

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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ARTICLE INFO

Article history:

Received 4 February 2011
Revised 25 March 2011
Accepted 27 March 2011
Available online 3 April 2011

Keywords:

Cortisol
Adrenocortical cells
Selenomethionine
Sodium selenite
Oxidative stress
Trout

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 trout (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_2SO_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, facilitates maintenance of homeostasis when fish are exposed to stressors, including 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, and 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 salmonids, provided additional evidence for species-specific differences in sensitivity to Se which should be considered when setting thresholds and water quality guidelines.

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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 et al., 2004), have higher hepatic glutathione (GSH) reserves than brook trout (*Salvelinus fontinalis*) (Miller et al., 2009), and have lower phase I biotransformation V_{max} for sulfoxidation than channel catfish (*Ictalurus punctatus*) or tilapia (*Oreochromis* sp.) (Gonzalez 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 et al., 2003; Niyogi and Wood, 2004). Species-specific sensitivity to toxicants in fish have been also documented for pesticides (VanDolah et al., 1997; Quinn et al., 2010), pharmaceuticals (Gonzalez et al., 2009), and selenium (Holm 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 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 et al., 2010). Selenite is the most acutely toxic form of Se to fish, followed by selenate and organic selenomethionine (Se-Met). Selenium is teratogenic in fish (Coyle et al., 1993; Hamilton et al., 2005; Rigby 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

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the environment (Holm 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 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 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 metabolized to methylselenol by methioninase, then methylselenol reacts with GSH to produce the ROS (Palace et al., 2004). Thus GSH, usually acting as an antioxidant protecting cells from damage (Kelly 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 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 et al., 2005; Fuzzen 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 signaling pathway to stimulate, via the StAR protein, the uptake of cholesterol by the mitochondria (Fig. 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 signaling 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 1–39), collagenase/dispase, DNAase, minimal essential medium (MEM), bovine serum albumin (BSA), sodium bicarbonate (NaHCO_3), L-buthionine-[S,R]-sulfoximine (BSO), N-acetyl-L-cysteine (NAC), sodium chloride (NaCl), calcium chloride (CaCl_2), potassium chloride (KCl), dextrose, sodium selenite (Na_2SeO_3), selenomethionine (Se-Met), potassium phosphate (KH_2PO_4), ethanol, ethylenediaminetetraacetic acid (EDTA), metaphosphoric acid, 2,6-di-*tert*-butyl-4-methylphenol (BHT), 3-amino-1,2,4-triazole (ATA), sodium diethyldithiocarbamate

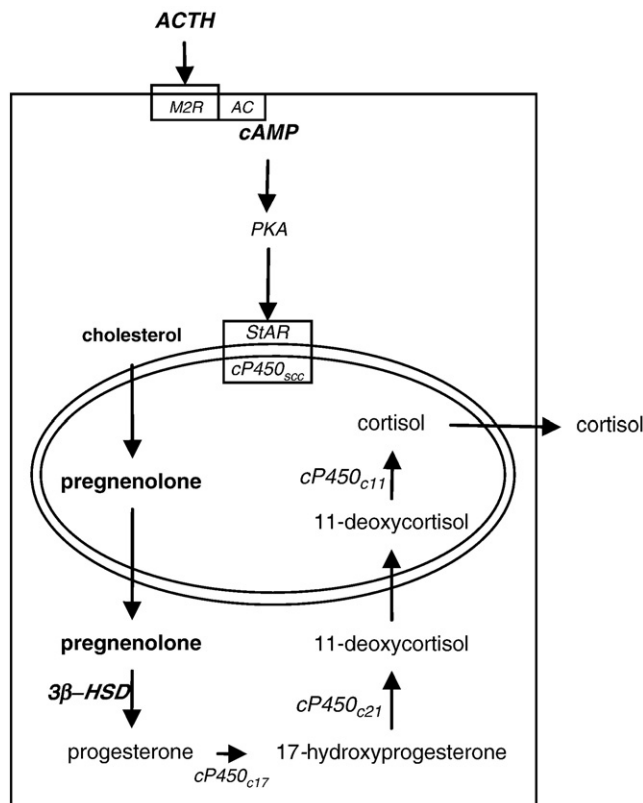


Fig. 1. Cortisol biosynthesis pathway in an adrenocortical steroidogenic cell of the teleost fish. ACTH = adrenocorticotrophic hormone, M2R = melanocortin 2 receptor, AC = adenylyl cyclase, cAMP = cyclic adenosine monophosphate, PKA = protein kinase A, StAR = steroidogenic acute regulatory protein, cP450_{sc} = 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. Compounds in bold were used in the pathway experiment.

Adapted from Hontela (2005) and Hontela and Vijayan, (2009).

trihydrate (DETCA), α-tocopherol, trypan blue, retinol, pregnenolone, $\text{N}^6,2'$ -o-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP), and OH-cholesterol were purchased from Sigma-Aldrich (Oakville, Ontario). Heps 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, *O. mykiss*, (109.9 ± 4.9 g) and juvenile brook trout, *S. 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 sacrificed 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 ~1 mm³). 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 μ L) was plated in a 96-well microplate and incubated at 15 °C. After 2 h, the microplate was centrifuged (233 \times 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, and 100 mg/L) and Se-Met (0, 30, 100, 1000, and 2000 mg/L) in Ringer solution (550 M $CaCl_2$, 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 \times 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 EC_{50} (rainbow trout = 12 mg/L; brook trout = 90 mg/L) for 1 h in Ringer. The microplate was centrifuged (233 \times 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 μ M; brook trout, 1.0 μ M), 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 μ L/well), as described in the Cell culture section above. The plate was centrifuged (233 \times 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, 1 h) to Ringer only (Control) or 12 mg/L Na_2SeO_3 (RT EC_{50}) 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; 1 h). The supernatant was collected and frozen for later cortisol analyses and the cells were washed with 50 mM KH_2PO_4 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 (μ mol GSH/mg protein) in the supernatant of half the wells for each treatment and post-exposure lipid peroxidation (LPO) was measured (μ mol malondialdehyde and 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 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 \times 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 the 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 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. 2A). The EC_{50} for BT cells was 90.49 mg/L with lower and upper confidence intervals of 40.85 mg/L and 545.65 mg/L (Fig. 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).

Table 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 adjusted	Intercept	Slope	p-value
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

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

Effect of selenomethionine on cortisol secretion *in vitro* (EC₅₀)

There were no significant relationships between cortisol secretion (probit units) and Se-Met concentrations (Fig. 3) for RT (n = 13) or BT (n = 14) adrenocortical cells (p > 0.05). Refer to Table 1 for regression parameters. The EC₅₀s could not be determined in either species, with EC₅₀ > 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: F₄ = 0.72, p = 0.5811; data not shown) or BT cells (one-way ANOVA: F₄ = 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: F₄ = 2.33, p = 0.0691; data not shown) or BT (one-way ANOVA: F₄ = 0.81, p = 0.5225; data not shown) adrenocortical cells.

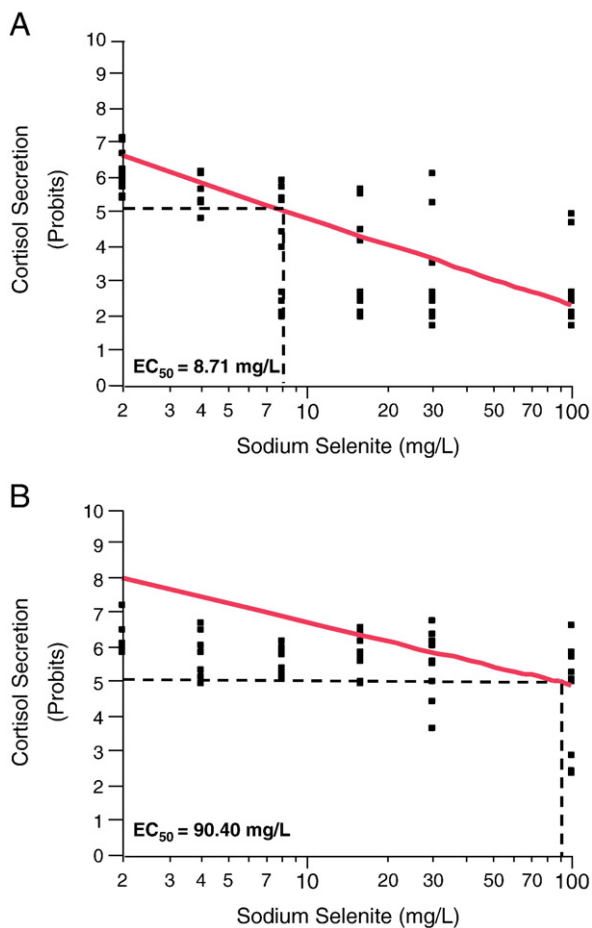


Fig. 2. The effect of sodium selenite (Na₂SeO₃) on cortisol secretion of (A) rainbow trout (n = 14) and (B) brook trout (n = 16) adrenocortical cells. EC₅₀ = concentration of toxicant that impairs 50% of ACTH-stimulated cortisol secretion. Refer to Table 2 for regression parameters. Exposure to positive control (Roundup) significantly decreased cortisol secretion, but did not alter cell viability (*t*-test, data not shown).

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 ± 6.32% of control; BT cells = 26.65 ± 5.54% of control; *t*-test: p < 0.05), but did not alter cell viability (RT cells: 100.17 ± 0.55%; BT cells = 100.06 ± 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 μM of pregnenolone stimulated cortisol secretion in RT adrenocortical cells (Table 2). Addition of OH-cholesterol (0.5 mM–2 mM) or 0.5 mM pregnenolone did not stimulate cortisol secretion (Table 2). Thus, 1 U/mL ACTH, 2 mM dbcAMP, 0.5 mM cholesterol, 0.5 mM OH-cholesterol + 1 U/mL ACTH, and 3 μM 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 μM of pregnenolone stimulated cortisol secretion in BT adrenocortical cells and were used in the subsequent experiments (Table 2). Brook trout adrenocortical cells had significantly higher basal (MEM treatment) and OH-cholesterol stimulated cortisol secretion than RT cells (Table 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. 4A; nested ANOVA: F₅ = 11.64, p < 0.0001). In BT

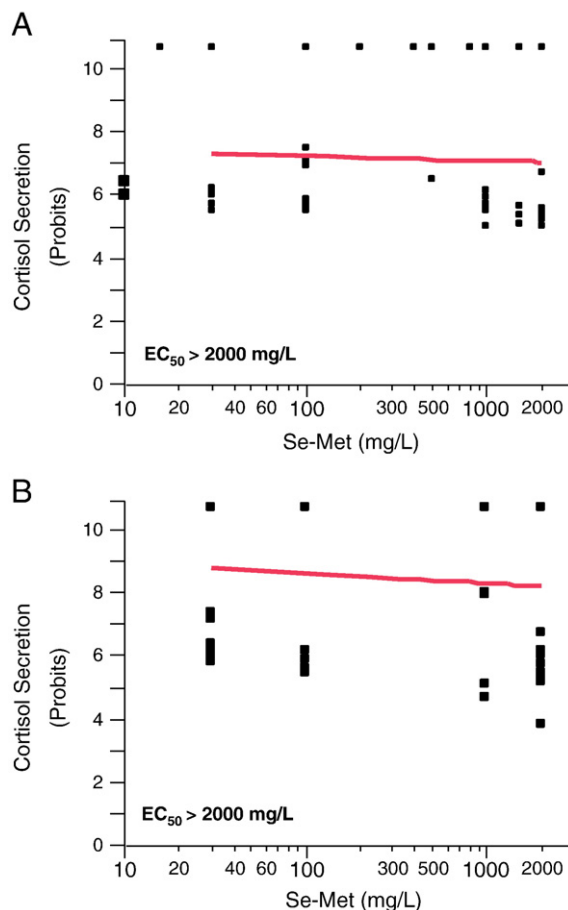


Fig. 3. The effect of selenomethionine (Se-Met) on cortisol secretion of (A) rainbow trout (n = 13) and (B) brook trout (n = 14) adrenocortical cells. See Fig. 2 for details.

Table 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: $F_5 = 1.41$; $p = 0.3805$).

* Significant difference between species (*t*-test, $p < 0.05$).

($n = 14$) adrenocortical cells, selenite exposure significantly decreased ACTH-, dbcAMP-, OH-cholesterol-, and OH-cholesterol + ACTH stimulated cortisol secretion (Fig. 4B; nested ANOVA: $F_5 = 12.73$, $p < 0.0001$). Addition of pregnenolone restored cortisol secretion in presence of selenite (Fig. 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 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. 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. 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 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. 5B). Exposure to DETCA significantly decreased cortisol secretion compared to exposure to sodium selenite alone ($n = 8$; Fig. 5B); however, exposure to ATA, Vit A, and Vit E did not alter the sodium selenite-induced impairment of cortisol secretion (Fig. 5B). LPO levels in the adrenocortical cells did not significantly vary with ATA, DETCA, Vit, A or Vit E treatments (Table 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).

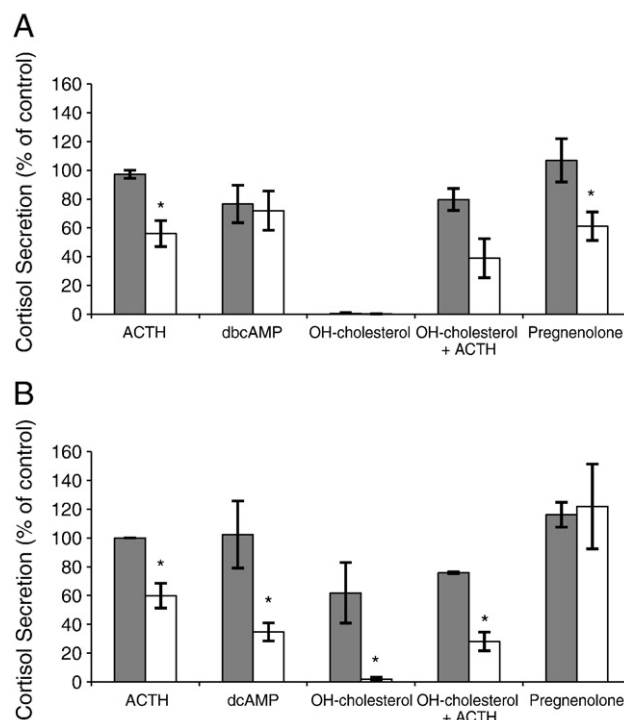


Fig. 4. Cortisol secretion (mean % of ACTH-stimulated control ± SE) of (A) rainbow trout ($n = 8$), and (B) brook trout ($n = 14$) adrenocortical cells exposed to sodium selenite (clear bars) at EC_{50} (12 mg/L for RT cells, 90 mg/L for BT cells) or control (shaded bars, no toxicant) and stimulated 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).

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 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 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 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 et al., 2004) and larvae of *Chironomus*, an aquatic invertebrate (Maier

Table 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.

Treatment	GSH experiment			Antioxidant experiment	
	Pre-exposure GSH ^a	Post exposure and stimulation GSH ^b	Post exposure and stimulation LPO ^c	Treatment	Post exposure and 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: $df_M = 5$, $F = 0.09$, $p = 0.9929$; $n = 6$.^c One-way ANOVA for treatment: $df_M = 5$, $F = 1.41$, $p = 0.2546$; $n = 5$.^d One-way ANOVA for treatment: $df_M = 5$, $F = 1.39$, $p = 0.2415$; $n = 10$.

and Knight, 1993). In the aquatic environment, all animals are exposed to Se-Met through their diet (Janz et al., 2010) while selenite exposure is primarily waterborne through the gills (Hodson 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 et al., 2000). Selenite may be reduced to selenide by thiols, before it is absorbed by cells (Ganyc and Self, 2008; Olm et al., 2009), but Se-Met uptake is controlled by the same amino acid transporters as methionine (Bakke 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 et al., 2004) generate ROS and oxidative damage when reduced by GSH;

however, selenite is reduced directly (Tarze et al., 2007; Gabel-Jensen and Gammelgaard, 2010) and Se-Met must be first metabolized to methylselenol by methioninase (Palace et al., 2004). Although there is some evidence to suggest that methioninase is present in fish embryos (Palace et al., 2004), and its activity has been detected in RT hepatocytes (Misra 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 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 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. OH-cholesterol, a key precursor in cortisol biosynthesis, did not stimulate cortisol secretion to the same extent as the other compounds used, possibly due to lower conversion rates of the exogenous substrate (Lacroix and Hontela, 2001). Some species differences in the basal, ACTH-, and OH-cholesterol stimulated cortisol secretion were detected, with higher secretion in BT than RT cells. Similar to other studies designed to identify the target(s) of specific toxicants in the steroidogenic pathway, including cadmium (Laskey and Phelps, 1991; Mathias et al., 1998) and some pesticides (Bisson and Hontela, 2002; Dorval et al., 2003), restoration of cortisol synthesis following substitution of specific precursors or signaling 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 signaling 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 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.

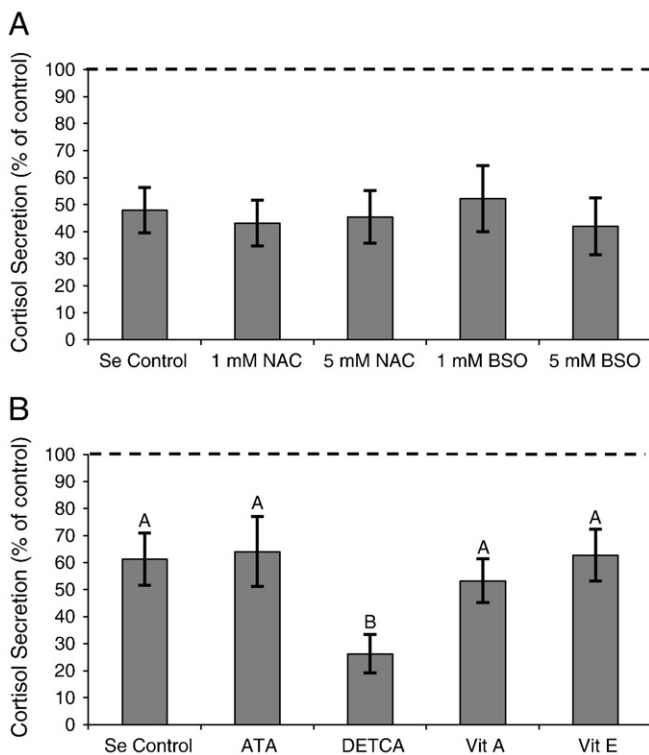


Fig. 5. Cortisol secretion (mean % of control \pm SE) in adrenocortical cells of rainbow trout exposed to 12 mg/L sodium selenite (EC_{50} Na_2SeO_3) treated with (A) NAC or BSO ($n = 9$), and (B) ATA, DETCA, Vit A or Vit E ($n = 8$). The dashed line indicates cortisol secretion (100%) of control cells 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 = diethylthiocarbamate trihydrate; Vit A = retinol; Vit E = tocopherol.

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 et al., 2004), generates oxidative radicals which will indiscriminately attack proteins, DNA or lipids in their vicinity (Kelly 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 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 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 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 defense against selenite toxicity in fish cells, since decreasing the cell's ability to scavenge the superoxide anion when exposed to selenite further inhibited cortisol secretion. LPO levels were not increased by the DETCA treatment, possibly because other cellular antioxidant defenses were still functional. It has been previously shown that SOD decreases Se-induced ROS production *in vitro* in a chemiluminescent assay (Spallholz 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 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 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 threshold 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.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgments

This project was funded by the Natural Science and Engineering Research Council's (NSERC) Metals In The Human Environment Strategic Network (MITHE-SN), NSERC DG to A. Hontela, and an Alberta Ingenuity (now part of Alberta Innovates – Technology Futures) PhD. Scholarship to L.L. Miller. Fish were a gift from the Allison Creek Brood Trout Station (Blairmore, Alberta, Canada). We would also like to thank R. Royer, H. Bird, A. Dann, and R. Flitton for their assistance with this project.

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