# Effects of acute and subchronic exposures to waterborne selenite on the physiological stress response and oxidative stress indicators in juvenile rainbow trout

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## Abstract

Selenium (Se) is an essential element that may bioaccumulate to toxic levels. In fish, the major toxicity symptom is larval teratogenic deformities, but little is known about the effect of Se on other systems such as the physiological stress response and oxidative stress. To test the hypothesis that Se is a chemical stressor that causes toxicity through oxidative stress, juvenile rainbow trout were exposed to waterborne sodium selenite, and physiological stress response and stress-related parameters (plasma cortisol, glucose, T3 and T4, gill $Na^+/K^+$-ATPase, the ability of the head kidney to secrete cortisol, and condition factor) and hepatic oxidative stress indicators (reduced glutathione, glutathione peroxidase, and lipid peroxidation) were measured after 96 h (acute exposure to 0–2.67 mg/L Se) and 30 days (sub-chronic exposure to 0–0.16 mg/L).

Acute exposure to waterborne sodium selenite significantly increased plasma cortisol levels (control = $0.01 \pm 0.0$ ng/mL, and 2.52 mg/L Se = $73.5 \pm 22$ ng/mL) and plasma glucose levels (control = $0.75 \pm 0.1$ mg/mL, and 3.60 mg/L Se = $1.64 \pm 0.2$ mg/mL), but gill $Na^+/K^+$-ATPase activities, plasma T3 and T4 levels, and condition factor were unchanged. The 96 h acute selenite exposure decreased hepatic reduced glutathione levels (control = $18.4 \pm 1.5$ μmol/mg protein, and 3.60 mg/L Se = $12.4 \pm 1.1$ μmol/mg protein). Lipid peroxidation levels (0.03–0.08 U/mg protein) and glutathione peroxidase (3.7–6.0 mU/mg protein) activities significantly varied with treatment. The 30 days sub-chronic exposure increased plasma cortisol, T3, and T4, but there was no effect on plasma glucose levels, gill $Na^+/K^+$-ATPase activity, the ability to secrete cortisol, and condition factor. The 30 days sub-chronic exposure to selenite did not alter antioxidant activities or lipid peroxidation levels.

These experiments show, for the first time, that exposure to waterborne selenite up to 0.1 mg/L, activates the physiological stress response in fish but does not impair cortisol secretion after 30 days. The decrease in reduced glutathione in juvenile rainbow trout subjected to the acute sodium selenite exposure suggests that oxidative stress may play an important role in the effects of Se in fish.
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## 1. Introduction

Selenium (Se) is an essential element that bioaccumulates and becomes toxic at concentrations slightly above the homeostatic requirement (Bowie et al., 1996). High concentrations can be found in soil derived from black shales and phosphate rocks (Haygarth, 1994); major anthropogenic sources of Se include coal and uranium mine runoff, coal fired power plant fly ash, irrigation drainwater and fossil fuel processing operations (Lemly,

1999). Selenium is a constituent of the enzyme antioxidant glutathione peroxidase and of deiodinase, the enzyme required for the conversion of thyroxine (T4) to triiodothyronine (T3), the more active form of the hormone (Köhrle et al., 2005). It is also a component of thioredoxin reductase, which is involved in DNA synthesis, oxidative stress defence, and protein repair (Arnér and Holmgren, 2000). In fish, deficiency symptoms include growth depression (Watanabe et al., 1997), abnormal swimming patterns (Bell et al., 1986), and liver and muscle degeneration (Hilton et al., 1980).

The toxicity of Se varies among teleost fish species, the forms or species of Se, and the life stages of fish. Selenium can exist in four oxidation states or species, each with different chemi-

cal, biological and toxicological properties (Maier and Knight, 1994). The 96 h sodium selenite ($Na_2SeO_3$, +4 oxidation state) $LC_{50}$ for juvenile rainbow trout ranges from 4.2 to 9.0 mg/L, depending on the exposure system (Hodson et al., 1980; Buhl and Hamilton, 1991). Selenate (+6 oxidation state) is less toxic to the same species, with the 96 h $LC_{50}$ ranging from 32 to 47 mg/L for rainbow trout (Buhl and Hamilton, 1991).

The most significant effect of excess Se in fish is the accumulation of Se in eggs and subsequent larval deformities (Lemly, 1997, 2002; Holm et al., 2005; Muscatello et al., 2006). Other documented effects in fish include skin lesions, cataracts, swollen gill filament lamellae, myocarditis, and liver and kidney necrosis (Lemly, 1997, 2002; Lohner et al., 2001). Three major mechanisms have been suggested for Se toxicity: (1) substitution of Se for sulphur during the assembly of proteins, (2) inhibition of Se methylation metabolism resulting in hydrogen selenide accumulation, and (3) membrane and protein damage from a Se generated reactive oxygen species (ROS) (Spallholz et al., 2004). Most Se toxicity studies have focused on the reproductive effects, but the effects of Se on other systems, such as the physiological stress response (PSR), have not yet been investigated in fish.

The PSR enables fish to maintain the internal homeostasis that is required for survival, growth and reproduction in a changing environment. It consists of primary endocrine responses (secretion of ACTH, cortisol, and catecholamines), secondary metabolite and tissue responses, including an increase in plasma glucose, and tertiary or whole organism responses (Vijayan et al., 1988; Barton, 2002). Cortisol promotes protein degradation and glycogen deposition in the liver, it increases gill $Na^+/K^+$-ATPase activity, and suppresses the immune system, sex steroid secretion, and gonad maturation (Hontela, 1997). Interactions between the interrenal and thyroid axes also occur (Vijayan et al., 1988; Walpita et al., 2007). Many toxicants activate the PSR; however, chronic exposures may also exhaust or impair the capacity to secrete cortisol (Hontela, 2005; Vijayan et al., 2005). Acute exposure to Se increased cortisol and corticosterone levels in rats (Potmis et al., 1993) but it decreased corticosteroid levels in gray seals, *Halichoerus grypus* (Freeman and Sangalang, 1977) and eiders, *Somateria mollissima borealis* (Wayland et al., 2002). The effects of excess Se on the PSR have not been firmly established in teleosts. However, it is known that Se is a constituent of glutathione peroxidase (GPx), a key enzymatic antioxidant (Köhrle et al., 2005) and that some contaminants impair the PSR through oxidative stress (Dorval et al., 2003).

Oxidative stress occurs when ROS overwhelm the cellular defences and damage proteins, membranes, and DNA (Kelly et al., 1998). Reactive oxygen species are by-products of electron transport chains, enzymes and redox cycling (Kelly et al., 1998) and their production may be enhanced by xenobiotics (Winston and Di Giulio, 1991). Reactive oxygen species are removed by cellular defences such as GPx, superoxide dismutase, catalase (CAT), reduced glutathione (GSH), Vitamin E, and carotenoids (Kelly et al., 1998). However, when ROS become too numerous, damage to membranes, proteins, and DNA results. Selenium can both cause and defend against oxidative stress. It is required for GPx, an enzyme that removes organic peroxides

(Kelly et al., 1998), but it can also generate ROS (Spallholz et al., 2004).

The objectives of this study were to: (1) determine the effect of selenite on the PSR and stress-related responses (as measured by plasma cortisol, glucose, and thyroid hormones T3 and T4, gill $Na^+/K^+$-ATPase activity, the ability of the head kidney to secrete cortisol, and condition factor), (2) determine if selenite causes oxidative damage (as measured by LPO) or changes the antioxidant status (as measured by hepatic GPx activity, and GSH reserves), and (3) test the hypothesis that Se is a chemical stressor that causes toxicity through induction of oxidative stress.

## 2. Materials and methods

### 2.1. Chemicals

Sodium selenite ($Na_2SeO_3$), sucrose, EDTA, imidazole-HCl, NaCl, KCl, oubain, $Na_2ATP$, $MgCl_2 \cdot 6H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $H_2SO_4$, potassium phosphate ($KH_2PO_4$, Tris–HCl, 2-mercaptoethanol, metaphosphoric acid, butylated hydroxytoluene (BHT), $H_2O_2$, GSH, NADPH, GSH reductase, $NaN_3$, porcine ACTH I-39, MEM, bovine serum albumin, $NaHCO_3$, and Bradford reagent were purchased from Sigma–Aldrich (Oakville, Ontario). MS-222 was purchased from MPBiomedicals (Solon, Ohio). Ultra pure nitric acid was purchased from Fischer Scientific (Ottawa, Ontario).

### 2.2. Fish and exposures

Animal-use protocols have been approved by the University of Lethbridge Animal Care Committees in accordance with national guidelines. Juvenile rainbow trout, *Oncorhynchus mykiss* (avg. weight = $18.1 \pm 1.2$ g) were obtained from the Allison Creek Provincial Hatchery (Blairmore, Alberta). Fish were kept in a 1000 L tank at 14 °C (semi-static system, 8% daily water renewal, 7 mg/L oxygen) for a minimum of 2 weeks and then moved to the experimental exposure tanks (50 L, semi-static system, 25% daily water renewal, 12 °C) and allowed 7 days to acclimate before exposures were begun. Fish were fed extruded floating steelhead food (Nelson's Silver Cup Fish Feed, Allison Creek Provincial Hatchery, Blairmore, Alberta) between 900 and 1000 h ad libidum.

*Acute exposure*: Fish were exposed to waterborne sodium selenite ($Na_2SeO_3$) at 0%, 10% (0.72 mg/L Se), 25% (1.80 mg/L Se), 35% (2.52 mg/L Se), and 50% (3.60 mg/L Se) of the 96 h $LC_{50}$ (7.2 mg/L) for juvenile rainbow trout (Hodson et al., 1980). Sodium selenite was chosen because it is more toxic to rainbow trout than the selenate form of Se. Eight fish were randomly assigned to each tank; temperature, dissolved oxygen, pH, and conductivity were monitored daily. Water samples for total Se analysis were also collected daily and then analysed as described below. After 96 h all fish were sacrificed and sampled (see Section 2.3).

*Subchronic exposure*: Fish were exposed to waterborne sodium selenite for 30 days at 0%, 1% (0.07 mg/L Se), and 5% (0.36 mg/L Se) of the 96 h $LC_{50}$. Twenty-four fish were

randomly assigned to each treatment; each treatment consisted of three tanks with eight fish per tank. More fish were used in the subchronic exposure than the acute exposure, as recommended for longer term studies (Hontela, 1997). Water samples for total Se analysis were collected twice a week and analysed as described below. On days 8 and 22, all fish were lightly anesthetised with MS-222 (0.1 g/L), a blood sample was taken from the caudal vasculature for cortisol and glucose analyses, and fish were returned to their respective tanks. After 30 days all fish were sacrificed and sampled (see Section 2.3).

*Water Se analysis*: Unfiltered water samples were collected in acid washed bottles and composites of the daily water samples collected from each tank in the acute exposure were acidified with 0.5% ultra pure nitric acid and analysed for total Se at the Ultra-Clean Trace Elements Laboratory, University of Manitoba. Weekly composites (20 mL) were similarly analysed for each tank in the subchronic exposure. Total Se was measured by inductively coupled plasma-mass spectrometry (ICP-MS) on an Elan DRC-II ICP-MS with $CH_4$ as the reaction gas. The method has a detection limit of 0.01 μg/L. NIST 1640 (NIST, USA) and TM-Rain 95 (Environment Canada) were used as the certified reference materials for the Se analysis. Further QA/QC was done through the Ecosystem Proficiency Testing QA Program of Environment Canada.

### 2.3. Somatic indices and biochemical analyses

Fish were removed from tanks and immediately anesthetised in MS-222 (0.1 g/L). Fork length, weight, and liver weight were recorded to calculate the condition factor ($K$ = (weight (g) × 100)/length $(cm)^3$) and liver somatic index (LSI = liver weight (g)/body weight (g) × 100). Blood samples were taken from the caudal vasculature and fish were sacrificed by a spinal transection. Whole blood was centrifuged at $16,000 \times g$ for 5 min to obtain plasma which was then flash frozen in liquid nitrogen. Plasma and tissues were stored at $-80\,°C$ until analysis.

#### 2.3.1. Plasma cortisol, glucose, T3 and T4

Cortisol (Catalogue #07-221102), T3 (Catalogue #06B-254215), and T4 (Catalogue #06B-254011) were measured with radioimmunoassay kits purchased from Medicorp (Montréal, Québec). Assay characteristics, including intra- and inter-assay variability, were assessed with internal standards, as previously described in Levesque et al. (2003). Glucose was determined by incubating plasma samples (60 min, 23 °C) with the GOD-PAP reagent (Roche Diagnostic, Laval, Québec) and measuring the absorbance at 510 nm.

#### 2.3.2. Gill $Na^+/K^+$-ATPase activity

$Na^+/K^+$-ATPase activity, expressed as μmol $PO_4$ liberated per mg of protein in a gill homogenate, was measured by liberating $PO_4$ from a hydrolysis reaction with ATPase, as described previously (Morgan et al., 1997; Levesque et al., 2003). Gills were homogenized in a buffer (pH 7.2) containing 250 mM sucrose, 6 mM EDTA, and 20 mM imidazole-HCl. The homogenate was incubated (10 min,

30 °C) in a medium without oubain, an ATPase inhibitor, (167 mM NaCl, 50 mM KCl, 33 mM imidazole-HCl, pH 7.2) and with oubain (237 mM NaCl, 1.67 mM oubain, 33 mM imidazole-HCl, pH 7.2). Phosphate was liberated during an incubation period (30 min at 30 °C) with ATP (25 mM $Na_2$ATP, 50 mM $MgCl_2 \cdot 6H_2O$). Phosphate forms a blue complex with Bonting solution (8.1 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 176 mM $FeSO_4 \cdot 7H_2O$, 560 mM $H_2SO_4$) during a 20 min incubation at room temperature, and was measured with a spectrophotometer (700 nm). $Na^+/K^+$-ATPase activity is expressed as μmol $PO_4$ liberated per mg of protein. Protein was measured using a spectrophotometric assay (595 nm) and the Bradford reagent.

#### 2.3.3. Oxidative stress parameters

A portion of the liver was homogenized in 10 volumes of 50 mM potassium phosphate buffer with 1 mM EDTA, pH 7.4 for GSH, and LPO determination. A second portion of liver was homogenized in 10 volumes of 50 mM Tris–HCl buffer with 5 mM EDTA and 1 mM 2-mercaptoethanol for GPx determination. Metaphosphoric acid was added to a portion of the first homogenate for GSH analysis (final concentration 0.209 M) and BHT was added to a portion of the first homogenate for LPO analysis (final concentration 5 mM). Homogenates were centrifuged at 4 °C at $3270 \times g$ for 20 min and supernatants removed. GSH was assayed within an hour, and supernatants for GPx and LPO were kept at $-80\,°C$ for a maximum of 2 days before analysis.

*GSH*: Reduced glutathione was determined using the Bioxytech GSH 400 kit (Catalogue #21011) purchased from Medicorp (Montréal, Québec). GSH forms a thioether that reacts with a reagent forming a thione which was measured on a UV–vis absorption spectrophotometer at 400 nm. GSH was expressed as μM GSH per mg protein.

*GPx*: Total cellular GPx activity was determined by measuring the decrease in absorbance (340 nm) due to the decline in NADPH at 23–25 °C (Lorentzen et al., 1994). The sample (70 μL) was added to 350 μL phosphate buffer, 350 μL 0.25 mM hydrogen peroxide and 350 μL of a reagent: the reagent contained 1 mM GSH, 0.2 mM NADPH, 1 unit GSH reductase and 2 mM sodium azide in a phosphate buffer (described previously). Absorbance was monitored for 3 min at 340 nm. The activity of GPx was expressed as mU/mg protein and 1 mU was defined as 1 nmol of NADPH consumed/min/mL of sample.

*LPO*: Lipid peroxidation was determined using the Bioxytech LPO-596 kit (Catalogue #21012) purchased from Medicorp (Montréal, Québec). The kit measured the reaction of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HA), end products of the LPO process, with *n*-methyl-2-phenylindole at 45 °C and 586 nm. LPO is expressed as U/mg protein, where one unit was one μM MDA and 4-HA.

#### 2.3.4. Adrenocortical challenge

The ability of fish to secrete cortisol was tested by stimulating cortisol production in a coarse head kidney homogenate with ACTH in vitro, as described previously (Levesque et al., 2003). The homogenate was preincubated at 15 °C for 2 h to remove any cortisol secretion due to handling of the fish, then the incu-

bation medium was either replaced with MEM (basal cortisol secretion), or with 2 units of ACTH in MEM (stimulated cortisol secretion) for each fish. The ability of the head kidney to secrete cortisol was expressed as the change in cortisol secretion (ng/mL) after stimulation with 2 units of ACTH (stimulated cortisol–basal cortisol).

## 2.4. Statistical analysis

Data were analyzed using JMP IN 5.1.2 (1989–2004 SAS Institute Inc.). Data that were not normally distributed were log transformed to respect normality. Treatment groups were compared using a one-way ANOVA and a Tukey–Kramer HSD. If data could not be transformed to normality a Kruskal–Wallis test was used to compare treatment groups, followed by multiple post hoc Wilcoxon tests with the Bonferroni correction to compare each exposure group to the control. The change in plasma cortisol and glucose levels in the subchronic exposure were calculated (level at day 30–level at day 8) and the treatment groups were compared as discussed above. Samples below the detection limit (0.01 ng/mL) of the radioimmunoassays were entered as 0.01 ng/mL. All the statistical tests use $\alpha = 0.05$ unless otherwise stated.

## 3. Results

### 3.1. Exposure characteristics

Temperature, dissolved oxygen, pH, conductivity, and Se water concentrations during exposures are presented in Table 1. The observed water Se concentrations during the acute 96 h exposure were similar to nominal values, although the concentration for the group exposed to 3.60 mg/L of Se was slightly higher than expected (Table 1). Measured selenium exposures ranged from 0.00 mg/L (control) to 2.67 mg/L (nominal 3.60 mg/L Se exposure).

Water quality parameters in the 30 days subchronic sodium selenite exposures were similar to those measured in the acute exposures (Table 1). Water Se concentrations ranged from 0.00 mg/L (control) to 0.16 mg/L (0.36 mg/L Se exposure) and the observed and nominal values were very similar.

### 3.2. Physiological stress response

Plasma cortisol levels were significantly higher ($p < 0.05$) in groups exposed to 2.52 mg/L and 3.60 mg/L Se for 96 h than in the control (Fig. 1A). Plasma glucose levels (Fig. 1B) were greater ($p < 0.05$) than the control group in the 96 h exposures to 1.80 and 3.60 mg/L, with the same trend in the 2.52 mg/L group, although the latter was not statistically different. Gill $Na^+/K^+$-ATPase (Table 2), plasma T3 (mean of all treatments $= 2.23 \pm 0.36$ ng/mL plasma, data not shown), T4 (mean of all treatments $= 23.04 \pm 2.40$ ng/mL plasma, data not shown), and T3/T4 (mean of all treatments $= 0.11 \pm 0.02$, data not shown) were not significantly influenced by a 96 h exposure to sodium selenite. Condition factor and LSI (Table 2) were also not significantly affected by the acute sodium selenite exposure.
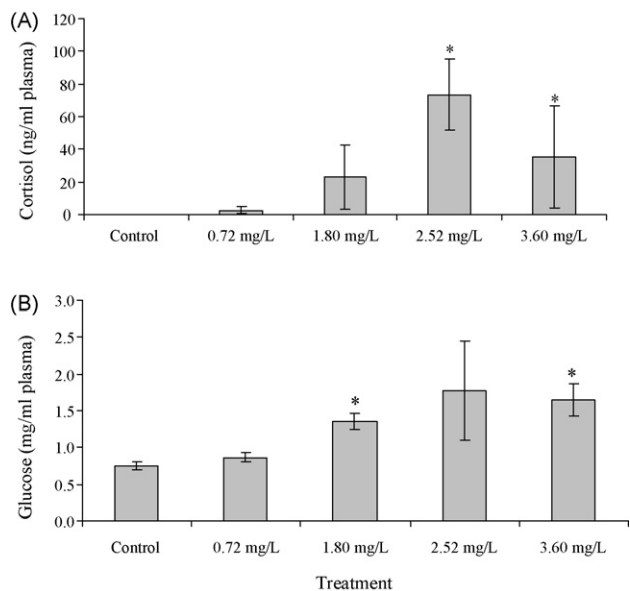


Fig. 1. Plasma cortisol (A) and plasma glucose (B) of juvenile rainbow trout (mean ± S.E.) exposed for 96 h to waterborne sodium selenite ($Na_2SeO_3$), $n = 8$. *Significant difference from the control group (Kruskal–Wallis).

After the 30 days (subchronic) sodium selenite exposure, plasma cortisol levels were higher ($p < 0.05$) in the group exposed to 0.36 mg/L Se than to 0.07 mg/L (Fig. 2A). The change in plasma cortisol between day 30 and day 8 was sig-
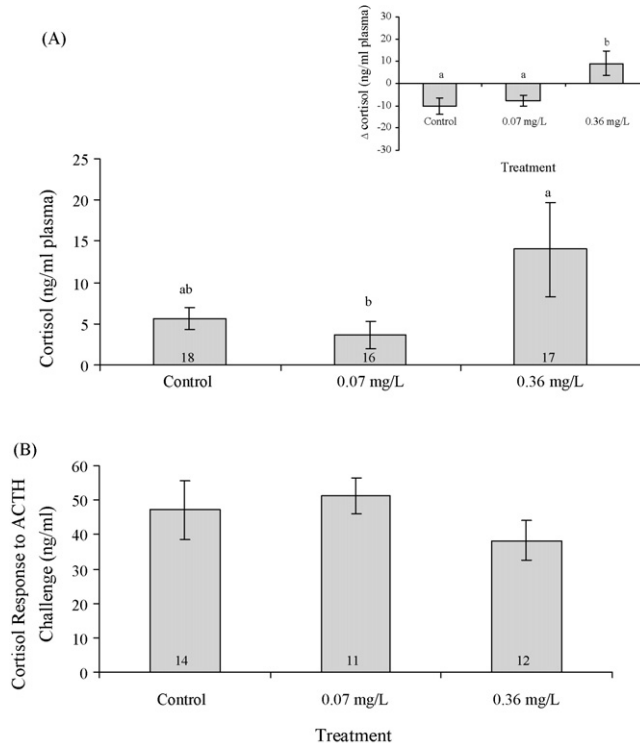


Fig. 2. Plasma cortisol levels and △cortisol (in the inset graph) expressed as change in plasma cortisol levels between day 30 and day 8 (A) and the ability of the head kidney to secrete cortisol (B) in juvenile rainbow trout (mean ± S.E.) exposed for 30 days to waterborne sodium selenite ($Na_2SeO_3$). Groups with different letters are significantly different (Kruskal–Wallis) in A. No significant differences were observed in B (ANOVA). Sample size indicated in the bars.

Table 1
Water quality characteristics (mean ± S.E.) and water Se concentrations for the 96 h and 30 days sodium selenite ($Na_2SeO_3$) exposures

| Experiment | Treatment | Temperature[a] (°C) | Oxygen[a] (mg/L) | pH[a] | Conductivity[a] ($\mu S$) | Nominal $Na_2SeO_3$ (mg/L) | Nominal Se (mg/L) | Measured Se[b,c] (mg/L) |
|---|---|---|---|---|---|---|---|---|
| 96 h (acute) | Control | 13.0 ± 0.1 | 6.8 ± 0.3 | 7.9 ± 0.16 | 318 ± 42 | 0.00 | 0.00 | 0.00 |
| | 10% $LC_{50}$ | 13.4 ± 0.2 | 6.5 ± 0.5 | 8.0 ± 0.06 | 293 ± 1.9 | 0.72 | 0.33 | 0.39 |
| | 25% $LC_{50}$ | 13.3 ± 0.3 | 6.5 ± 0.2 | 7.9 ± 0.10 | 310 ± 41 | 1.80 | 0.82 | 0.99 |
| | 35% $LC_{50}$ | 12.2 ± 0.3 | 7.6 ± 0.5 | 7.9 ± 0.27 | 323 ± 60 | 2.52 | 1.15 | 1.15 |
| | 50% $LC_{50}$ | 12.4 ± 0.5 | 7.5 ± 0.8 | 7.5 ± 0.40 | 284 ± 1.2 | 3.60 | 1.64 | 2.67 |
| 30 days (subchronic) | Control | 12.6 ± 0.1 | 9.3 ± 0.4 | 7.6 ± 0.04 | 276 ± 6.1 | 0.00 | 0.00 | 0.00 |
| | 1% $LC_{50}$ | 12.2 ± 0.1 | 8.7 ± 0.3 | 7.7 ± 0.03 | 287 ± 7.5 | 0.07 | 0.03 | 0.05 |
| | 5% $LC_{50}$ | 11.9 ± 0.1 | 8.5 ± 0.2 | 7.7 ± 0.03 | 290 ± 2.0 | 0.36 | 0.16 | 0.16 |

[a] $n = 4$.
[b] Composite of daily water samples for acute exposure ($n = 3$).
[c] Composite of weekly (2 samples/week) water samples for subchronic exposure ($n = 4$).

Table 2
Somatic indices and gill $Na^+/K^+$-ATPase activity (mean ± SE) of juvenile rainbow trout exposed for 96 h or 30 days to waterborne sodium selenite ($Na_2SeO_3$)

| Experiment | Treatment (mg/L Se) | Sample size | Condition factor[a] | Liver somatic index[b] | Gill $Na^+/K^+$-ATPase[c] |
|---|---|---|---|---|---|
| 96 h (acute) | Control | 8 | 1.11 ± 0.03 | 1.21 ± 0.12 | 1.15 ± 0.33 |
| | 0.72 | 8 | 1.14 ± 0.08 | 1.19 ± 0.16 | 1.66 ± 0.32 |
| | 1.80 | 8 | 1.16 ± 0.02 | 1.78 ± 0.13 | 1.83 ± 0.24 |
| | 2.52 | 8 | 1.09 ± 0.04 | 1.20 ± 0.16 | 1.98 ± 0.42 |
| | 3.60 | 8 | 1.09 ± 0.04 | 1.21 ± 0.17 | 2.27 ± 0.14 |
| 30 days (subchronic) | Control | 21 | 1.10 ± 0.02 | 1.14 ± 0.04 | 0.76 ± 0.19 |
| | 0.07 | 19 | 1.10 ± 0.02 | 1.17 ± 0.05 | 0.92 ± 0.13 |
| | 0.36 | 18 | 1.10 ± 0.02 | 1.11 ± 0.05 | 0.72 ± 0.16 |

No significant differences were observed.
[a] $K = w_f \times 100/l^3$, where $K$ is condition factor, $w_f$ the final weight and $l$ is the fork length.
[b] $LSI = lv/w_f \times 100$, where LSI is the liver somatic index, lv the liver weight and $w_f$ is the final weight.
[c] Activity expressed in units (U/mg protein), where one unit is 1 $\mu$mol of liberated $PO_4^{3-}$.

nificantly greater ($p < 0.05$) in the 0.36 mg/L exposure than the other two treatment groups (Fig. 2A inset). Plasma cortisol in the control and 0.07 mg/L groups decreased over the 30 days, but increased in the 0.36 mg/L group. The ability of the head kidney to respond to an ACTH challenge did not change significantly with exposure to sodium selenite for 30 days (Fig. 2B). There were no significant differences between treatment groups in plasma glucose levels after 30 days (mean of all treatments = 0.78 ± 0.01 ng/mL plasma, data not shown) or in the change of plasma glucose levels (mean of all treatments = 0.13 ± 0.04 ng/mL plasma, data not shown). Sodium selenite did not significantly change gill $Na^+/K^+$-ATPase activity after 30 days (Table 2). The group exposed to 0.36 mg/L of sodium selenite had significantly higher plasma T3 and T4 levels ($p < 0.05$) than the control on day 30 of the subchronic exposure (Fig. 3); however, the T3/T4 ratio was not significantly influenced by treatment (mean of all treatments = 0.19 ± 0.01, data not shown). Condition factor and LSI were not different among treatment groups (Table 2), indicating that all the fish, including those exposed to selenium, were feeding.

### 3.3. Oxidative stress biomarkers

Hepatic oxidative stress biomarkers were modified by acute (96 h) sodium selenite exposure. Lipid peroxidation levels were

significantly ($p < 0.05$) lower in the group exposed to 2.52 mg/L sodium selenite than in the control group (Table 3). Rainbow trout had significantly less hepatic GSH reserves ($p < 0.05$) in the 2.52 mg/L and 3.60 mg/L exposures compared to the 1.80 mg/L treatment (Fig. 4). Hepatic GPx activity ($p < 0.05$) was signif-
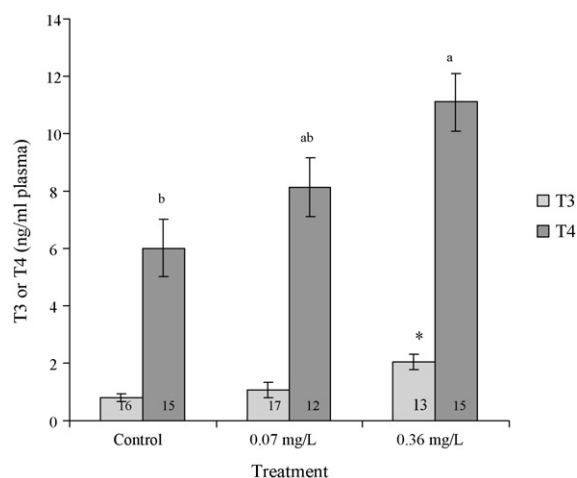


Fig. 3. Plasma T3 and T4 levels (mean ± S.E.) in juvenile rainbow trout exposed to 30 days of waterborne sodium selenite ($Na_2SeO_3$). *Significant difference from the control (Kruskal–Wallis), groups with different letters are significantly different (ANOVA). Sample size indicated in bars.

Table 3
Oxidative stress biomarkers (mean ± S.E.) in juvenile rainbow trout exposed for 96 h or 30 days to waterborne sodium selenite (Na$_2$SeO$_3$)

| Experiment | Treatment (mg/L Se) | GSH[a] (μmol/mg protein) | GPx[b] (mU/mg protein) | LPO[c] (U/mg protein) |
|---|---|---|---|---|
| 96 h (acute)[d] | Control | | 4.88 ± 0.50a | 0.08 ± 0.004 |
| | 0.72 | | 3.74 ± 0.86ab | 0.07 ± 0.011 |
| | 1.80 | | 5.99 ± 1.37a | 0.08 ± 0.003 |
| | 2.52 | | 2.27 ± 0.66b | 0.03 ± 0.006[*] |
| | 3.60 | | 5.50 ± 1.70ab | 0.05 ± 0.009 |
| 30 days (subchronic)[e] | Control | 25.75 ± 2.09 | 4.07 ± 0.62 | 0.05 ± 0.006 |
| | 0.07 | 23.59 ± 2.03 | 4.16 ± 0.88 | 0.05 ± 0.005 |
| | 0.36 | 27.81 ± 2.86 | 3.32 ± 0.62 | 0.06 ± 0.005 |

[a] Refer to Fig. 4 for the 96 h exposure GSH results.

[b] 1 mU is 1 nmol NADPH consumed per minute; groups with different letters (a and b) are significantly different (ANOVA).

[c] 1 U is 1 μmol MDA and 4-HA; [*]significant difference from the control (Kruskal–Wallis).

[d] Number of fish per group, $n = 5$–8.

[e] Number of fish per group, $n = 9$–12; no significant differences observed.

icantly lower in the group exposed to 2.52 mg/L selenite than in the control (Table 3). Hepatic oxidative stress parameters did not significantly change (Table 3) with 30 days of subchronic sodium selenite exposure.

## 4. Discussion

An important feature of the present study was the use, during the subchronic (30 days) exposures, of Se water concentrations that approach environmental exposures. Streams receiving runoff from coal mines in northern Alberta contain 0.001–0.032 mg/L Se and developmental deformities have been observed in the resident rainbow trout population (Holm et al., 2005). In lentic systems, reproductive effects have been documented in fish exposed to water Se concentrations as low as 0.005 mg/L (Lemly, 1999). The lowest Se exposure achieved in this study was 0.05 mg/L. This concentration approaches the Se levels measured in water in coal mine-impacted streams where reproductive effects have been documented; therefore, the Se concentrations used in our subchronic laboratory study are environmentally relevant.

Acute (96 h) exposure to sodium selenite activated the PSR in juvenile rainbow trout. It raised plasma cortisol and plasma glucose levels; however, other stress-related responses (gill



Fig. 4. GSH concentrations in the liver of juvenile rainbow trout (mean ± S.E.) exposed for 96 h to waterborne sodium selenite (Na$_2$SeO$_3$). Groups with different letters are significantly different (ANOVA). Sample size indicated in the bars.

Na$^+$/K$^+$-ATPase activity and plasma T3 and T4 levels), including tertiary responses (condition factor and LSI) were not affected. Numerous other studies have reported that acute exposures to contaminants such as crude oil (Kennedy and Farrell, 2005), and copper or cadmium (Pelgron et al., 1995) increase plasma cortisol in fish. However, the present study provides the first evidence that elevated Se activates the PSR in fish and increases plasma cortisol. A similar response to Se was reported in male rats (Potmis et al., 1993), but Se decreased plasma corticosterone levels in gray seals (Freeman and Sangalang, 1977) and female eiders (Wayland et al., 2002).

The subchronic (30 days) exposure to sodium selenite also activated the PSR. Plasma cortisol levels increased significantly in the 0.36 mg/L exposure group, although the acute exposure raised cortisol higher. This indicates exposures to waterborne Se between 1.15 and 2.67 mg Se/L for 96 h are more stressful to the fish than 0.16 mg Se/L for 30 days. Plasma T4 and T3 levels increased in a exposure-dependent pattern, but the other stress indicators (plasma glucose levels, gill Na$^+$/K$^+$-ATPase activity), and the tertiary responses (condition, LSI) were not altered. In chronic exposures to contaminants, an increase in plasma cortisol is generally followed by a decrease as the fish acclimates (Barton, 2002) or, as has been reported for contaminants such as Cd, the cortisol secretory response becomes impaired (Hontela, 1997). In the present study, plasma cortisol increased between day 8 and day 30 in the highest exposure (0.36 mg/L) group, but it decreased in the control and 0.07 mg/L groups. This pattern suggests Se is still activating the stress response after 30 days and the fish did not acclimate to Se before the end of the exposure. Our results also indicate that the interrenal cells were not impaired by the 30 days exposure, since the ability of the head kidney to secrete cortisol in response to ACTH stimulation in vitro was not significantly altered by Se exposure in vivo.

The present study provided evidence, for the first time, that subchronic exposures to waterborne Se increase plasma T3 and T4 levels. Exposure to Se increased plasma cortisol and it has been documented that cortisol influences thyroid hormone metabolism (Brown et al., 1991). Moreover, Se is an integral part of the deiodinase enzymes involved in thyroid hormone synthesis (Köhrle et al., 2005). Our data show that a 30 days exposure
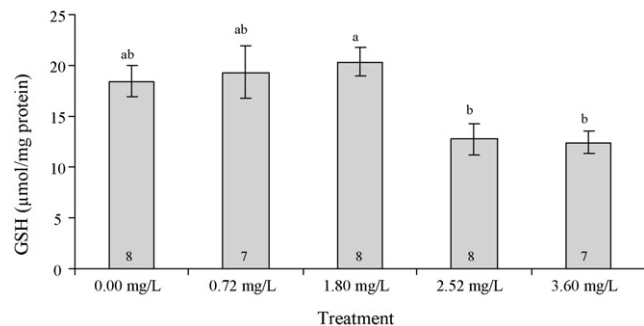
to 0.16 mg/L (5% LC50) of sodium selenite alters the thyroid status in rainbow trout; however, the relationship between availability of selenium, the activity of the deiodinase enzyme and thyroid status in fish is not understood at present.

The links between physiological effects of Se exposure and oxidative stress were investigated in the present study. In the acute exposure, sodium selenite decreased liver LPO in the two highest exposure groups, although only the group exposed to 3.60 mg/L was significantly lower than the control group. These results were unexpected as most contaminants including metals (Roberts and Oris, 2004), pulp and paper effluent (Oakes et al., 2004), agricultural runoff (Dorval et al., 2005), copper (Berntssen et al., 2000), and hydrocarbons (Di Giulio et al., 1993) increase LPO in fish tissues. Orun et al. (2005) did report that exposure for 72 h to 2–6 mg/L sodium selenite decreased, compared to controls, malondialdehyde levels (indicator of oxidative damage) in the liver, as well as other organs, in rainbow trout. Lipid peroxidation may decrease with increased Se exposure because Se is a constituent of the antioxidants GPx and thioredoxin reductase (Steinbrenner et al., 2006), and at lower levels protects fish from oxidative damage. In the present study, both LPO and GPx activity were significantly lower, compared to control, in the group exposed to 2.52 mg/L of selenite, and both increased (towards control levels) at the higher selenite exposure. The effect of Se on GPx activity, and the link to LPO must be further investigated, to understand the role of Se in oxidative stress. Selenium had an effect on hepatic antioxidant levels in rainbow trout exposed for 96 h; it decreased liver GSH concentrations in the two highest exposure groups. There is substantial evidence that GSH is a powerful antioxidant and antitoxicant as it binds to many different toxicants, inactivating them (Kelly et al., 1998). Chronic exposure to Se has been shown to deplete GSH and decrease the ratio of GSH to oxidized GSSG in rainbow trout fed Se-methionine in the laboratory (Holm, 2002). The GSH reserves also decreased with exposure to endosulfan (Dorval et al., 2003), agricultural runoff (Dorval et al., 2005), copper (Ahmad et al., 2005), cadmium (Berntssen et al., 2000), and mercury (Payne et al., 1998).

In addition to acting as an antioxidant, GSH may also act as a prooxidant in the presence of Se. Reduced glutathione has been shown to react with methylselenol (a Se metabolite), generating Se-ROS in rainbow trout embryos (Palace et al., 2004). Reduced glutathione's ability to act as a prooxidant or an antioxidant has also been identified in human hepatoma cells (Shen et al., 2000). Damage indicators (LPO, protein peroxidation, DNA damage) should increase if GSH and Se produce ROS in the liver; however, in the present study LPO decreased in the 2.52 mg/L exposure group, but not in the higher Se exposure group. Whether this type of dose response (suggesting a U shaped curve) results from GSH acting as an antioxidant inactivating Se, then at higher exposures (3.6 mg/L) acting as a prooxidant generating ROS in conjunction with Se, warrants further investigation.

The pattern observed for LPO was also evident for GPx activity, suggesting the effects of Se may be concentration-dependent. As with LPO, GPx activity was significantly lower in fish exposed to 2.52 mg/L sodium selenite, increasing in the group

exposed to 3.36 mg/L. Orun et al. (2005) reported an increase in hepatic GPx in rainbow trout at exposures over 4 mg/L sodium selenite. Other studies where GPx activity was measured in fish suggest that the response of GPx is toxicant-dependent, with some toxicants (e.g. β-naphtholflavone or copper) increasing the activity (Ahmad et al., 2005; Sanchez et al., 2005), and others (dichlorophenol or agricultural runoff) decreasing it (Zhang et al., 2005; Dorval et al., 2005). The particular pattern of the effects of Se reported in the present study requires further investigation to understand the interactions between GSH, LPO and GPx in teleosts, and to identify the threshold at which Se ceases to be an essential element and becomes a toxicant.

In contrast to the acute Se exposure, the subchronic exposure to sodium selenite did not alter LPO or antioxidants (GPx, GSH). The exposures may not have been high or long enough to cause oxidative stress and alter antioxidant levels and activities, or the fish may have acclimated to the Se within 30 days. Oxidative stress defences are important in the acclimation process. For example, acclimation to low levels of copper protected fish from oxidative stress by oxidizing the ferrous ion before it could produce free radicals (Pandey et al., 2001); however, at higher levels copper can cause oxidative stress (Berntssen et al., 2000). Selenium may act in a similar manner. At higher levels (similar to those present in the 96 h acute exposure) Se may begin to alter antioxidant status, but at lower levels it may protect the liver from damage. Determining acclimation patterns by measuring oxidative damage and antioxidant activity at different times during the 30 days period could not be done in this study as fish were sacrificed after 30 days.

## 5. Conclusions

This study provided evidence that sodium selenite activates the physiological stress response in fish subjected to acute (96 h) and sub-chronic (30 days) exposures, and does not impair, following the subchronic exposures, the ability of the head kidney tissue to secrete cortisol. Moreover, subchronic exposure to sodium selenite increased plasma thyroid hormone levels. The importance of oxidative stress in Se-induced responses was investigated and a decrease of hepatic GSH content was detected following acute exposures, along with a U shaped pattern for LPO and GPx activities. Future studies will examine the relationship between the Se concentration, and the antioxidant and prooxidant actions of this element in teleosts.

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