = 1.86 ± 0.66 µg/g dw) and experimentally

stocked into reference and Se-contaminated pit lakes as previously described (Miller, Rasmussen et al. submitted). Fish were sampled 5, 12, 17, and 24 months after the initial stocking with one hour gill net sets (22 to 62 mm mesh), euthanized with clove oil (160 ppm, emulsified in ethanol), bled from the caudal vessels, and kept on ice during transportation to the field laboratory. No RT were caught in Lake 24, but sample sizes ranged from 5 to 29 at each sampling time for RT and BT in the other pit lakes. Dissections began 2.5 – 3.0 hours after the last net was pulled to standardize the travel times from all sites. At the field laboratory, plasma was recovered (centrifuged for 5 minutes, 16,000 x g) and frozen for later analyses. Gills and liver were removed and flash frozen in liquid nitrogen for later analyses. Head kidneys were also removed for an adrenocortical challenge, which tests the ability of the fish to secrete cortisol by stimulating cortisol production with 2 units ACTH in a fresh coarse head kidney homogenate as previously described (Levesque, Dorval et al. 2003; Miller, Rasmussen et al. 2009). The ability of head kidney to secrete cortisol is expressed as the change in cortisol secretion (Δ ng/ml) after stimulation with ACTH (stimulated cortisol – basal cortisol).

Biochemical analyses

Cortisol (07-221102), total T3 (06B-254215) and total T4 (06B-254011) were measured with radioimmunoassay kits from Medicorp (Montréal, Québec) as previously described (Levesque, Dorval et al. 2003). Plasma testosterone (# 402510) and estradiol (# 402110) were measured using ELISA kits from Neogen Corporation (Lexington, Kentucky). Samples were extracted with ethyl ether under a stream of N₂ and assayed according to kit protocol. Gill Na⁺/K⁺ ATPase activity was measured by liberating PO₄ from ATP with ATPase, as previously described (Morgan, Henry et al. 1997; Miller, Wang et al. 2007). Activity was expressed as μ mol PO₄

liberated per mg of protein in a gill homogenate. Protein was measured using the Bradford reagent (510 nm). Refer to Table 5.1 for details of QA/QC.

Livers were homogenized in a 50 mM potassium phosphate buffer as previously described (Miller, Wang et al. 2007). GSH was assayed within the hour and LPO was stored at -80°C until analyses the next day. GSH and LPO levels were determined using the Bioxytech kits GSH-400 (Catalogue #21011) and LPO-596 (Catalogue #21012) respectively (Medicorp, Montréal, Québec) (Miller, Wang et al. 2007). GSH was expressed as μmol GSH per mg protein. LPO was expressed as units/mg protein, where one unit is 1 μM of malondialdehyde and 4-hydroxyalkenals. Refer to Table 5.1 for QA/QC information. Vitamin E (tocopherol) and vitamins A (retinol, dehydroretinol, and retinyl esters) were measured by HPLC in the liver, as previously described (Palace and Brown 1994) and modified (Palace, Brown et al. 1998).

Statistical analyses

All statistical analyses were performed using the JMP 7.0.2 software package, used $\alpha = 0.05$, and data was log transformed to respect normality. One-way analyses of variance (ANOVAs) with *post hoc* Tukey-Kramer HSDs were used to investigate the effect of site and species on water, stomach content, and muscle Se levels. Analyses of covariance (ANCOVA) were used to investigate the effect of Se on the biochemical parameters. Covariates included muscle Se (indicator of exposure), food availability, species, months after stocking, sex, and fork length. Only covariates that explained a significant amount of variation were included in the final model.

Results and discussion

Selenium exposure

Experimental stocking of juvenile RT and BT into Se-contaminated and reference pit lakes was an excellent system to study the chronic effects of environmentally relevant concentrations of Se. Selenium concentrations in the water were significantly different among the pit lakes experimentally stocked (Lake 24 < Lake 44 < Lake C4 < Luscar Lake; Table 5.2); thus, Lakes 24 and 44 were classified as reference lakes ($\text{Se} \leq 2 \mu\text{g/L}$) and Lake C4 and Luscar Lake as Se-contaminated lakes ($\text{Se} > 10 \mu\text{g/L}$), as previously described (Miller, Rasmussen et al. submitted). Water concentrations in Lakes 44, C4, and Luscar Lake were all greater than the Canadian Water Quality Guidelines of $1 \mu\text{g/L}$ (<http://mst.ccme.ca>). Water Se levels are within the range observed for other contaminated systems including Belews Lake ($10 \mu\text{g/L}$) and the Elk River Valley ($50 \mu\text{g/L}$) (Young, Finley et al. 2010). No other trace elements in the pit lakes exceeded water quality guidelines (Miller, Rasmussen et al. submitted).

Selenium levels of the stomach contents also differed significantly among lakes (Lake 44 > Lake 24 > Lake C4 > Luscar Lake; Table 5.2). Stomach content Se levels were similar to other Se contaminated systems, such as Belews lake (Young, Finley et al. 2010), and were lower in the reference pit lakes than the Se-contaminated pit lakes. Lakes 44 and C4 were classified as lakes with poor food availability, and Lake 24 and Luscar Lake as lakes with rich food availability as previously described (Miller, Rasmussen et al. submitted). Muscle Se levels in both RT and BT followed water Se levels (Table 5.2). Fish from Lake 24 had the lowest Se levels, followed by Lake 44, Lake C4, and Luscar Lake. In Luscar Lake, BT had significantly higher muscle Se levels than RT (Table 5.2), suggesting that BT accumulate more Se when exposed to high levels than RT.

Endocrine system biomarkers

For the first time endocrine disrupting effects of chronic Se exposure were observed the thyroid axis of juvenile RT and BT. Muscle Se levels explained a significant amount of variation in RT and BT T3 and T4 levels (Table 5.3). Fish with higher muscle Se levels had lower plasma T3 and T4 levels, but this effect was more pronounced for T4, than T3 (Figure 5.1). Decreases in plasma thyroid hormones with Se exposure have also been observed in calves (Kumar, Rampal et al. 2008). Selenium-linked impairment of thyroid hormones have not been documented previously in fish; however a dose dependant increase in thyroid hormones was observed in juvenile RT exposed to selenite in the laboratory for 30 days (Miller, Wang et al. 2007). Both plasma T3 and T4 levels were also significantly influenced by food supply, month of exposure, and length of the fish (Table 5.3). A decrease in both T3 and T4 may be due to decreased production of T4 or increases in clearance of the hormones. The ratio of T3 to T4 was not significantly influenced by Se (Table 5.3), suggesting the conversion of the inactive form (T4) to the active form (T3) was not affected by Se exposure.

Brook trout had greater plasma T3 levels than RT, suggesting species differences in the basic physiology of RT and BT exist. This difference may be driven by species differences in the deiodinase enzymes that convert the inactive T4 to the active form T3 (Blanton and Specker 2007), as no differences were observed in plasma T4 levels. While deiodinase levels were not directly measured in this experiment, the ratio of T3:T4 is also greater in BT than RT, further supporting the hypothesis that differences in deiodinase activity drive the observed differences in plasma thyroid hormones. A similar pattern was observed for growth and maturation, as BT had greater specific growth rates and matured earlier than RT (Chapter 4). Thyroid hormones influence many processes, including growth and maturation (Blanton and Specker 2007), suggesting the differences in T3 levels observed (Table 5.3) may partially control species differences in growth and maturation rates.

Previous research has shown that selenite impairs cortisol secretion in both RT and BT adrenocortical cells (Miller and Hontela 2011); thus, other hormones derived from cholesterol, such as, testosterone, and estradiol, may also be affected. However, this was not observed in the pit lake experiment; instead, effects of species, length of exposure, sex, and fish length were observed (Table 5.3). Brook trout had greater levels of plasma testosterone and estradiol than RT (Table 5.3) as expected as BT mature more quickly than RT and BT had begun to mature by the end of this experiment (Chapter 4). Additionally, as expected, larger fish had greater testosterone and estradiol levels than smaller fish, males had higher testosterone levels than females, and females had higher estradiol levels than males (Table 5.3).

Similar to estradiol and testosterone, Se exposure had no effect on the ability to secrete cortisol in RT and BT from this experiment (Table 5.3). Previous research has shown that selenite, but not selenomethionine can inhibit cortisol secretion *in vitro* in both RT and BT (Miller and Hontela 2011). The fish in the pit lakes were exposed to some selenite present in the water (Miller, Rasmussen et al. submitted); however, the primary Se exposure was to protein bound forms, such as selenomethionine, through the diet (Janz, DeForest et al. 2010). Unlike selenite, selenomethionine does not impair cortisol secretion *in vitro* (Miller and Hontela 2011). Similarly, a field study also found no effect of Se on the ability to secrete cortisol in RT and BT from Se-contaminated streams (Miller, Rasmussen et al. 2009).

Oxidative stress biomarkers

Biomarkers of oxidative stress may be used as screening tools for effects of aquatic pollution (Hinck, Blazer et al. 2007), but they may also be used to elucidate toxicity mechanisms (Dorval and Hontela 2003; Miller and Hontela 2011). In this experiment none of the oxidative stress biomarkers were significantly influenced by Se exposure; however, effects of species, food

availability, sex, month of exposure, and fish size were observed (Table 5.3). Hepatic GSH levels increased with the size of the fish, and LPO levels increased with month of exposure, suggesting that as trout age, GSH is up-regulated, and oxidative damage to lipids accumulates. Similarly, larger RT and BT from Se-contaminated and reference streams had greater liver LPO levels than the smaller fish from the same streams (Miller, Rasmussen et al. 2009), protein carbonyls (marker of protein peroxidation) increased with age of brown trout (Almroth, Johansson et al. 2010), and GSH levels of RT increased with age (Otto and Moon 1996). No significant species differences in GSH and LPO were observed, but fish from food-rich lakes had higher GSH levels than those from food-poor lakes suggesting site-specific factors such as food supply must be considered to avoid confounding the results.

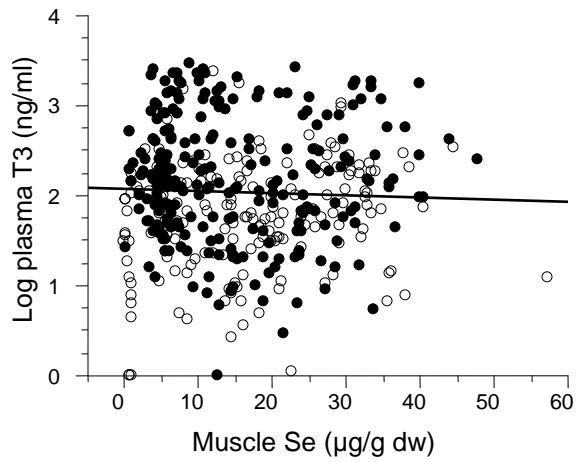
Similarly, food supply also influenced retinol and dehydroretinol levels in fish, as fish from food-poor lakes had lower retinol (vitamin A1) and dehydroretinol levels (vitamin A2), than those from the food-rich lakes (Table 5.3). Fish cannot synthesize Vitamin A; thus, it or its precursor, β -carotene, must be obtained from their diet (Palace and Werner 2006). The results from this experiment suggest that prey items readily available in food-poor lakes had lower β -carotene levels than those from the food-rich lakes. In addition to the effect of food availability, species differences in vitamin A concentrations were detected. Rainbow trout had greater hepatic retinol and dehydroretinol levels (the active forms) than BT; but similar amounts of total retinyl esters (storage form of Vitamin A), and tocopherol (Vitamin E). This suggests that RT have more active vitamin A to act as an antioxidant, protecting the liver, than BT. Also, it may indicate a species difference in enzymes such as retinol ester hydrolase which release vitamin A from the storage form and can be inhibited by contaminants such as polychlorinated biphenyl (Ndayibagira and Spear 1999); however, more research is needed to define species differences in vitamin metabolism and its implications to toxicology.

The experimental stocking of juvenile RT and BT into reference and Se-contaminated lakes for two years provided a system for a comparative study of the effects of chronic environmental Se exposure on the endocrine disrupting potential and oxidative stress biomarkers in two trout species. Species differences detected in plasma T3 levels may be linked to growth rates (Chapter 4) as both were greater in BT than RT, while differences in hepatic vitamin A levels may reflect different diets between RT and BT. Selenium exposure lowered thyroid hormone in RT and BT; however, plasma cortisol, testosterone and estradiol levels were unaffected. No effect of Se was observed on any of the oxidative stress biomarkers, but biomarkers were altered by other factors such as food availability, age, and sex suggesting that future experimental designs should consider these confounding factors.

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(A)



(B)

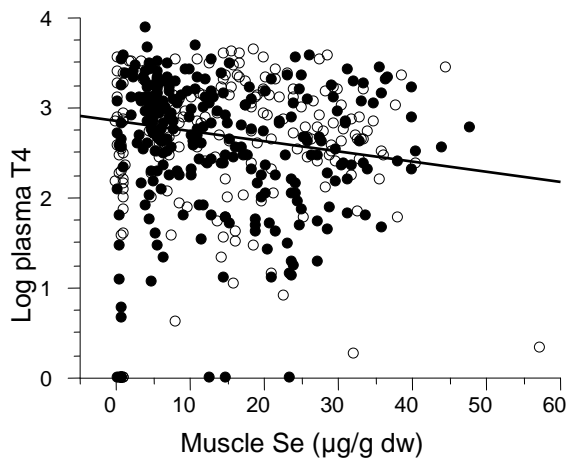


Figure 5.1. Effect of Se exposure on plasma (A) T3 and (B) T4 levels in rainbow trout (open circles) and brook trout (shaded circles). Lines represent contribution of muscle Se to the models described in Table 5.3 (Se effect in ANCOVA: T3 $p = 0.0050$, and T4 $p = 0.0295$).

Table 5.1. QA/QC parameters of cortisol, T3, T4, estrogen, testosterone, GSH, and LPO assays.

| | Expected Low | Observed Low ^a | Expected High | Observed High ^a | Sensitivity ^b |
|--|-----------------|------------------------------|------------------|-------------------------------|--------------------------|
| Cortisol (ng/ml plasma) | 20.0 | 19.0 (12%) | 200.0 | 208 (13%) | 5.8 |
| Total T3 (ng/ml plasma) | 0.5 | 0.6 (6%) | 5.0 | 4.9 (6%) | 0.2 |
| Total T4 (ng/ml plasma) | 20.0 | 21 (19%) | 150.0 | 152 (10%) | 5.2 |
| Testosterone (ng/ml plasma) | 0.0020 | 0.003 (20%) | 0.0800 | 0.095 (7%) | 0.0001 |
| Estradiol (ng/ml plasma) | 0.04 | 0.05 (26%) | 1.00 | 1.05 (7%) | 0.03 |
| Gill Na ⁺ /K _i ATPase activity (μmol PO ₄ /ml) | 2.0 | 1.9 (25%) | 10.0 | 10.6 (18%) | 1.2 |
| GSH concentration (μmol/ml) | 20.0 | 18.6 (21%) | 80.0 | 78.1 (10%) | 7.8 |
| LPO concentration (U/ml) | 0.50 | 0.46 (20%) | 3.00 | 2.96 (6%) | 0.19 |

^a parentheses contain the % co-variation of internal standards from all assay runs.

^b lowest detectable dose = two standard deviations from zero dose response.

Table 5.2. Exposure characteristics (mean \pm SE for all sampling times) of pit lakes experimentally stocked with rainbow trout and brook trout.

| | Lake 24 | Lake 44 | Lake C4 | Luscar Lake |
|--|------------------------------|------------------------------|-------------------------------|--------------------------------|
| Water [Se] ($\mu\text{g/L}$) | 0.44 \pm 0.08 ^A | 2.00 \pm 0.17 ^B | 16.72 \pm 1.33 ^C | 50.47 \pm 10.07 ^D |
| Diet [Se] ($\mu\text{g/g dw}$) | 6.37 \pm 0.33 ^A | 3.58 \pm 0.20 ^B | 8.15 \pm 0.38 ^C | 13.46 \pm 0.43 ^D |
| Rainbow trout muscle [Se] ($\mu\text{g/g dw}$) | - | 6.35 \pm 0.78 ^A | 18.77 \pm 0.79 ^B | 25.44 \pm 1.41 ^{C*} |
| Brook trout muscle [Se] ($\mu\text{g/g dw}$) | 6.87 \pm 0.37 ^A | 7.96 \pm 0.50 ^A | 21.63 \pm 0.79 ^B | 29.41 \pm 1.06 ^C |
| Lake classification | reference | reference | Se-contaminated | Se-contaminated |
| Food Availability | rich | poor | rich | poor |

Refer to (Miller, Rasmussen et al. submitted) for a more detailed characterization of the pit lakes. Different letters indicate a significant difference among sites (1-way ANOVA for site, $p < 0.05$). * indicates a significant difference between species at that site (1-way ANOVA for species, $p < 0.05$).

Table 5.3. Analyses of covariance models that describe a significant ($p < 0.05$) amount of variation in the endocrine and oxidative stress parameters measured in rainbow trout (RT) and brook trout (BT) experimentally stocked into reference and Se-contaminated pit lakes for two years.

| Log Transformed Parameters | Adjusted R ² | $F_{df(model), df(error)}$ | Effect of ANCOVA Factor ^a | | | | | |
|---|-------------------------|----------------------------|--------------------------------------|-------------------|----------------------|---------------------|------------------|---------------------|
| | | | Se | Food ^b | Species ^c | months ^d | sex ^e | length ^f |
| Plasma T3 | 0.5087 | $F_{3, 394} = 60.59$ | Fig. 1A | R > P | BT > RT | 17 < 5, 12, 24 | NS | ↑ |
| Plasma T4 | 0.4688 | $F_{3, 397} = 60.29$ | Fig. 1B | R > P | NS | 5 < 12 < 24 < 17 | NS | ↑ |
| Plasma T3:T4 | 0.1426 | $F_{4, 413} = 18.34$ | NS | NS | BT > RT | 24 < 12, 17 < 5 | NS | NS |
| Plasma testosterone | 0.5080 | $F_{7, 269} = 41.72$ | NS | NS | BT > RT | 17 > 5 > 12 > 24 | M > F > I | ↑ |
| Plasma estradiol | 0.4406 | $F_{7, 269} = 32.35$ | NS | NS | BT > RT | 17 > 5, 12, 24 | F > I, M | ↑ |
| Adrenocortical challenge | 0.0360 | $F_{3, 278} = 4.50$ | NS | NS | NS | 12 > 24, 5 (17) | NS | NS |
| Gill Na ⁺ /K ⁺ ATPase | 0.1343 | $F_{6, 424} = 12.12$ | NS | R > P | NS | 5 > 12, 17, 24 | M < F, I | NS |
| Hepatic GSH | 0.6914 | $F_{7, 406} = 133.17$ | NS | R > P | NS | 24, 12 > 17 > 5 | M > I, F | ↑ |
| Hepatic LPO | 0.5748 | $F_{3, 418} = 190.72$ | NS | NS | NS | 24 > 17 > 12 > 5 | NS | NS |
| Hepatic tocopherol | 0.1117 | $F_{2, 136} = 9.68$ | NS | NS | NS | 12 > 17 (5) | NS | NS |
| Hepatic retinol | 0.4289 | $F_{4, 136} = 27.28$ | NS | P > R | RT > BT | 5, 17 > 12 | NS | NS |
| Hepatic dehydroretinol | 0.3335 | $F_{6, 134} = 1.61$ | NS | P > R | RT > BT | 5, 17 > 12 | M > F, I | NS |
| Hepatic total retinyl esters | 0.0858 | $F_{4, 132} = 4.19$ | NS | NS | NS | NS | I > F (M) | ↓ |

^a NS = not significant; differences indicated tested with *post hoc* Students *t*-tests for food, species, months and sex (data not shown; $\alpha = 0.05$).

^b R = food-rich pit lakes; P = food-poor pit lakes

^c Rainbow trout = RT; brook trout = BT

^d parentheses indicate that sampling time is intermediate

^e M = male; F = female; I = immature

^f Arrows indicate the slope of the parameter vs. length regression.

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CHAPTER 6. SPECIES DIFFERENCES IN SE TOXICITY: A SUMMARY

Selenium (Se), an essential element that can be toxic when requirements for homeostasis are exceeded, increases the rate of teratogenesis in oviparous vertebrates, but this effect is species-specific. While much research has been conducted on the reproductive effects of Se, relatively little attention has been focused on other physiological systems. To fill this knowledge gap and further investigate the species-specific sensitivity to Se, the objectives of this project were to:

- 1) Compare the sensitivity of juvenile rainbow trout and brook trout to Se exposure.
- 2) Determine the effects of environmentally relevant Se exposure on juvenile rainbow trout and brook trout.
- 3) Elucidate the role of oxidative stress in selenium toxicity.
- 4) Link the effects seen at the cellular level, to effects at the individual and population levels.

A meta-analysis (Chapter 1) collected reference or control data from 202 studies found in the scientific literature, including baseline levels of oxidative stress biomarkers, energy reserves, physiological stress response parameters, and condition and growth indices of juvenile rainbow trout and brook trout. This meta-analysis identified:

- 1) That juvenile rainbow trout have higher normal plasma T4 levels and whole-body lipid levels than juvenile brook trout, but all other parameters were similar.
- 2) Means and ranges of each parameter for juvenile rainbow trout and brook trout that may be used to determine effects or predict species-specific vulnerability of toxicants.

To investigate the species-specific effect of Se and elucidate the toxicity mechanism at the cellular level, adrenocortical cells from juvenile rainbow trout and brook trout were exposed to selenite and selenomethionine *in vitro* (Chapter 2). These experiments showed that:

- 1) Selenite, but not selenomethionine, disrupts cortisol secretion in both rainbow trout and brook trout adrenocortical cells.
- 2) Rainbow trout cells are more sensitive to selenite-induced cortisol impairment than brook trout cells.
- 3) Selenite impairs cortisol secretion at different steps in the biosynthetic pathway of rainbow trout and brook trout adrenocortical cells.
- 4) Superoxide dismutase may protect adrenocortical cells from selenite-induced cortisol impairment.

To investigate the effect of Se at the individual and population levels, a novel experimental approach was used. Two reference and two Se-contaminated pit lakes, formed by open pit coal mining, were selected and experimentally stocked with un-exposed hatchery reared juvenile rainbow trout and brook trout (Chapters 3 – 5). Fish were sampled over a two year period and the experiments showed that:

- 1) Selenium accumulation was similar between rainbow trout and brook trout.
- 2) Whole-body: muscle Se regressions, similar between the two trout species, and may be used for non-lethal monitoring activities.
- 3) After two years of exposure, fish muscle Se levels were not yet at equilibrium with their environment.

- 4) Selenium acts as an energetic stressor and lowers liver glycogen reserves, condition, and growth; however, this effect can be mitigated by adequate food supplies. This effect was greater in rainbow trout and most apparent in the spring.
- 5) Winter stress syndrome may occur in rainbow trout and brook trout, but only if fish are exposed to Se and poor food supplies.
- 6) Brook trout matured earlier and increased liver glycogen reserves more than rainbow trout, but rainbow trout were in better condition than brook trout.
- 7) Selenium exposure may decrease thyroid hormone levels, as muscle Se concentrations explained a significant amount of the variation in plasma T3 and T4 levels.
- 8) Selenium exposure did not alter the ability of fish from the pit lakes to secrete cortisol, or plasma testosterone and estradiol levels.
- 9) Selenium exposure did not alter liver oxidative stress biomarkers; however, biomarkers were altered by size, species, food availability, and sex suggesting these factors must be considered in all studies using oxidative stress biomarkers.

To link the effects of Se observed at the cellular level to the individual level, the GSH content of adrenocortical cells and the head kidney tissue from fish sampled from the pit lakes was altered and then function (ability to secrete cortisol) was assessed (Appendix C). To link the individual effects with population effects, a simple matrix population model was built using the catch per unit effort data from the pit lake experiment (Appendix C).

- 1) Altering the GSH content of both the adrenocortical cells and head kidney tissue had no effect on cortisol secretion at the Se exposure levels tested.
- 2) No effects of site and species were observed in the modeled population growth rate or the percentage of fish remaining in the lakes after 12 years.

Overall, the major conclusions and management implications of this project were:

- 1) Selenium has the potential to disrupt the endocrine system (cortisol, thyroid hormones) of juvenile fish; thus, endocrine parameters should be monitored at exposed sites.
- 2) Rainbow trout are more sensitive to Se toxicity than brook trout; thus, species-specific risk assessments should be considered.
- 3) The energetic effects of Se on juvenile fish may be mitigated by adequate food supplies; thus, site-specific risk assessments are needed.

The research described above has identified a number of different knowledge gaps that could be addressed in the future:

- 1) Role of antioxidants and oxidative stress in selenite-induced cortisol disruption of brook trout adrenocortical cells.
- 2) Effect of selenium exposure on adrenocortical cells of other salmonids, including cutthroat trout and bull trout.
- 3) Effect of selenium on the thyroid axis of fish.
- 4) Build a population model that includes fecundity to determine if the species-specific reproductive effects of Se have the potential to alter population level parameters.

APPENDIX A. SUPPORTING INFORMATION FOR CHAPTER 3

Supporting information submitted with Chapter 3 to Environmental Toxicology and Chemistry, April 2011.

Table A.A.1. Water quality parameters and ion concentrations for the pit lakes in June 2009.

| Parameter | Lake 24 | Lake 44 | Lake C4 | Luscar Lake |
|---|---------|---------|---------|-------------|
| <i>Water Quality</i> | | | | |
| Conductivity ($\mu\text{S}/\text{cm}$) | 357 | 442 | 801 | 1119 |
| Hardness (as CaCO_3) (mg/L) | 66 | 72 | 175 | 112 |
| Hydroxide (as CaCO_3) (mg/L) | 0 | 0 | 0 | 0 |
| P-Alkalinity (as CaCO_3) (mg/L) | 2 | 0 | 0 | 5 |
| pH | 8.33 | 8.29 | 8.26 | 8.38 |
| Total Alkalinity (as CaCO_3) (mg/L) | 153 | 172 | 213 | 382 |
| Total Dissolved Solids (calculated) (mg/L) | 224 | 285 | 528 | 733 |
| <i>Ions</i> | | | | |
| Calcium (mg/L) | 14.0 | 18.3 | 29.6 | 17.9 |
| Bicarbonates (mg/L) | 179 | 210 | 260 | 444 |
| Bromides (mg/L) | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Carbonates (mg/L) | 3 | 0 | 0 | 9 |
| Chlorides (mg/L) | 0.3 | 0.4 | 0.9 | 0.7 |
| Fluorides (mg/L) | 0.09 | 0.15 | 0.16 | 0.36 |
| Iron (mg/L) | < 0.03 | < 0.03 | < 0.03 | < 0.03 |
| Magnesium (mg/L) | 7.6 | 6.4 | 24.6 | 16.3 |
| Manganese (mg/L) | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| Nitrates as N (mg/L) | < 0.02 | < 0.02 | 5.1 | 4.3 |
| Nitrites as N (mg/L) | < 0.02 | < 0.02 | < 0.02 | < 0.02 |
| $\text{NO}_3 + \text{NO}_2$ as N (mg/L) | < 0.02 | < 0.02 | 5.1 | 4.3 |
| Potassium (mg/L) | 3.5 | 4.3 | 2.8 | 2.2 |
| Sodium (mg/L) | 63 | 85 | 135 | 251 |
| Sulfates (mg/L) | 44 | 67 | 202 | 211 |
| Sum of Cations | 4.15 | 5.26 | 9.44 | 13.20 |
| Sum of Anions | 3.97 | 4.86 | 8.86 | 12.31 |
| Ion Balance | 1.05 | 1.08 | 1.06 | 1.07 |
| TDS/EC Ratio | 0.63 | 0.64 | 0.66 | 0.65 |
| Sodium Absorption | 3.37 | 4.36 | 4.44 | 10.33 |
| Saturation Index | 0.24 | 0.37 | 0.59 | 0.69 |

Table A.A.2. Surface water trace metal concentrations for the pit lakes in June 2009.

| Concentration ($\mu\text{g/L}$) | Lake 24 | Lake 44 | Lake C4 | Luscar Lake |
|-----------------------------------|---------|------------------|--------------------|--------------------|
| Aluminum | 10.8 | 29.1 | 12.0 | 63.2 |
| Antimony | <0.3 | < 0.3 | 2.4 | 2.5 |
| Arsenic | < 0.04 | <0.04 | < 0.04 | < 0.04 |
| Barium | 56.5 | 75.5 | 54.3 | 69.5 |
| Beryllium | <0.05 | < 0.05 | < 0.05 | < 0.05 |
| Boron | 15.6 | 42.1 | 40.6 | 26.7 |
| Cadmium | <0.05 | < 0.05 | < 0.05 | < 0.05 |
| Chromium | <0.1 | < 0.1 | < 0.1 | 0.1 |
| Cobalt | <0.1 | < 0.1 | 0.1 | 0.2 |
| Copper | 0.5 | 0.4 | 3.5 | 0.8 |
| Lead | <0.1 | < 0.1 | < 0.1 | < 0.1 |
| Mercury | < 0.05 | < 0.05 | < 0.05 | < 0.05 |
| Molybdenum | 0.1 | 11.2 | 14.4 | 21.5 |
| Nickel | 0.6 | 1.2 | 10.9 | 2.6 |
| Phosphorus | 7.5 | 2.0 | 2.0 | 6.0 |
| Selenium | 0.2 | 1.5 ^a | 13.3 ^{ab} | 32.1 ^{ab} |
| Silver | <0.04 | < 0.04 | < 0.04 | < 0.04 |
| Strontium | 326 | 635 | 1356 | 894 |
| Tellurium | <0.07 | < 0.07 | < 0.07 | < 0.07 |
| Thallium | < 0.03 | < 0.03 | < 0.03 | < 0.03 |
| Thorium | <0.03 | <0.03 | < 0.03 | < 0.03 |
| Tin | < 0.02 | < 0.02 | < 0.02 | < 0.02 |
| Titanium | 0.1 | 0.4 | 0.1 | 0.4 |
| Tungsten | 0.07 | 0.1 | 0.1 | 0.2 |
| Uranium | 0.9 | 2.1 | 5.0 | 5.3 |
| Vanadium | 0.2 | 0.1 | 0.1 | 0.3 |
| Zinc | < 0.2 | < 0.2 | 1 | 0.3 |
| Zirconium | <0.01 | < 0.01 | < 0.1 | 0.1 |

^a exceeds the CCME Canadian Water Quality Guidelines for the protection of aquatic life (Se: 1 $\mu\text{g/L}$)

^b exceeds the USEPA Aquatic Life Criteria for (Se: 5 $\mu\text{g/L}$)

Table A.A.3. Statistical results of ANOVA and ANCOVA tests used.

| Model Factors | Parameter | df _M | df _E | F - statistic | p-value |
|---|---|-----------------|-----------------|---------------|-----------|
| Site | Water selenite | 3 | 16 | 4.87 | 0.0136* |
| | Water selenate | 3 | 16 | 17.50 | < 0.0001* |
| | Surface O ₂ | 3 | 15 | 0.11 | 0.9501 |
| | pH | 3 | 16 | 0.37 | 0.3699 |
| | Water temperature: summer | 3 | 8 | 0.36 | 0.7849 |
| | Water temperature: fall | 3 | 3 | 0.88 | 0.5407 |
| | Surface conductivity | 3 | 15 | 9.71 | 0.0008* |
| | Secchi disk depth | 3 | 16 | 13.22 | < 0.0001* |
| | Littoral invertebrate Se | 3 | 27 | 4.12 | 0.0158* |
| | Stomach content Se: detritivores | 3 | 9 | 9.15 | 0.0043* |
| | Stomach content Se: misc. | 3 | 9 | 24.15 | < 0.0001* |
| | Stomach content Se: omnivores | 3 | 10 | 2.16 | 0.1557 |
| | Stomach content Se: predators | 3 | 4 | 0.46 | 0.7230 |
| | Stomach content Se: terrestrial | 1 | 1 | 0.03 | 0.8971 |
| | Stomach content Se: herbivores | 3 | 5 | 0.80 | 0.5437 |
| | Muscle Se: all fish | 4 | 478 | 273.36 | < 0.0001* |
| | Water Total Se | 4 | 19 | 20.88 | < 0.0001* |
| Pelagic order richness | 3 | 16 | 4.46 | 0.0185* | |
| Species | Muscle Se: hatchery | 1 | 54 | 0.94 | 0.3375 |
| Months of Exposure (ANCOVA) ^a | Muscle Se: Lake 24 brook trout | 1 | 73 | 60.46 | < 0.0001* |
| Species x Months of Exposure ^a (ANCOVA) ^a | Muscle Se: Lake 44 | 3 | 109 | 24.38 | < 0.0001* |
| | Muscle Se: Lake C4 | 3 | 131 | 12.55 | < 0.0001* |
| | Muscle Se: Luscar Lake | 3 | 100 | 14.30 | < 0.0001* |
| Site x Species | Se bioaccumulation factor | 6 | 20 | 15.9 | < 0.0001* |
| | Se biomagnification factor | 6 | 20 | 0.85 | 0.5482 |
| | Condition Factor at 24 months | 6 | 91 | 26.71 | < 0.0001* |
| | Specific Growth Rate at 24 months | 6 | 91 | 151.16 | < 0.0001* |
| Phosphorous | Pelagic invertebrate biomass | 1 | 2 | 121.44 | 0.0081* |
| Species(site) | % Predators in stomach contents | 3 | 426 | 3.20 | 0.0234* |
| | % Detritivores in stomach contents | 3 | 426 | 4.12 | 0.0067* |
| | % Filter Feeders & Herbivores in stomach contents | 3 | 426 | 1.89 | 0.1299 |
| | % Terrestrial in stomach contents | 3 | 426 | 0.74 | 0.5272 |
| | % Omnivores in stomach contents | 3 | 426 | 0.11 | 0.9567 |
| | % Miscellaneous in stomach contents | 3 | 426 | 6.58 | 0.0002* |

* indicates a significant effect in the model on the parameter of interest. ^a hatchery data was excluded from this analysis.

Table A.A.4. Oxygen, conductivity and temperature in the end pit lakes during the 0, 5, 12, 17, and 24 month Se exposure sampling periods.

| Site | Depth (m) | Oxygen Levels (mg/L) | | | | | Conductivity (μ S) | | | | | Water Temperature ($^{\circ}$ C) | | | | |
|-------------|-----------|----------------------|-----|------|-----|------|-------------------------|-----|------|-----|-----|-----------------------------------|-----|------|-----|------|
| | | months of exposure | | | | | months of exposure | | | | | months of exposure | | | | |
| | | 0 | 5 | 12 | 17 | 24 | 0 | 5 | 12 | 17 | 24 | 0 | 5 | 12 | 17 | 24 |
| Lake 24 | 0 | 8.4 | 8.4 | 8.7 | 9.5 | 10.3 | 336 | 297 | 248 | 269 | 308 | 14.7 | 6.9 | 11 | 5.7 | 15.7 |
| | 5 | 10.5 | 7.7 | 7.5 | 9.1 | 9.8 | 347 | 301 | 315 | 274 | 308 | 9.2 | 6.9 | 5.3 | 5.9 | 6.1 |
| | 10 | 4.3 | 7.7 | 3.7 | 1.8 | 3.3 | 319 | 303 | 331 | 363 | 314 | 4.9 | 6.9 | 4.5 | 6.4 | 4.8 |
| Lake 44 | 0 | 7.0 | 7.5 | 9.9 | 9.6 | 9.6 | 425 | 350 | 367 | 324 | 351 | 13.9 | 7.5 | 12.4 | 4.7 | 10.1 |
| | 5 | 7.4 | 7.7 | 11.6 | 9.4 | 9.5 | 385 | 350 | 341 | 327 | 351 | 9.1 | 7.7 | 7.2 | 4.6 | 7.2 |
| | 10 | 5.7 | 7.9 | 6.9 | 8.9 | 8.5 | 357 | 350 | 360 | 329 | 354 | 6.1 | 7.9 | 4.7 | 4.6 | 4.9 |
| Lake C4 | 0 | 8.5 | 9.1 | 8.7 | 9.6 | 9.1 | 604 | 980 | 452 | 613 | 681 | 15 | 5.4 | 10 | 2.1 | 13.4 |
| | 5 | 10.6 | 9.4 | 6.8 | 9.1 | 10.6 | 683 | 627 | 665 | 633 | 746 | 5.5 | 5.3 | 3.5 | 3.1 | 5.6 |
| | 10 | 9.1 | 6.0 | 3.7 | 8.1 | 7.6 | 669 | 716 | 703 | 648 | 706 | 4.1 | 4.7 | 3.9 | 3.5 | 4.1 |
| Luscar Lake | 0 | 9.1 | 6.6 | 10.5 | - | 8.8 | 440 | 738 | 1135 | - | 900 | 14.1 | 4.4 | 10.5 | - | 12.1 |
| | 5 | 9.3 | 7.8 | 9.5 | - | 8.7 | 930 | 741 | 811 | - | 800 | 12.1 | 4.2 | 9.5 | - | 8.1 |
| | 10 | 6.1 | 8.1 | 7.6 | - | 7.4 | 715 | 741 | 797 | - | 700 | 11.2 | 4.1 | 7.6 | - | 7.2 |

Table A.A.5. Surface pH and Secchi disk transparency in the pit lakes during the 0, 5, 12, 17, and 24 month Se exposure sampling periods.

| Site | pH | | | | | Secchi Disk Transparency (m) | | | | |
|-------------|--------------------|------|------|------|------|------------------------------|------|------|------|------|
| | months of exposure | | | | | months of exposure | | | | |
| | 0 | 5 | 12 | 17 | 24 | 0 | 5 | 12 | 17 | 24 |
| Lake 24 | 8.45 | 8.30 | 7.95 | 7.37 | 7.22 | 9.00 | 4.24 | 6.00 | 7.00 | 5.25 |
| Lake 44 | 8.49 | 8.23 | 8.20 | 7.46 | 7.61 | 1.25 | 5.00 | 2.50 | 2.50 | 2.00 |
| Lake C4 | 8.48 | 8.25 | 8.24 | 7.49 | 7.41 | 1.00 | 2.50 | 2.25 | 2.25 | 3.00 |
| Luscar Lake | 8.28 | 8.22 | 6.79 | 7.78 | 7.34 | 1.75 | 2.00 | 1.25 | 3.00 | 0.75 |

Table A.A.6. Mean (\pm SE)^a selenium content ($\mu\text{g/g dw}$) and bioaccumulation factors (BAF) of invertebrates in the pit lakes (June 2009 kick samples).

| Order | Lake 24 | | Lake 44 | | Lake C4 | | Luscar Lake | |
|----------------|------------------------------|-------|---------------------------|------|---------------------------|------|---------------------------|------|
| | Se ($\mu\text{g/g dw}$) | BAF | Se ($\mu\text{g/g dw}$) | BAF | Se ($\mu\text{g/g dw}$) | BAF | Se ($\mu\text{g/g dw}$) | BAF |
| Amphipoda | 4.37 \pm 0.35 | 5.99 | 7.79 | 3.91 | - | - | 20.97 | 0.60 |
| Arachnidia | - | - | 11.88 | 5.93 | - | - | 2.19 | 0.06 |
| Cladocera | 3.81 \pm 0.73 | 5.22 | 3.65 | 1.85 | - | - | 17.34 \pm 4.56 | 0.50 |
| Coleoptera | 3.39 \pm 0.17 | 5.33 | - | - | 1.89 | 0.14 | - | - |
| Copepoda | 2.61 | 3.57 | - | - | 7.10 | 0.52 | - | - |
| Diptera | 45.49 | 62.3 | 6.92 | 3.48 | 14.76 | 1.08 | 11.32 | 0.32 |
| Emphemeroptera | 5.75 | 7.88 | 8.95 \pm 0.05 | 4.50 | 10.41 | 0.76 | 19.76 \pm 3.81 | 0.56 |
| Gastropoda | 1.85 | 2.53 | - | - | - | - | 5.86 | 0.17 |
| Hemiptera | - | - | 1.04 | 0.52 | - | - | - | - |
| Hirudinae | 11.01 | 15.08 | - | - | - | - | - | - |
| Odonata | 6.60 | 9.04 | 5.62 | 2.82 | - | - | 21.55 | 0.62 |
| Pelecypoda | 0.76 | 1.04 | - | - | - | - | 2.01 | 0.06 |
| Plecoptera | - | - | - | - | - | - | 7.08 | 0.20 |
| Tricoptera | 4.98 \pm 1.09 | 6.82 | - | - | - | - | 23.67 \pm 8.79 | 0.68 |
| <i>Mean</i> | 4.51 \pm 0.91 ^b | - | 6.55 \pm 1.34 | - | 8.54 \pm 2.72 | - | 13.18 \pm 2.67 | - |

^a Mean \pm SE is presented when enough material was collected for multiple samples; ^b does not include Diptera, an outlier

APPENDIX B. SUPPORTING INFORMATION FOR CHAPTER 4

Supporting information to be submitted with Chapter 4 to Environmental Science and Technology.

Chemicals

Isopropanol, sodium citrate, free glycerol reagent, triglyceride reagent, glycerol standard, potassium hydroxide, sodium acetate trihydrate, glacial acetic acid, amyloglucosidase, and glycogen were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Clove oil was purchased from Nutter's Bulk and Natural Foods (Lethbridge, Alberta, Canada). GOD-PAP reagent was purchased from Roche Diagnostic (Laval, Québec, Canada).

Table A.B.1. Internal standards and sensitivity of glycogen and triglyceride assays used to analyse rainbow trout and brook trout samples from the pit lakes.

| | Expected Low | Observed Low ¹ | Expected High | Observed High ¹ | Sensitivity ² |
|------------------------------------|-----------------|------------------------------|------------------|-------------------------------|--------------------------|
| Liver Glycogen (mg/g ww) | 0.200 | 0.201 (11%) | 2.000 | 2.040 (8%) | 0.040 |
| Muscle triglycerides (mg/mg ww) | 0.250 | 0.281 (18%) | 2.250 | 2.318 (8%) | 0.100 |

¹ Parentheses contain the % co-variation of internal standards from all assay runs.

² lowest detectable dose = two standard deviations from zero dose response.

Table A.B.2. *F*-statistics and *p*-values of statistical tests (* indicates a significant effect; $\alpha = 0.05$).

| Test | Parameter | $F_{df(model), df(error)}$ | <i>p</i> -value |
|---|-----------------------------|----------------------------|-----------------------|
| 1-way ANOVA: site | Water total Se ¹ | $F_{3, 16} = 18.45$ | < 0.0001* |
| | Benthic Invertebrate Se | $F_{3, 27} = 4.12$ | 0.0158* |
| | Stomach Content Se | $F_{5, 56} = 7.57$ | 0.0002* |
| | Water pH | $F_{3, 16} = 0.37$ | 0.7758 |
| | Water Temperature: Spring | $F_{3, 8} = 0.36$ | 0.7849 |
| | Water Temperature: Fall | $F_{3, 3} = 0.88$ | 0.5407 |
| | Surface Oxygen | $F_{3, 15} = 0.11$ | 0.9501 |
| | 2-way ANOVA: site x species | Muscle Se | $F_{6, 420} = 126.08$ |
| Liver glycogen: spring ¹ | | $F_{6, 242} = 11.53$ | < 0.0001* |
| Liver glycogen: fall ¹ | | $F_{6, 167} = 6.32$ | < 0.0001* |
| Muscle triglycerides: spring ¹ | | $F_{6, 250} = 12.89$ | < 0.0001* |
| Muscle triglycerides: fall ¹ | | $F_{6, 166} = 5.91$ | < 0.0001* |
| GSI: spring ¹ | | $F_{4, 266} = 1.66$ | < 0.1605 |
| GSI: fall ¹ | | $F_{6, 174} = 15.45$ | < 0.0001* |
| Condition factor: spring | | $F_{6, 264} = 45.85$ | < 0.0001* |
| Condition factor: fall | | $F_{6, 178} = 28.74$ | < 0.0001* |
| Specific growth rate: spring ¹ | | $F_{6, 264} = 44.07$ | < 0.0001* |
| Specific growth rate: fall ¹ | | $F_{6, 178} = 10.46$ | < 0.0001* |
| HSI: spring ¹ | | $F_{6, 251} = 25.18$ | < 0.0001* |
| HSI: fall ¹ | $F_{6, 166} = 23.11$ | < 0.0001* | |

¹ log transformed to respect normality

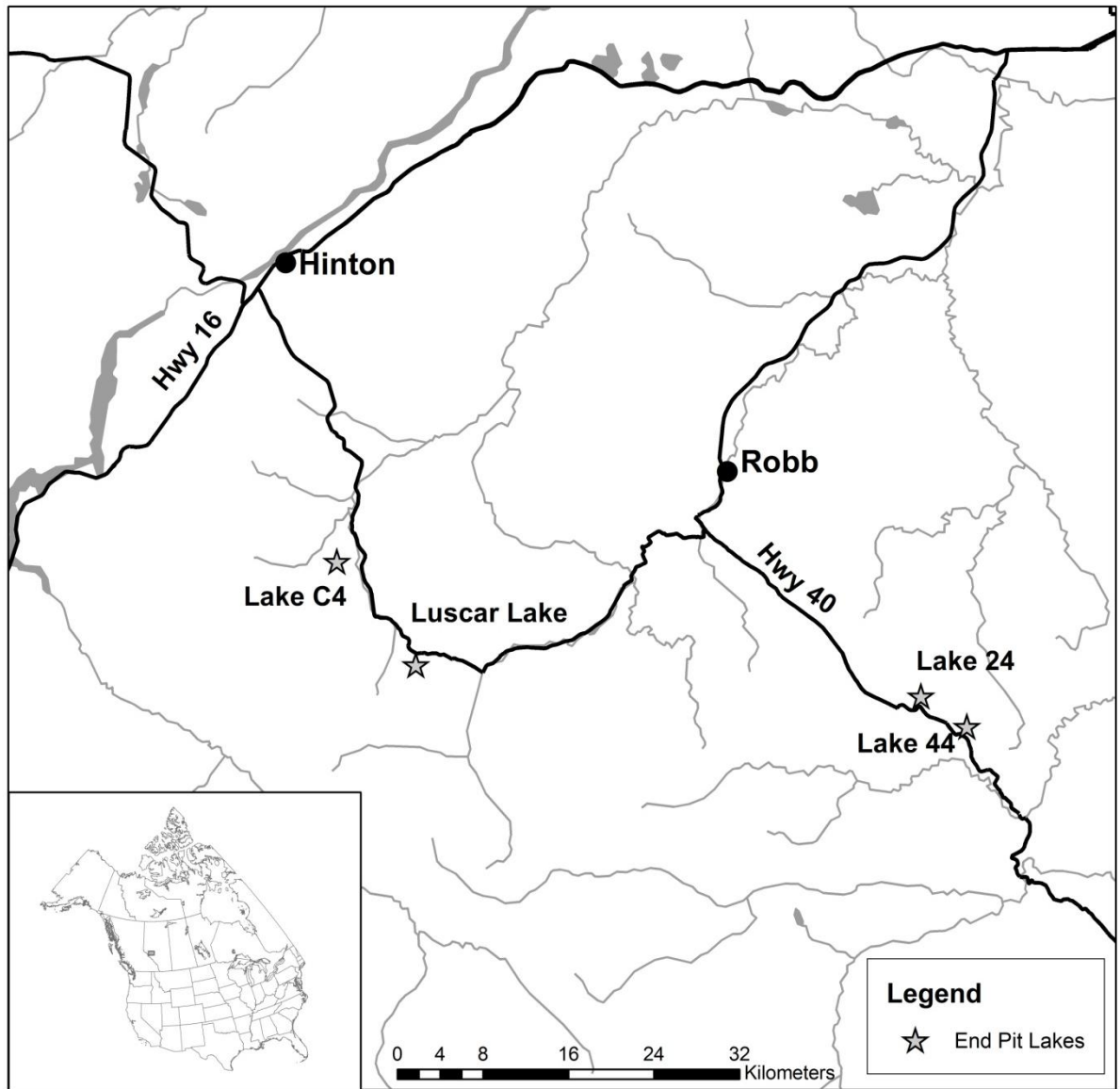


Figure A.B.1. Locations of pit lakes located in Alberta, Canada, experimentally stocked with rainbow trout and brook trout in June 2007 and June 2008.

APPENDIX C: LINKING THE EFFECT OF SELENIUM ON CELLULAR RESPONSES TO INDIVIDUAL AND POPULATION EFFECTS IN JUVENILE RAINBOW TROUT AND BROOK TROUT

Introduction

When evaluating the effects of toxicants, such as selenium (Se), different levels of biological organization may be considered; however, data collected at each level has different implications. Experiments at the cellular level may collect data on toxicity mechanisms, but often these experiments use concentrations of the toxicant that are much larger than those found in the environment. For example, selenium levels used in the adrenocortical cell experiments (Chapter 2, (Miller and Hontela 2011)) were 2000 times greater than the highest Se levels observed in the pit lakes (Chapter 3, (Miller, Rasmussen et al. submitted)), but the adrenocortical cell experiments were able to show that oxidative stress plays a role in selenite toxicity (Chapter 2, (Miller and Hontela 2011)). Conversely, experiments at the population level may be environmentally relevant, but may not help elucidate the mechanisms driving the effects observed. Studies at Belews Lake (Young, Finley et al. 2010) showed that Se exposure altered the fish community structure, completely eliminating some species; however, this population and community level data did not identify teratogenesis as the mechanism. The best risk or effect assessments take into account multiple lines of evidence from multiple levels of organization (Adams, Greeley et al. 2000). To link the levels of organization investigated (Chapters 2-5), the objectives of this appendix were to (1) build a population model to determine if chronic Se exposure alters juvenile survival, and (2) determine if links between conclusions drawn at the cellular and individual levels of juvenile rainbow trout and brook trout can be linked to population level responses.

Materials and methods

Study site and fish collection

Juvenile rainbow trout, *Oncorhynchus mykiss*, (weight = 10.64 ± 2.25 g; muscle Se = 1.25 ± 0.62 $\mu\text{g/g dw}$) and brook trout, *Salvelinus fontinalis*, (weight = 3.13 ± 0.41 g; muscle Se = 0.60 ± 0.07 $\mu\text{g/g dw}$) were stocked into four pit lakes (2 reference and 2 Se-contaminated) as previously described (Chapter 3, (Miller, Rasmussen et al. submitted)). Fish were collected 5, 12, 17, and 24 months after the initial stocking in June 2007, bled, euthanized and the gills, liver, and muscle were removed and flash frozen for later analyses as previously described (Chapter 3 - 5). Head kidneys were also removed to test the effect of GSH on cortisol secretion.

Effect of GSH on cortisol secretion

To test the effect of GSH on cortisol secretion of fish exposed to Se for two years, a modified adrenocortical challenge test was used. The adrenocortical challenge tested the ability of the fish to secrete cortisol by stimulating cortisol production with ACTH in a coarse head kidney homogenate as previously described (Chapter 5). In the modified assay, an additional treatment was added that was pre-incubated for 2 hours with 5 mM NAC (Sigma-Aldrich, Oakville Ontario) in minimal essential media (MEM; pH = 7.4, supplemented with 5 g/l bovine serum albumin and 2.2 g/l sodium bicarbonate; Sigma-Aldrich, Oakville, Ontario), and then stimulated with 1U/ml ACTH for one hour. Cortisol was measured in the supernatant by radioimmunoassay as previously described (Chapter 5). The effect of GSH elevation on the ability of head kidney to secrete cortisol is expressed as percent of the ACTH stimulated control.

Population model

Population size projections were modeled using a Leslee matrix model (Figure A.C.1) with three stages: age 0, age 1, and age 2+ corresponding to the age of the fish caught. The initial conditions of the model were the number of juvenile rainbow trout stocked into each of the pit lakes. Fecundity rates were zero as there was no spawning habitat present in the pit lakes. Survival rates (S) were calculated from plots of catch per unit effort (number of fish caught per 100 m² net per hour) vs. time (1 – slope). The probability of a fish remaining in each stage (P) was zero for Age 0 and Age 1, but as no gill catch data was collected after two years, the probability of remaining in Age 2+ stage (P₂₊) was assumed to be the survival rate from Age 1 to Age 2+ (S₁). The model (exponential growth; $N_t = e^{rt}N_0$) was iterated for 60 time steps and the population growth rate ($\lambda = e^r$) and the % of fish remaining 12 years after stocking (modeled number of fish left after 12 years/total number of fish stocked) were calculated. The standard error from the catch data was used in a Monte Carlo analyses (n = 500) to provide an estimate of variability in the population parameters modeled.

Statistical analyses

Data was analysed with the JMP 7.0.2 software. The data from the cortisol secretion experiment and the population model were tested using a 2-way Analyses of Variance (species x site x species*site). Data was log transformed to respect normality and α set to 0.05.

Results and discussion

Cortisol secretion

Experiments with rainbow trout and brook trout adrenocortical cells showed that Se, in the form of selenite, inhibits cortisol secretion (Chapter 2, (Miller and Hontela 2011)); however, chronic Se exposure did not alter the ability of rainbow trout and brook trout from the pit lakes to

secrete cortisol (Chapter 5). The EC_{50s} for selenite from the adrenocortical cell experiments were 20 times greater than Se concentrations in the Se-contaminated pit lakes and (Chapter 3, (Miller, Rasmussen et al. submitted)) suggesting there was not enough selenite present in the water column to impair cortisol secretion. However, the main exposure route for wild fish is the diet, not the water column (Janz, DeForest et al. 2010). Dietary exposures consist mainly of organic Se compounds such as selenomethionine (Janz, DeForest et al. 2010), but similarly, Se-Met exposure did not alter the function or viability of rainbow trout or brook trout adrenocortical cells (Chapter 2, (Miller and Hontela 2011)).

While being metabolized, both selenite and Se-Met may react with GSH to create reactive oxygen species (ROS) that can damage cellular components and tissues if the antioxidant defences are not sufficient (Kelly, Havrilla et al. 1998). Altering the GSH content of adrenocortical rainbow trout cells did not alter their vulnerability to selenite-induced cortisol impairment (Chapter 2, (Miller and Hontela 2011)). Similarly, no effect of site or species was observed on the ability to secrete cortisol in the NAC supplemented head kidney of fish experimentally stocked into the end pit lakes for two years (Table A.C.1).

Population model

To link the effects seen at the individual level to potential population level effects, a simple population model was built. Effects of chronic Se exposure at the individual level observed in the pit lake experiment were altered glycogen storage, growth, and condition of juvenile rainbow trout and brook trout. Using the catch per unit effort data from the same experiment, no significant differences in population growth rate or % of fish remaining after 12 years was observed (Table A.C.1). This suggests that the effects observed at the individual level on juvenile rainbow trout and brook trout were not severe enough to significantly alter juvenile survival, as assessed by this model. While the main effect of Se on populations is the declines in recruitment due to Se-induced teratogenesis, large fish kills have also been documented (e.g. Belews Lake and Hyco Lake (Young, Finley et al. 2010)). The selenium exposures in the pit lake experiment did not cause such fish kills, but this may be due to differences in species sensitivities among coldwater and warmwater fish, or differences in the severity of the exposures.

Fish were not able to reproduce during the course of this experiment; thus, the effect of Se on the reproductive potential of the individuals and its effect on population dynamics was not assessed. Differences in the rate of Se-induced teratogenesis have been observed in rainbow trout and brook trout (Holm, Palace et al. 2005); thus, it is expected that if reproduction could have been evaluated in fish from these lakes, an effect of both species and Se-exposure would have been observed. Future research that documents fry survival rates of both rainbow trout and brook trout exposed to Se, could be added to the present model to improve its applicability.

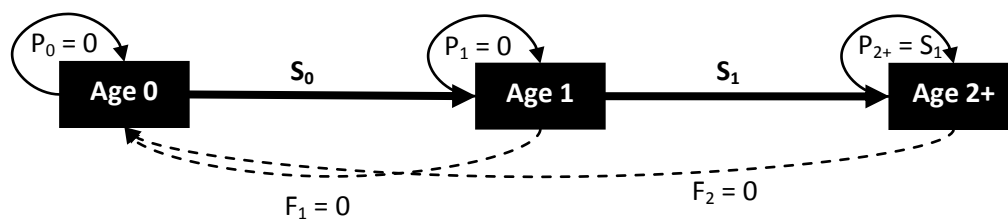


Figure A.C.1. Stage-classified matrix model used to calculate projected population growth rates of juvenile rainbow trout and brook trout stocked into reference and Se-contaminated pit lakes. Black boxes indicate the different age classes of fish, S = survival rate, F = fecundity rate, P = probability of remaining in the age class.

Table A.C.1. The effect of site and species on the NAC supplemented head kidney cortisol secretion, population growth rate, and % fish remaining after 12 years.

| | | Cortisol Secretion after NAC exposure (% of ACTH control) ¹ | Population growth rate ² | Fish Remaining after 12 years (%) ³ |
|-------------|----|---|--|--|
| Lake 24 | RT | - | - | - |
| | BT | 123.6 ± 20.9 | 0.941 ± 0.002 | 38 ± 1 |
| Lake 44 | RT | 135.8 ± 34.5 | 0.938 ± 0.002 | 38 ± 1 |
| | BT | 117.6 ± 19.4 | 0.941 ± 0.002 | 39 ± 1 |
| Lake C4 | RT | 139.6 ± 23.1 | 0.938 ± 0.002 | 37 ± 1 |
| | BT | 101.4 ± 18.1 | 0.941 ± 0.002 | 39 ± 1 |
| Luscar Lake | RT | 110.5 ± 12.9 | 0.937 ± 0.002 | 39 ± 1 |
| | BT | 129.8 ± 19.5 | 0.940 ± 0.002 | 40 ± 1 |

¹No significant differences observed (2-way ANOVA: $F_{6, 105} = 0.91$; $p = 0.4949$).

²No significant difference observed (2-way ANOVA: $F_{6, 500} = 1.19$; $p = 0.3063$).

³No significant difference observed (2-way ANOVA: $F_{6, 500} = 0.39$; $p = 0.8872$).

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