The Effects of Acute Waterborne Exposure to Sublethal Concentrations of Molybdenum on the Stress Response in Rainbow Trout, *Oncorhynchus mykiss*

Chelsea D. Ricketts, William R. Bates, Scott D. Reid

Department of Biology, Irving K. Barber School of Arts and Sciences, University of British Columbia, Okanagan campus, 3333 University Way, Kelowna, British Columbia, Canada, V1V 1V7

Abstract

To determine if molybdenum (Mo) is a chemical stressor, fingerling and juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne sodium molybdate (0, 2, 20, or 1,000 mg l⁻¹ of Mo) and components of the physiological (plasma cortisol, blood glucose, and hematocrit) and cellular (heat shock protein [hsp] 72, hsp73, and hsp90 in the liver, gills, heart, and erythrocytes and metallothionein [MT] in the liver and gills) stress responses were measured prior to initiation of exposure and at 8, 24, and 96 h. During the acute exposure, plasma cortisol, blood glucose, and hematocrit levels remained unchanged in all treatments. Heat shock protein 72 was not induced as a result of exposure and there were no detectable changes in total hsp70 (72 and 73), hsp90, and MT levels in any of the tissues relative to controls. Both fingerling and juvenile fish responded with similar lack of apparent sensitivity to Mo exposure. These experiments demonstrate that exposure to waterborne Mo of up to 1,000 mg l⁻¹ did not activate a physiological or cellular stress response in fish. Information from this study suggests that Mo water quality guidelines for the protection of aquatic life are highly protective of freshwater fish, namely rainbow trout.

Introduction

Molybdenum (Mo), as molybdate (MoO₄²⁻), is a common component of natural freshwaters due to erosion and weathering of ores from igneous and sedimentary rock, especially shale [1,2]. In unimpacted areas freshwater levels of Mo rarely exceed 0.02 mg l⁻¹ [1]. Anthropogenic loading of Mo into aquatic and terrestrial ecosystems occurs by Mo mining, milling, and smelting, combustion of fossil fuels, uranium and copper mining and milling, oil refining, shale oil production, and Mo containing fertilizers [1, 3–8]. Elevated levels of Mo such as 1.4 mg l⁻¹ [9], 11.4 mg l⁻¹ [10], 32.5 mg l⁻¹, [11], and 100 mg l⁻¹ [12] have been reported in freshwaters receiving mining discharge. There is potential for additional release and distribution of Mo into the aquatic environment as world production of this valued natural resource, which is primarily used as an alloying agent, continues to increase [13, 14].
Molybdenum is an essential micronutrient and forms the catalytic center of over 50 enzymes including xanthine oxidase, sulphite oxidase, aldehyde oxidase and DMSO reductase [15–17]. In domestic animals, deficiency symptoms include depressed growth, impaired reproduction, poor fetal survival, and decreased molybdoenzyme activities [18, 19]. Deficiency signs and requirements of Mo remain to be established in fish [20].

Little is known about the effects of elevated Mo levels on living systems, especially fish. Most of the available information is based on gross morphological changes or readily visible symptoms in domestic animals [1,4]. Toxicity tests in freshwater fish have reported 96 h LC50 Mo (added as sodium molybdate) concentrations ranging from >50 to >10,000 mg l⁻¹ [9, 21–26]. Manifestations that have been reported as a result of Mo exposure include fused gill lamellae, gut and pyloric caeca hemorrhaging, pale livers with hemorrhaging along liver margins, pale kidneys [22], inhibition of spermatogenesis, decreased gonadosomatic index [27], darker appearance, increased mucus production, higher ventilatory frequencies, post-exercise loss of equilibrium, and exercised-induced delayed mortality [26]. According to these studies, it appears that Mo, at high concentrations, does have physiological effects and can result in death. Therefore, more studies regarding the effect of Mo on other systems, such as the stress response, need to be investigated.

The stress response enables fish to avoid or cope with environmental, physical, or biological changes and thus maintain internal homeostasis. Fish, like other vertebrates, exhibit both a physiological and a cellular stress response. The physiological stress response consists of primary endocrine responses (secretion of cortisol and catecholamines), secondary metabolic responses, including an increase in glucose and hematocrit, and tertiary or whole organisms responses [28]. Elevated cortisol and glucose levels have been reported for fish exposed to a variety of physical [29, 30] and chemical stressors [31–33], including metals [34–36].

The cellular stress response is facilitated by the action of various stress proteins such as the heat shock protein (hsp) and the metallothionein (MT) families. Heat shock proteins are molecular chaperones that function by regulating cellular homeostasis through ensuring proper folding, transport, and degradation of proteins. The two main hsp families are hsp70, consisting of the constitutively expressed hsp73 and the stress inducible hsp72, and hsp90. In fish, these proteins are induced in cell lines, primary cell cultures, and whole organisms by a variety of stressors including industrial effluents [37–39], pesticides [40, 41], pathogens [42, 43], and metals [44–49]. Exposure to stress causes proteins to denature, misfold, or unfold, ultimately exposing hydrophobic regions and causing protein aggregation. The heat shock response is therefore essential to maintain proper protein structure and cellular function. Metallothionein is a metal binding protein with a high affinity for groups Ia and Ib transition metals [50]. One of the suggested functions of MT is to regulate essential trace metals like zinc and copper and detoxify metals such as cadmium and copper. Synthesis of MT is induced to a greatest degree by exposure to metals and to a lesser degree by hormones, cytokines, and organic contaminants [51].

The objectives of the present study were to determine the effect of sublethal concentrations of waterborne Mo on (1) the physiological stress response, as measured by plasma cortisol, blood glucose, and hematocrit, and (2) the cellular stress response, as measured by total hsp70 (72 and 73), hsp72, hsp90, and MT induction in rainbow trout, a species that has proven to be a very useful model system for understanding the effects of many other metals [52].

Materials and Methods

Animals

This study was approved by the University of British Columbia Animal Care Committee (AUP A10–0301) and conforms to guidelines outlined by the Canadian Council on Animal Care. Juvenile (mass range 215–509 g) and fingerling (mass range 2–18 g) rainbow trout,
Onchorhynchus mykiss, were obtained from Campbell Lake Trout Hatchery (Little Fort, BC, Canada; Mo ≤ 1.0 μg l⁻¹) and transferred to the University of British Columbia Okanagan (Kelowna, BC, Canada). Fish were kept indoors under a 12 h light/dark photoperiod in polyethylene tanks: 600 l for juveniles and 70 l for fingerlings. Tanks received a constant supply of aerated, dechlorinated City of Kelowna (Glenmore-Ellison Improvement District; Mo ≤ 1.0 μg l⁻¹) tap water at a temperature range of 10 to 13°C. Trout were acclimated and held under these conditions for 3 months prior to experimentation. The fish were fed 2% body weight every other day with a commercial feed (Aqua Pride Trout Pellets; Hi-Pro Feeds LP), but received no food for 48 h prior to and throughout all experiments.

Cannulation procedure
Juvenile rainbow trout were anesthetized with 0.1 g l⁻¹ MS-222 (ethyl m-aminobenzoate methanesulfonate) adjusted to pH 7.5 with NaHCO₃ and then placed onto an operating table to allow continuous retrograde irrigation of the gills with anesthetic solution. An indwelling cannula (Clay-Adams PE 50 tubing) was implanted into the dorsal aorta according to standard techniques [53]. Fish were revived on the operating table by irrigation of the gills with aerated water and then placed into individual chambers (see section 2.3). Fish were allowed to recover from the effects of anesthesia and surgery for 48 h before experimentation commenced. Cannulas were flushed daily with 0.2–0.3 ml of heparinized (100 i.u. ml⁻¹ ammonium heparin) Cortland saline to prevent clotting [54].

Exposure system
Forty-eight hours prior to Mo exposure fish were randomly transferred to black plexiglass chambers (3.0–3.5 l; 1 juvenile/chamber or 8 fingerlings/chamber) in order to recover from surgery and to acclimate to the experimental system. Each chamber was supplied with a flow of water and constant aeration. All chambers were half-submerged on a continuously flowing wet table to maintain water temperature during the static exposure. At time 0, flow to the chambers was stopped and water was replaced with the desired Mo concentration. In doing so, one-third of the chamber was allowed to drain, fish were always fully submerged, and then the chamber was flushed with 18 L of solution before being refilled. Fish were exposed to waterborne sodium molybdate dihydrate (Na₂MoO₄·2H₂O; Fisher Scientific, Pittsburgh, PA, USA) for 96 h at 0 (existing Mo in the water was ≤ 0.0010 mg l⁻¹), 2, 20, or 1000 mg l⁻¹ of Mo. The lowest exposure concentration of 2 mg l⁻¹ was chosen as it is the water quality limit for the protection of freshwater aquatic life proposed by Swain [55] and adopted by the province of British Columbia. When included, the highest exposure concentration of 1000 mg l⁻¹ was chosen as it was 50% of the 96 h LC₅₀ estimate for freshwater fish native to Okanagan water (Reid 2002). Furthermore, chosen exposure concentrations overlap with molybdenum concentrations that we have previously shown to accumulate internally, gill and liver, in freshwater fish held and exposed under nearly identical conditions (0–250 mg l⁻¹, [26]). To ensure that water quality and Mo levels were adequately maintained water was changed, as previously described, every 8 h. Temperature (10–13°C), general hardness (140 ± 12 mg l⁻¹ CaCO₃), pH (8.0 ± 0.2), and ammonia (0.92 ± 0.11 mg l⁻¹) were constantly monitored.

Treatments and sampling
Juvenile fish. Non-cannulated (318.3 ± 20.9 g, N = 18) juvenile fish were exposed to 0, 2, or 20 mg l⁻¹ of Mo. The experiment was conducted three times and each experiment used two fish per treatment. At 96 h, fish were quickly captured with minimal disturbance and stunned by a cephalic blow. Blood (0.5 ml) was withdrawn by caudal puncture using a syringe previously rinsed with heparinized Cortland saline. Immediately upon collection, whole blood was
centrifuged at 10,000 rpm for 2 min to separate the plasma from the erythrocytes. Both plasma and erythrocyte samples were stored at -80°C. Following blood collection, the gills were perfused with Cortland saline and excised from both the fish and the gill arch. The liver was removed and the heart excised without the bulbous arteriosus. All tissues were flash frozen in liquid nitrogen and stored at -80°C until analysis.

**Cannulated juvenile fish.** Cannulated juvenile fish (342.1 ± 16.9 g, N = 18) were exposed to 0, 2, or 20 mg l⁻¹ of Mo; six fish per treatment. Fish had their dorsal aortic cannulas sampled prior to initiation of exposure (0 h) and at 8, 24, and 96 h during exposure. Blood (0.5 ml) was sampled using a syringe previously rinsed with heparinized Cortland saline and processed as previously described. Plasma samples were stored at -80°C until analysis. To partially maintain blood volume, approximately 0.2–0.3 ml of Cortland saline was reinjected into the cannula after each blood collection.

**Fingerling fish.** Fingerling fish (11.1 ± 0.9 g, N = 96) were exposed to 0, 2, 20, or 1,000 mg l⁻¹ of Mo. The experiment was done three times at 16 fish per treatment. Fish were sampled with minimal disturbance from each chamber prior to initiation of exposure (0 h) and at 8, 24, and 96 h during exposure. Fish were sacrificed from a cephalic blow. Less than 0.1 ml of blood was collected into a heparinized hematocrit tube by means of capillary action after severing the caudal peduncle. The gills, excluding the arch, and liver were excised, flash frozen in liquid nitrogen, and stored at -80°C until analysis.

**Determination of tissue molybdenum content: ICP-MS**

To verify that our molybdenum exposures resulted in significant accumulation tissue molybdenum in trout we measured plasma, gill, white muscle and liver molybdenum content from juvenile fish using ICP-MS; sample quantities were insufficient to do so with fingerlings. Tissues samples for ICP-MS analysis were weighed and placed in constant temperature drying oven until a constant dry weight was obtained; approximately one week. A 50 mg aliquot of dry tissue was placed in an acid-washed 15 ml Teflon test tube. 2 ml of concentrated trace metal free nitric acid was pipetted into each tube and placed in a 75°C water bath for 24 h. Tubes were allowed to cool to room temperature then 0.5 ml of peroxide was added and the samples placed back a 46°C water bath for 18 h to complete sample oxidation. The lower temperature was required for this portion of the digestion to avoid sample loss due to over-active bubbling. The temperature was then increased to 80°C for an additional 24 h. Once digestion was complete, samples were removed from the bath and placed in a rack beneath an air manifold for drying. Once dry, samples were reconstituted in 10 ml of 1% trace metal free nitric acid and left to settle overnight. 100 μl aliquots of digests were drawn off and placed into a fresh, acid-washed 15 ml Falcon tube and topped off to 10 ml with 1% trace metal free nitric acid. Each sample then received 100 μl of Yttrium (1 ppm stock solution, diluted from 1000 ppm standard) for ICP-MS analysis. Yttrium was added to all samples as an internal standard and to correct for any potential matrix discrepancies. A standard curve was prepared in the same concentrated trace metal free nitric acid matrix (0, 1.0, 10.0 and 100.0 ppb; μg l⁻¹). These standards also contained 10 ppb (μg l⁻¹) Yttrium as an internal standard. The standard curve was used to convert the output of the ThermoFisher Element XR double-focusing ICP-MS from counts per second to a concentration in parts per billion (ppb). We measure the molybdenum isotope Mo⁹⁷ because it was one of two isotopes (Mo⁹⁵, Mo⁹⁷) that did not have any possibility for interference from other elements that were in our samples and it yielded the most consistent results.

**Determination of hematocrit, blood glucose, and plasma cortisol**

Hematocrit levels were measured in duplicate by means of micro hematocrit tubes. Tubes were spun at 2,510 rpm for 5 min on a clinical centrifuge (International Equipment Company,
Needham, MA, USA) fitted with a hematocrit head. Blood glucose levels were measured in duplicate using a Precision Xtra glucose meter (Abbott Laboratories, Abbott Park, IL, USA). Total plasma cortisol levels were determined in duplicate using an enzyme-linked immunosorbent assay kit (Neogen Corporation, Lexington, KY, USA), read at 630 nm on an OpsysMR microplate reader (Dynex Technologies, Chantilly, VA, USA).

**Protein extraction and quantification**

Tissues and erythrocytes were sonicated in ice-cold lysis buffer (1:1 w/v) containing 100 mM Tris-HCl pH 7.5, 0.1% SDS (sodium dodecyl sulfate), and a SigmaFAST protease inhibitor tablet (Sigma-Aldrich, St. Louis, MO, USA). The lysates were cleared in a microcentrifuge at 10,000 g for 3 min at room temperature. A 10 μl aliquot of supernatant was taken for protein determination with the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as a standard. Standards and samples were read at 490 nm. The remaining supernatant was mixed 1:1 with SDS-sample dilution buffer [56] and boiled for 3 min. In order to equalize total protein concentration samples were further diluted in 1X SDS-sample dilution buffer before being frozen at -80°C.

**Hsp70, hsp90, and MT analysis**

Protein levels were measured using the discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) method of Laemmli [56]. For hsp analysis, total protein (10 μg) from heart, gill, liver, and erythrocyte samples was resolved with a 4% stacking gel and a 12% separating gel. For MT analysis, 25 μg of liver and 27.5 μg of gill tissue were resolved with a 4% stacking gel and a 15% separating gel. Samples were run alongside Precision Plus Protein dual-color standards (Bio-Rad, Hercules, CA, USA). For hsp72 analysis, a liver sample from the same heat-shocked fish was loaded, in at least duplicate, onto each gel to allow direct comparison among gels. A purified bovine brain standard (H-9776; Sigma-Aldrich) and a purified human hsp90 standard (SPP-770; Assay Designs, Ann Arbor, MI, USA), and a liver sample from the same control fish were used for total hsp70 (72 and 73), hsp90, and MT analysis, respectively. Gels were run in a Mini-Protein II electrophoretic cell (Bio-Rad) containing 1X running buffer at 75 V until samples reached the separating gel at which time the voltage was increased to 150 V for approximately 1 h. Gels were then transferred to Hybond-P PVDF membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) for 1 h at 100 V for hsp analysis or 80 V for MT analysis. After transfer, the membrane was stained with Ponceau-S stain [0.5% (w/v) Ponceau-S red and 1% (v/v) acetic acid] for 5 min to determine the success of the transfer and equal loading. Membranes containing erythrocyte protein did not stain well with Ponceau-S; therefore, equal protein loading was detected by immunoblotting using a monoclonal mouse anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) primary antibody (CSA-335; Assay Designs).

Membranes were blocked with 5% skim milk powder in TBS-T (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20; pH 7.6) for 1 h. Membranes were washed in TBS-T and then incubated for 1 h in primary antibodies diluted in TBS-T. Rabbit anti-salmonid hsp72 (AS05061; Agrisera, Vännäs, Sweden), mouse anti-bovine total hsp70 (72 and 73) (H-5147; Sigma-Aldrich), or rabbit anti-salmonid hsp90 (AS05063; Agrisera) were all used at 1:5,000 for liver, heart, and erythrocyte samples and at 1:20,000, 1:5,000, and 1:20,000, respectively, for gill samples. The rabbit anti-cod (KH-1) MT primary antibody (M04406210–500; Biosense Laboratories, Bergen, Norway) was used at 1:1,000 for liver samples and 1:500 for gill samples. Membranes were then washed twice for 10 min each in TBS-T and, for hsp72, incubated for 1 h in secondary antibody (SAB-100 goat anti-mouse IgG HRP conjugate; Assay Designs) at a
1:10,000 dilution in TBS-T. For total hsp70 and hsp90 detection, membranes were incubated for 1 hr in goat anti-rabbit IgG HRP conjugate (SAB-300; Assay Designs) at a 1:1000 dilution in liver, heart, and erythrocyte samples and 1:40,000 dilution in gill samples. The same goat anti-rabbit antibody was used for MT detection at 1:5,000 for liver samples and 1:6,000 in gill samples. After this final incubation, membranes were washed 3 times for 10 min in TBS-T. All incubations and washes were done on an orbital shaker.

Densitometry
Proteins were detected by enhanced chemiluminescence using the ECL Western Blotting Analysis System (GE Healthcare) and Hyperfilm (GE Healthcare). The autoradiography film was developed using KODAK GBX Developer and Replenisher and Fixer and Replenisher (Sigma-Aldrich). Several different exposure times were taken for each blot to ensure linearity of band densities. Bands were scanned using an AGFA SnapScan e50 scanner and quantified using Quantity One software (Version 4.6.3; Bio-Rad). Sample band density was divided by the average positive control sample band density (run two or three times in the same gel) to give relative band density.

Calculations and statistics
Total protein concentrations were calculated by multiplying protein concentration in the initial homogenate by homogenate volume and dividing by tissue weight.

All data are presented as means ± SEM. Data were analyzed using JMP IN statistical software (Version 7.0.1; SAS Institute, Cary, NC, USA). P values of less than 0.05 were considered significant for all statistical tests. Tissue molybdenum data were analyzed using a Student’s 2 tailed t-test. Data from juvenile non-cannulated fish were submitted to a one-way ANOVA. Pre-exposure data from fingerling fish were submitted to a one-way ANOVA to test for exposure chamber effects while exposure data (8, 24, and 96 h) from fingerling fish were subjected to a two-way (main effects of treatment and sampling time) ANOVA. A Tukey-Kramer HSD test was applied to discern differences among means for those treatments that had statistically significant differences. Data from cannulated fish were analyzed by a repeated measures factorial design ANOVA with between subjects factors (characterizing variation within a fish and not the population). When necessary, post hoc contrasts were used to discern differences among means for those treatments that had statistically significant differences.

Results
Tissue Molybdenum
Tissue molybdenum for gill, plasma, white muscle and liver of fish from the control group (0 mg l⁻¹ Mo) to be 6.0 ± 3.46, 10.5 ± 2.02, 46.5 ± 1.44 and 227 ± 2.02 ng g⁻¹ dry weight, respectively (Fig. 1A). Following 96 h exposure to 20 mg l⁻¹ molybdenum, these tissues in juvenile rainbow trout were found to contain significantly greater molybdenum concentrations with gill and plasma showing the greatest increases (Fig. 1B). Gill and plasma molybdenum concentrations increased by 3,000 and 800 times, respectively, compared to the control group. Liver and white muscle experienced less dramatic, yet significant, increases in molybdenum concentration with a 23 fold and a 38% increases, respectively compared to the control group. The findings shown in Fig. 1 are consistent with those for tissue molybdenum concentrations from all juvenile fish exposed to elevated water molybdenum.
Physiological stress response parameters

Plasma cortisol levels remained unchanged in all groups of juvenile fish after 96 h of exposure to 2 or 20 mg l\(^{-1}\) of Mo (Fig. 2A). There was also no detectable effect of Mo, at the same concentrations, on plasma cortisol at 8, 24, or 96 h in the cannulated juvenile fish (data not shown). The 96 h pooled treatment means in non-cannulated (33.6 ± 7.7 ng ml\(^{-1}\)) and cannulated (26.1 ± 5.6 ng ml\(^{-1}\)) fish were not significantly different.

In concordance with plasma cortisol data, Mo exposure to 2 or 20 mg l\(^{-1}\) did not have a significant effect on plasma glucose levels in juvenile fish when measured after 96 h (Fig. 2B). Similar results were observed during the time course (8, 24, and 96 h) studies in cannulated...
juvenile fish exposed up to 20 mg l^{-1} of Mo and fingerling fish exposed up to 1,000 mg l^{-1} (data not shown). However, in these two studies there was a decrease in glucose levels over time. No significant differences between the 96 h pooled treatment means in non-cannulated juvenile
fish (5.3 ± 1.0 mM), cannulated juvenile fish (4.5 ± 1.0 mM), and fingerling fish (3.6 ± 0.2 mM) were detected.

At 96 h, hematocrit levels in juvenile fish exposed to 2 or 20 mg l⁻¹ of Mo were not significantly different than control fish (Fig. 2C). Similar results were observed in the time course studies using cannulated juvenile fish and fingerling fish (data not shown). There was a significant difference in 96 h pooled treatment means between cannulated fish (16.0 ± 1.4%) and non-cannulated fish: juvenile fish (30.3 ± 2.9%) and fingerling fish (33.9 ± 1.9%).

**Cellular stress response parameters**

Total liver, gill, heart, and erythrocyte protein concentrations from juvenile fish sampled after 96 h of exposure to 2 or 20 mg l⁻¹ of Mo showed no statistically significant differences when compared to controls (Fig. 3A). Similar results were observed in fingerling fish (Fig. 3 B, C). There were no significant differences in liver or gill total protein concentration in fish sampled from each of the exposure chambers (0, 2, 20, and 1,000 mg l⁻¹) prior to exposure. During the exposure period all fingerlings responded with a lack of change in total protein in both tissues.

Immunoblotting was carried out with a panel of hsp antibodies on cell free extracts of liver, gill, heart, and erythrocyte samples from fish exposed to Mo. There was no detectable induction of hsp72 under control conditions or after 96 h of exposure to 2 or 20 mg l⁻¹ in the liver, gills, or heart of juvenile fish. The erythrocytes, however, in both the control group and the Mo exposure groups expressed basal levels of hsp72 at about 30–50% of the heat shocked positive control (Fig. 4). Even at 1,000 mg l⁻¹ there was no detectable induction of hsp72 in the liver or gills of fingerling fish.

Figs. 5 and 6 show that the expression of total hsp70 (72 and 73) and hsp90 in the liver (A), gills (B), heart (C), and erythrocytes (D) of juvenile rainbow trout remained at control levels after the 96 h exposure to 2 or 20 mg l⁻¹. Fingerling fish sampled prior to addition of Mo did not differ in their liver and gill total hsp70 (Fig. 7A, B) or liver hsp90 (Fig. 8) levels, and these levels did not change when sampled at 8, 24, and 96 h during exposure.

Analysis of MT band density in liver and gill tissue of juvenile fish did not reveal any significant differences between control fish and Mo exposed (2 or 20 mg l⁻¹) fish after 96 h of exposure (Fig. 9). Similar results were observed in fingerling fish exposed to up to 1,000 mg l⁻¹ when liver (Fig. 10) and gill tissue were examined prior to exposure and at 8, 24, and 96 during exposure.

**Discussion**

Fish in nature are exposed to a variety of stressors that can adversely affect their health. In order to cope with stress, fish can respond to it by eliciting a physiological or a cellular stress response. Such responses result in biochemical, hematological, and cellular changes that can be used as biomarkers to allow for the assessment and management of stress in fish. While there have been many studies correlating various stress related biomarkers with exposure to metals such as cadmium [57], copper [58], and zinc [59], information concerning Mo is extremely limited. This study investigated the effects of an acute, sublethal Mo exposure on the stress response of rainbow trout. Both fingerling and juvenile fish did not elicit a physiological or a cellular stress response when exposed to Mo nor were there any detectable differences in sensitivity between the two life stages despite the tissue accumulation of significant molybdenum (Fig. 1, this study; [26]).

Control plasma cortisol concentrations were within the range of values reported for unstressed rainbow trout [29, 32, 43, 60–63]. A Mo exposure of up to 20 mg l⁻¹ did not activate a plasma cortisol response in juvenile rainbow trout at 8, 24, and 96 h during exposure. Results
Fig 3. Tissue total protein concentration (mg g⁻¹ tissue wet weight) in rainbow trout after 96 h molybdenum exposure. Tissue total protein is shown for liver, gill, heart and erythrocyte total protein in juvenile trout exposed to 0, 2 or 20 mg l⁻¹ (A) and liver (B) and gill (C) in fingerling trout exposed to 0, 2, 20 or 1,000 mg l⁻¹ molybdenum. Fingerlings were sampled prior to (PE = pre-exposure) and at 8, 24, and 96 h of exposure. Data plotted as means ± 1 SEM (n = 6). No significant differences (p > 0.05) were found.

doi:10.1371/journal.pone.0115334.g003
from this study are consistent with data from a 168 h endpoint Mo exposure to 25 or 250 mg l\(^{-1}\) in kokanee salmon reporting no difference in plasma cortisol levels between Mo exposed and control fish [26].

It has been suggested that the effects of toxicants on the stress response vary with the nature of the chemical, its concentration, method of exposure, and duration of exposure. For example, a waterborne exposure to the 96 h LC\(_{50}\) of copper (0.25 mg l\(^{-1}\)) caused plasma cortisol levels to increase by ~280 ng ml\(^{-1}\) after 24 h of exposure [32]. Similarly, plasma cortisol levels increased ~140 ng ml\(^{-1}\) at 72 h during exposure to cadmium [0.01 mg l\(^{-1}\), 50% of the 96 h LC\(_{50}\) determined by Hollis et al. [64]; Chowdhury et al., 2004] and increased ~65 ng ml\(^{-1}\) after 96 h of exposure to selenite (2.52 mg l\(^{-1}\), 35% of the 96 h LC\(_{50}\) determined by [36, 65]). In contrast, but similar to the results of this study, waterborne exposure to lower doses of copper (0.016 mg l\(^{-1}\); 6.4% of the 96 h LC\(_{50}\) determined by Syvokiene et al. [66]) for 72 h [61] and selenite (0.72 mg l\(^{-1}\), 10% of the 96 h LC\(_{50}\) determined by Hodson et al. [65]) for 96 h [36] did not elevate plasma cortisol levels. All of the aforementioned exposure studies used rainbow trout as the test species. Therefore, it is likely that in this study 20 mg l\(^{-1}\), 0.27% of the 96 h LC\(_{50}\) reported by Bentley [21] whose study used rainbow trout of similar size to rainbow trout in this present study, was not concentrated enough to elicit a response.

Control glucose and hematocrit levels were within the range previously reported in unstressed rainbow trout [29, 35, 67–70]. The lower hematocrit values observed in the cannulated fish versus the non-cannulated fish are characteristic of cannulated fish [71–74]. Explanations may be blood loss due to the cannulation procedure and mild hemodilution, caused by repeated sampling and injection of saline after each sampling. In the present study, the absence of hyperglycemia is consistent with the lack of elevated cortisol. Although hyperglycemia was not
observed throughout the 96 h exposure period, a hypoglycemia was observed. The decline in glucose is most likely a result of withholding feed during acclimation and experimentation and the exhaustion of energy stores. Brown et al. [75] reported a similar alteration in plasma glucose in catheterized rainbow trout and attributed it to withholding feed. There was no effect of waterborne Mo exposure on hematocrit. The results of this study are in concordance with data from a chronic (1 year) waterborne exposure of up to 17 mg l\(^{-1}\) reporting no change in hematocrit in various life stages of rainbow trout [73]. Findings outlined by McConnell [73] regarding observations of fused gill lamellae in rainbow trout and by Reid [26] regarding increased ventilation and mucus production in kokanee salmon during Mo exposure, however, would preclude one to think that these manifestations would have an effect on hematocrit. According to
Heath [76], any pollutant that results in gill damage and subsequent internal hypoxia can be expected to increase hematocrit. This indicates that waterborne Mo, despite irritating the gills, does not induce internal hypoxia. This is also true of the metal lead. When Hodson et al. [77] and Martinez et al. [78] exposed rainbow trout and Prochilodus lineatus to waterborne lead they observed that although the metal caused changes in gill morphology hematocrit remained unaffected.

Exposure of rainbow trout to Mo failed to upregulate expression of hsp72, hsp73, and hsp90 (Figs. 4–8). There was no response in the liver, gills, heart, or erythrocytes of juveniles exposed to a maximum of 20 mg l\(^{-1}\) or in the liver or gills of fingerlings exposed to a maximum of 1000 mg l\(^{-1}\). As a result, there appears to be no utility of these proteins as measures of Mo exposure. There is
confidence that the lack of induction in response to acute Mo exposure in trout does not reflect a
diminished capacity of fish to activate a heat shock response. In this study, heat shocked fish re-
sponded by synthesizing hsp72 and in previous studies that used the same antibodies heat shocked
fish responded with inductions in hsp72 and hsp90 in rainbow trout liver, heart, and erythrocytes
[79, 80]. Heat shock in rainbow trout has also lead to increases in hsp70 mRNA in the liver, gills,
heart, and blood [81]. The lack of hsp induction by Mo is also not due to metal load sequestering
by MT because, as discussed later, there was no induction of MT in response to Mo exposure.

Fig 7. Liver (A) and gill (B) total hsp70 levels in fingerling rainbow trout after a 96 h molybdenum
exposure to 0, 2, 20 or 1,000 mg l−1. Fish were sampled prior to exposure (PE = pre-exposure) and at 8, 24
and 96 h during exposure. Data plotted as means ± 1 SEM (n = 6). No significant differences (p > 0.05)
were found.

doi:10.1371/journal.pone.0115334.g007
Molybdenum is not the only stressor that is incapable of stimulating hsp70 production. Neither anesthesia administration nor handling induced hsp70 levels in the liver, gills, heart, or muscle of rainbow trout [82]. Furthermore, various forms of husbandry stress such as anesthesia, hypoxia, capture, crowding, feed deprivation, and cold stress had no affect on hsp70.

Fig 8. Liver hsp90 levels (relative to positive control) in fingerling rainbow trout exposed to 0, 20, or 1,000 mg l\(^{-1}\) of molybdenum prior to exposure (PE = pre-exposure) and during exposure (8, 24 and 96 h). Data presented as means ± 1 SEM (n = 6). No significant differences (p > 0.05) were found.

doi:10.1371/journal.pone.0115334.g008

Fig 9. Liver and gill MT levels (relative to positive control) of juvenile rainbow trout exposed for 96 h to waterborne Mo at concentrations of 0, 2, or 20 mg l\(^{-1}\). Data were plotted as means ± 1 SEM (n = 6). There were no significant differences (p > 0.05) between groups.

doi:10.1371/journal.pone.0115334.g009
mRNA levels in the gills of Atlantic salmon [83]. The commonality of these stressors is that none of them have been demonstrated to denature proteins. Therefore, it can be assumed that Mo, at concentrations tested in this study, does not cause detectable proteotoxicity. Although a number of metals induce MT synthesis, there is a general assumption that Mo does not have this ability. Jakobsen et al. [84] reported MT induction in the liver of rats implanted with cobalt-chromium-molybdenum alloys, yet the authors speculated the induction as a response to the presence of cobalt, chromium, manganese, iron, and/or nickel but not to Mo. Koizumi et al. [85] demonstrated that Mo did not elevate levels of MT mRNA; however, increases in mRNA are not always concomitant with increases in protein. This study is the first to suggest that Mo does not stimulate MT protein expression. Mo exposure of concentrations up to 1,000 mg l⁻¹ did not cause an up-regulation of MT in the liver or gills of rainbow trout (Figs. 9, 10), tissues that are known to possess high levels of MT [86] and accumulate Mo (Fig. 1, this study; [26]). This finding suggests that MT levels cannot be used as an indicator of previous environmental exposure to Mo. The lack of MT induction suggests that Mo neither induces MT directly through binding to MT nor indirectly through activation of an inflammatory response.

Molybdenum is a borderline, d(5) metal [87]. As such, the metal has significant oxide and sulphide chemistries as demonstrated by its formation of molybdenite (MoS₂) and wulfenite (PbMoO₄). As a result, Mo can be expected to bind to the negatively charged thiolate groups of MT if it exists as a cation. Other borderline d(5) metals such as chromium and manganese can bind to MT but with low affinity [88]. Lead, for example, has a high affinity for MT in vitro [89] but binds sparingly in vivo [90]. The lack of MT induction, however, suggests that Mo did not bind to MT. These findings would be expected if Mo, which exists as molybdate in the natural environment and has been shown to move across the gill as molybdate (J. Hoeskstra and S. D. Reid, pers. comm.) was distributed internally as molybdate. Although the speciation of Mo...
in fish body fluids has yet to be characterized, the interpretation of our MT findings are consistent with the study by Matsuura et al. [91] that demonstrated that Mo exists as molybdate inside salmon egg cytoplasm.

Exposure to chromium, iron, cobalt, nickel, arsenic [92, 93], cerium [94], and vanadate [95], metals which are unable to bind to MT, caused inductions in MT but indirectly though an inflammatory mediated response. These metals inflicted tissue injury triggering the production of cytokines such as interleukin-1 beta, interleukin-6, and tumor necrosis factor alpha, all known inducers of MT [96]. The fact that Mo did not increase MT expression also suggests that this metal is unable to induce an inflammatory response. Therefore, the toxic response of fused gill lamellae reported by McConnell [22] is probably not true tissue damage but rather a response to ionoregulatory disruption.

The findings from this study are consistent with previous studies demonstrating that an elevation in Mo is not perceived as a toxic threat by fish [22, 23, 26]. Collectively, these studies and the current one bring into question the Mo water quality guidelines for the protection of freshwater aquatic life of 0.073, 0.04, and 2 mg l⁻¹ set by Canada [2], Ontario [97], and British Columbia [55], respectively. It is therefore suggested that the current limits may be overly protective of rainbow trout and should be reevaluated. However, based on our limited finds we are not suggesting that the limit should be set to 1,000 mg l⁻¹. This study involved the acute exposure of only two life stages of a single species of freshwater fish to Mo, the analysis of an incomplete suite of physiological indicators of stress/toxicity and we have previously observed mortality in juvenile freshwater fish (kokanee) exposed to 2,000 mg l⁻¹ molybdenum under nearly identical conditions [26]. Furthermore, there is evidence that Mo in combination with additional environmental stressors could contribute to increased mortality in fish [23, 26, 98, 99]. Therefore a much or comprehensive examination of the toxicity of molybdenum is warranted and completed before any change in the Mo water quality guidelines are consider.

Acknowledgments

The authors would like to thank Luis O.B. Afonso and Tiago S.F. Hori (Memorial University) for sharing their technical expertise to C.D. Ricketts when conducting research at the National Research Council of Canada Institute for Marine Biosciences (Halifax, NS, Canada) and Courtney Ek for her technical work developing the ICP-MS protocol for the analysis of molybdenum in fish tissue.

Author Contributions

Conceived and designed the experiments: SDR WRB. Performed the experiments: CDR SDR. Analyzed the data: CDR SDR. Contributed reagents/materials/analysis tools: SDR WRB. Wrote the paper: CDR SDR.

References

1. Eisler R (1989) Molybdenum Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review. Biological Report 85(1.19). US Fish and Wildlife Service, Laurel, MD.
2. Canadian Council of Ministers of the Environment (1999) Canadian Water Quality Guidelines for the Protection of Aquatic Life. CCME, Ottawa, ON. PMID:25506965
3. Phillips GR, Russo RC (1978) Metal Bioaccumulation in Fishes and Aquatic Invertebrates: A Literature Review. EPA600/3–78–103. US Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN.
4. Chappell WR, Meglen RR, Moure-Eraso R, Solomons CC, Tsongas TA, et al. (1979) Human Health Effects of Molybdenum in Drinking Water. EPA-600/1–79–006. US Environmental Protection Agency, Health Effects Research Laboratory, Cincinnati, OH.
5. McNeely RN, Neimanis VP, Dwyer L (1979) Molybdenum. Water Quality Sourcebook: A Guide to Water Quality Parameters. Water Quality Branch, Inland Waters Directorate, Environment Canada, Ottawa, ON. PMID: 25121236

6. Goyer RA (1986) Toxic effects of metals. In: Klaassen C.D., Amdur MO, Doull J, editors. Casarett and Doull's Toxicology. 3rd ed. Macmillan Publications, New York, pp. 582–635. PMID: 11568356

7. Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air, water, and soils with trace metals. Nature 333: 134–139. PMID: 3285219

8. Reid SD (2011) Molybdenum and Chromium. In: Homeostasis and Toxicology of Essential Metals. Wood CM, Farrell AP, Brauner C, editors. Fish Physiology Vol. 31A. Elsevier, pp. 375–416.

9. Pyle GG, Swanson SM, Lehmkuhl DM (2001) Toxicity of uranium mine-receiving waters to caged fathead minnows, *Pimephales promelas*. Ecotoxicol Environ Saf 48: 202–214. PMID: 11161696

10. Jones CE (1999) Molybdenum in the environment: an overview of implications to the British Columbia mining industry. In: Price WA, Hart B, Howell C, editors. Proceedings of the 1999 Workshop on Molybdenum Issues in Reclamation. Bitech Publishers Ltd., Richmond, BC, pp. 1–14.

11. Whiting ER, Mathieu S, Parker DW (1994) Effects of drainage from a molybdenum mine and mill on stream macroinvertebrate communities. J Freshwater Ecol 9: 299–311.

12. Smith C, Brown KW, Deuel LE Jr (1987) Plant availability and uptake of molybdenum as influenced by soil type and competing ions. J Environ Qual 16: 377–382.

13. United States Geological Survey (2004) Mineral Commodity Summary: Molybdenum, Available at http://minerals.usgs.gov/minerals/pubs/commodity/molybdenum/molybmcs04.pdf [accessed (May 17 2008)].

14. United States Geological Survey (2009) Mineral Commodity Summary: Molybdenum, Available at http://minerals.usgs.gov/minerals/pubs/commodity/molybdenum/mcs-2009-molyb.pdf [accessed (March 22 2009)].

15. Hille R (1996) The mononuclear molybdenum enzymes. Chem Rev 96: 2757–2816. PMID: 11848841

16. Kisker C, Schindelin H, Rees DC (1997). Molybdenum-cofactor-containing enzymes: structure and mechanism. Annu Rev Biochem 66: 233–267. PMID: 9242907

17. Hille R, Hall J, Basu P (2014) The mononuclear molybdenum enzymes. Chem Rev 114: 3963–267. PMID: 24467397
doi: 10.1021/cr400443z PMID: 24467397

18. Nell JA, Annison EF, Balnave D (1980) The influence of tungsten on the molybdenum status of poultry. Br Poult Sci 21: 193–202. PMID: 6930313

19. Anke M, Groppel B, Grun M, (1985) Essentiality, toxicity, requirement, and supply of molybdenum in human and animals. In: Mills CT, Bremner I, Chesters JK, editors. Trace Elements in Man and Animals-TEMA 5. Commonwealth Agricultural Bureaux, Farnham Royal, London, pp. 154–157.

20. Watanabe T, Kiron V, Satoh S (1997) Trace minerals in fish nutrition. Aquaculture 151: 185–207.

21. Bentley RE (1973) Acute Toxicity of Sodium Molybdate to Bluegill (*Lepomis macrochirus*), Rainbow Trout (*Salmo gairdneri*), Fathead Minnow (*Pimephales promelas*), Channel Catfish (*Ictalurus punctatus*), Water Flea (*Daphnia magna*) and Scud (*Gammarus fasciatus*). Biomios, Wareham, MA. PMID: 4805339

22. McConnell RP (1977) Toxicity of molybdenum to rainbow trout under laboratory conditions. In: Chappell WR, Petersen KK, editors. Molybdenum in the Environment: The Geochemistry, Cycling, and Industrial Uses of Molybdenum. Marcel Dekker Inc., New York, pp. 725–730.

23. Hamilton SJ, Buhl KJ (1990) Acute toxicity of boron, molybdenum, and selenium to fry of chinook salmon and coho salmon. Arch Environ Contam Toxicol 19: 366–373. PMID: 2353835

24. Trucco RG, Inda J, Fernandez ML (1990) Acute toxicity and accumulation of copper, manganese, and molybdenum by *Basilichthys australis*. In: Chapman P, Bishay F, Power E, Hall K, Harding L, McLeay D, Nassichuk M, Knapp W, editors. Proceedings of the 17th Annual Aquatic Toxicity Workshop, November 5–7, Vancouver, BC, Can Tech Rep Fish Aquat Sci 1774, p. 102.

25. Hamilton SJ, Buhl KJ (1997) Hazard evaluation of inorganics, singly and in mixtures, to flannemouth sucker *Catostomus latipinnis* in the San Juan River, New Mexico. Ecotoxicol Environ Saf 38: 296–308. PMID: 9469884

26. Reid SD (2002) Physiological impact of acute molybdenum exposure in kokanee salmon (*Oncorhynchus nerka*). Comp Biochem Physiol C 133: 355–367. PMID: 12379420

27. Yamaguchi S, Miura C, Ito A, Agusa T, Iwata H. et al. (2007) Effects of lead, molybdenum, rubidium, arsenic and organochlorines on spermatogenesis in fish: monitoring at Mekong Delta area and in vitro experiment. Aquat Toxicol 83: 43–51. PMID: 17448548

28. Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish 9: 211–268.
29. Benfey TJ, Biron M (2000) Acute stress response in triploid rainbow trout (Oncorhynchus mykiss) and brook trout (Salvelinus fontinalis). Aquaculture 184: 167–176.

30. Ruane NM, Carballo EC, Komen J (2002). Increased stocking density influences the acute physiological stress response of common carp Cyprinus carpio (L.). Aquac Res 33: 777–784.

31. McBride JR, Donaldson EM, Derksen G (1979) Toxicity of landfill leachates to underlying rainbow trout (Salmo gairdneri). Bull Environ Contam Toxicol 23: 806–813. PMID: 519064

32. Carballo M, Muñoz MJ, Cuellar M, Tarazona JV (1995) Effects of waterborne copper, cyanide, ammonia, and nitrite on stress parameters and changes in susceptibility to saprolegniosis in rainbow trout (Oncorhynchus mykiss). Appl Environ Microbiol 61: 2108–2112. PMID: 16535039

33. Wood AW, Johnston BD, Farrell AP, Kennedy CJ (1996). Increased stocking density influences the acute physiological and cellular stress responses of juvenile rainbow trout (Oncorhynchus mykiss). Can J Fish Aquat Sci 53: 2424–2432.

34. Bleau H, Daniel C, Chevalier G, van Tra H, Hontela A (1996) Effects of acute exposure to mercury chloride and methylmercury on plasma cortisol, T3, T4, glucose and liver glycogen in rainbow trout (Oncorhynchus mykiss). Aquat Toxicol 34: 221–235.

35. Chowdhury MJ, Pane EF, Wood CM (2004) Physiological effects of dietary cadmium acclimations and waterborne cadmium challenge in rainbow trout: respiratory, ionoregulatory, and stress parameters. Comp Biochem Physiol C 139: 163–173.

36. Miller LL, Wang F, Palace VP, Hontela A (2007) Effects of acute and subchronic exposures to waterborne selenite on the physiological stress response and oxidative stress indicators in juvenile rainbow trout. Aquat Toxicol 83: 263–271. PMID: 17568967

37. Janz DM, McMaster ME, Munktittick KR, Van Der Kraak G (1997) Elevated ovarian follicular apoptosis and heat shock protein-70 expression in white sucker exposed to bleached kraft pulp mill effluent. Toxicol Appl Pharmacol 147: 391–398. PMID: 9439734

38. Vijayan MM, Pereira C, Krzynski G, Iwama GK (1998) Sublethal concentrations of contaminant induce the expression of hepatic heat shock protein 70 in two salmonids. Aquat Toxicol 40: 101–108.

39. Hori TSF, Avilez IM, Iwama GK, Johnson SC, Moraes G, et al. (2008) Impairment of the stress response in matrinxã juveniles (Brycon amazonicus) exposed to low concentrations of phenol. Comp Biochem Physiol C 147: 416–423. doi: 10.1016/j.cbpc.2008.01.003 PMID: 18308642

40. Sanders BM (1993) Stress proteins in aquatic organisms: an environmental perspective. Crit Rev Toxicol 23: 49–75. PMID: 8471160

41. Hassanein HMA, Banhawy MA, Soliman FM, Abdel-Rehim SA, Müller WEG, et al. (1999) Induction of hsp70 by the herbicide oxyfluorfen (goal) in the Egyptian Nile fish, Oreochromis niloticus. Arch Environ Contam Toxicol 37: 78–84. PMID: 10341045

42. Cho WJ, Cha SJ, Do JW, Choi JY, Lee JY, et al. (1997) A novel 90-kDa stress protein induced in fish cells by fish rhabdovirus infection. Biochem Biophys Res Commun 233: 316–319. PMID: 9144531

43. Ackerman PA, Iwama GK (2001) Physiological and cellular stress responses of juvenile rainbow trout to vibriosis. J Aquat Anim Health 13: 173–180.

44. Heikkila JJ, Schultz GA, Iatrou K, Gedamu L (1982) Expression of a set of fish genes following heat or metal ion exposure. J Biol Chem 257: 12000–12005. PMID: 7118927

45. Misra S, Zafarullah M, Price-Haughey J, Gedamu L (1989) Analysis of stress-induced gene expression in fish cell lines exposed to heavy metals and heat shock. Biochim Biophys Acta 1007: 325–333. PMID: 2467689

46. Sanders BM, Nguyen J, Martin LS, Howe SR, Coventry S (1995) Induction and subcellular localization of two major stress proteins in response to copper in the fathead minnow Pimephales promelas. Comp Biochem Physiol C 112: 335–343. PMID: 8836887

47. Williams JH, Farag AM, Stansbury MA, Young PA, Bergman HL, et al. (1996) Accumulation of hsp70 in juvenile and adult rainbow trout gill exposed to metal-contaminated water and/or diet. Environ Toxicol Chem 15: 1324–1328.

48. Duffy LK, Scofield E, Rodgers T, Patton M, Bowyer RT (1999) Comparative baseline levels of mercury, hsp70 and hsp60 in subsistence fish from the Yukon-Kuskokwim delta region of Alaska. Comp Biochem Physiol C 124: 181–186. PMID: 10622434

49. Boone AN, Vijayan MM (2002) Constitutive heat shock protein 70 (hsc70) expression in rainbow trout hepatocytes: effect of heat shock and heavy metal exposure. Comp Biochem Physiol C 132: 223–233. PMID: 12106899

50. Durnam DM, Palmiter RD (1981) Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. J Biol Chem 256: 5712–5716. PMID: 7240167
51. Kägi JHR (1993) Evolution, structure, and chemical activity of class I metallothioneins. In: Suzuki KT, Imura N, Kimura M, editors. Metallothionein III, Biological Roles and Medical Implications. Birkhauser Verlag, Basel, pp. 29–55.

52. Wood CM (2001) Toxic responses of the gill. In: Schlenk D, Benson WH, editors. Target Organ Toxicity on Marine and Freshwater Teleosts, Vol. I. Taylor and Francis, London, pp. 1–89.

53. Soivio A, Nyholm K, Westman K (1975) A technique for repeated blood sampling of the blood of individual resting fish. J Exp Biol 62: 207–217.

54. Wolf K (1963) Physiological salines for freshwater teleosts. Progr Fish-Cult 25: 135–140.

55. Swain LG (1986) Water Quality Criteria for Molybdenum: Technical Appendix. Ministry of Environment and Parks, Resource Quality Section, Water Management Branch, Victoria, BC. PMID: 25101439

56. Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227: 680–685. PMID: 5432063

57. McGeer JC, Niyogi S, Smith DS (2011) Cadmium. In: Homeostasis and Toxicology of Non-Essential Metals. Wood CM, Farrell AP, Brauner C, editors. Fish Physiology Vol. 31B. Elsevier, pp. 125–184.

58. Grosell M (2011) Copper. In: Homeostasis and Toxicology of Essential Metals. Wood CM, Farrell AP, Brauner C, editors. Fish Physiology Vol. 31A. Elsevier, pp. 53–133.

59. Hogstrand C (2011) Copper. In: Homeostasis and Toxicology of Essential Metals. Wood CM, Farrell AP, Brauner C, editors. Fish Physiology Vol. 31A. Elsevier, pp. 135–200.

60. Milligan CL, Wood CM (1987) Regulation of blood oxygen transport and red cell pH, after exhaustive activity in rainbow trout (Salmo gairdneri) and starry flounder (Platichthys stellatus). J Exp Biol 133: 263–282. PMID: 3430114

61. Dethloff GM, Schlenk D, Khan S, Bailey HC (1999) The effects of copper on blood and biochemical parameters of rainbow trout (Oncorhynchus mykiss). Arch Environ Contam Toxicol 36: 415–423. PMID: 10227861

62. Gregory TR, Wood CM (1999) The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. Physiol Biochem Zool 72: 286–295. PMID: 10223233

63. Basu N, Kennedy CJ, Iwama GK (2003) The effects of stress on the association between hsp70 and the glucocorticoid receptor in rainbow trout. Comp Biochem Physiol A 134: 655–663. PMID: 12600675

64. Hollis L, McGeer JC, McDonald DG, Wood CM (1999) Cadmium accumulation, gill Cd binding, acclimation, and physiological effects during long term sublethal Cd exposure in rainbow trout. Aquat Toxicol 46: 101–119.

65. Hodson PV, Spry DJ, Blunt BR (1980) Effects on rainbow trout (Salmo gairdneri) of chronic exposure to waterborne selenium. Can J Fish Aquat Sci 37: 233–240.

66. Syvokiene J, Mickeniene L, Svecevicius G (2006) Acute toxicity of copper-zinc mixture to rainbow trout (Oncorhynchus mykiss) and its impact on the bacterial flora of the digestive tract. Acta Zool Litu 16: 235–240.

67. McCarthy DH, Stevenson JP, Roberts MS (1973) Some blood parameters of the rainbow trout (Salmo gairdneri) Richardson. J Fish Biol 5: 1–8.

68. Ricard AC, Daniel C, Anderson P, Hortelau A (1998) Effects of subchronic exposure to cadmium chloride on endocrine and metabolic functions in rainbow trout Oncorhynchus mykiss. Arch Environ Contam Toxicol 34: 377–381. PMID: 9543058

69. Ruane NM, Nolan DT, Rotllant J, Costelloe J, Wendelaar Bonga SE (2000) Experimental exposure of rainbow trout Oncorhynchus mykiss (Walbaum) to the infective stages of the sea louse Lepeophtheirus salmonis (Krøyer) influences the physiological stress response to an acute stressor. Fish Shellfish Immunol 10: 451–463. PMID: 10994589

70. Pane EF, Richards JG, Wood CM (2003) Acute waterborne nickel toxicity in the rainbow trout (Oncorhynchus mykiss) occurs by respiratory rather than ionoregulatory mechanism. Aquat Toxicol 63: 65–82. PMID: 12615421

71. Wells RMG, Weber RE (1991) Is there an optimal haematocrit for rainbow trout, Oncorhynchus mykiss (Walbaum)? An interpretation of recent data based on blood viscosity measurements. J Fish Biol 38: 53–65.

72. Ron B, Zohar Y, Borski R, Young G, Grau EG (1995) Effects of dorsal aorta cannulation on cortisol and other stress parameters in the euryhaline tilapia, Oreochromis mossambicus. Aquaculture 135: 213–218.

73. Söhlberg S, Martinussen B, Horsberg TE, Søli NE (1996) Evaluation of the dorsal aorta cannulation technique for pharmacokinetic studies in Atlantic salmon (Salmo salar) in sea water. J Vet Pharmacol Ther 19: 460–465. PMID: 8971675
74. Mugnier C, Fostier A, Guezou S, Gaignon JL, Quemener L (1998) Effect of some repetitive factors on turbot stress response. Aquaculture 163: 33–45.

75. Brown SB, Eales JG, Hara TJ (1986). A protocol for estimation of cortisol plasma clearance in acid-exposed rainbow trout (Salmo gairdneri). Gen Comp Endocrinol 62: 493–502. PMID: 3770441

76. Heath AG (1995) Water Pollution and Fish Physiology. 2nd ed. Lewis, Boca Raton, FL. PMID: 25144093

77. Hodson PV, Blunt BR, Spry DJ (1978) Chronic toxicity of waterborne and dietary lead to rainbow trout (Salmo gairdneri) in Lake Ontario water. Water Res 12: 869–878.

78. Martinez CBR, Nagae MY, Zaia CTBV, Zaia DAM (2004). Acute morphological and physiological effects of lead in the neotropical fish Prochilodus lineatus. Braz J Biol 64: 797–807. PMID: 15744420

79. Rendell JL, Fowler S, Cockshutt A, Currie S (2006) Development-dependent differences in intracellular localization of stress proteins (hsp) in rainbow trout, Oncorhynchus mykiss, following heat shock. Comp Biochem Physiol D 1: 238–252. doi: 10.1016/j.cbd.2005.12.004 PMID: 20843255

80. Currie S, Reddin K, McGinn P, McConnell T, Perry SF (2008) β-adrenergic stimulation enhances the heat-shock response in fish. Physiol Biochem Zool 81: 414–425. doi: 10.1086/589095 PMID: 18507532

81. Currie S, Moyes CD, Tufts BL (2000) The effects of heat shock and acclimation temperature on hsp70 and hsp90 mRNA expression in rainbow trout: in vivo and in vitro comparisons. J Fish Biol 56: 398–408.

82. Washburn BS, Moreland JJ, Slaughter AM, Werner I, Hinton DE, et al. (2002) Effects of handling on heat shock protein expression in rainbow trout (Oncorhynchus mykiss). Environ Toxicol Chem 21: 557–560. PMID: 11878469

83. Zarte J, Bradley TM (2003) Heat shock proteins are not sensitive indicators of hatchery stress in salmon. Aquaculture 223: 175–187.

84. Jakobsen SS, Danscher G, Stoltenberg M, Larsen A, Bruun JM, et al. K (2007) Cobalt-chromium-molybdenum alloy causes metal accumulation and metallothionein up-regulation in rat liver and kidney. Basic Clin Pharmacol Toxicol 101: 441–446. PMID: 17971067

85. Koizumi T, Saito S, Yamane Y (1984) Effect of molybdenum on the acute toxicity of mercuric chloride. IV. Effect of molybdenum on mercury-mediated mRNA induction. Chem Biol Interact 51: 219–231. PMID: 6293938

86. Wu SM, Chen CC, Lee YC, Leu HT, Lin NS (2006) Cortisol and copper induce metallothionein expression in three tissues of tilapia (Oreochromis mossambicus) in organ culture. Zoo Biol 25: 363–370.

87. Nieboer E, Richardson DHS (1980) The replacement of the nondescript term ‘heavy metals’ by a biologically and chemically significant classification of metal ions. Environ Pollut B 1: 3–26

88. Suzuki Y, Yoshikawa H (1976) Induction of hepatic zinc-binding proteins of rats by various metals. Ind Health 14: 25–31.

89. Waalkes MP, Harvey MJ, Klaassen CD (1984) Relative in vitro affinity of hepatic metallothionein for metals. Toxicol Lett 20: 33–39. PMID: 6695394

90. Ulmer DD, Vallee BL (1969) Effects of lead on biochemical systems. In: Hemphill DD, editor. Trace Substances in Environmental Health, vol. II. University of Missouri Press, Columbia, pp. 7–27.

91. Matsuura H, Hasegawa T, Nagata H, Takatani K, Asano M, et al. (2003) Speciation of small molecules and inorganic ions in salmon egg cell cytoplasm by surfactant-mediated HPLC/ICP-MS. Anal Sci 19: 117–121. PMID: 12558034

92. Fleet JC, Golemboski KA, Dietert RR, Andrews GK, McCormick CC (1990) Induction of hepatic metallothionein by intraperitoneal metal injection: an associated inflammatory response. Am J Physiol 258: G926–933. PMID: 2360638

93. Albores A, Koropatnick J, Cherian MG, Zelazowski AJ (1992) Arsenic induces and enhances rat hepatic metallothionein production in vivo. Chem Biol Interact 85: 127–140. PMID: 1493605

94. Kobayashi K, Shida R, Hasegawa T, Satoh M, Seko Y, et al. (2005) Induction of hepatic metallothionein by trivalent cerium: role of interleukin 6. Biol Pharm Bull 28: 1859–1863. PMID: 16204935

95. Kobayashi K, Himeno S, Satoh M, Kuroda J, Shibata N, et al. (2006) Pentavalent vanadium induces hepatic metallothionein through interleukin-6-dependent and-independent mechanisms. Toxicology 228: 162–170. PMID: 16987576

96. De SK, McMaster MT, Andrews GK (1990) Endotoxin induction of murine metallothionein gene expression. J Biol Chem 265: 15267–15274. PMID: 2203773

97. Ministry of Environment and Energy (1994) Water Management Policies Guidelines: Provincial Water Quality Objectives. MOEE, Ottawa, ON. PMID: 25144107
98. Naddy RB, La Point TW, Klaine SJ (1995) Toxicity of arsenic, molybdenum, and selenium combinations to *Ceriodaphnia dubia*. Environ Toxicol Chem 14: 329–336.

99. Harper ER, St. Leger JA, Westberg JA, Mazzaro L, Schmitt T, et al. (2007) Tissue heavy metal concentrations of stranded California sea lions (*Zalophus californianus*) in Southern California. Environ Pollut 147: 677–682. PMID: 17116350