THE EFFECTS OF ACUTE WATERBORNE EXPOSURE TO SUB-LETHAL CONCENTRATIONS OF MOLYBDENUM ON THE STRESS RESPONSE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

by

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ABSTRACT

Molybdenum (Mo) is an essential metal that is increasing in popular demand as a valuable natural resource. Exploration activity in British Columbia, which hosts over 1350 molybdenum-bearing deposits, has exploded and there are over a handful of projects that have potential to begin operations. The metal's rapidly growing production and use represents a potential for increased release and distribution into the aquatic environment, especially in British Columbia. Although molybdenum is considered relatively non-toxic to fish, toxicity data are severely lacking and nothing is known about the effect of molybdenum on the stress response. To determine if molybdenum acts as a stressor, fingerling and juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne molybdenum (0, 2, 20, or 1000 mg/L) and components of the physiological (plasma cortisol, blood glucose, and hematocrit) and cellular [heat shock protein (HSP) 72, HSP73, HSP90, and metallothionein (MT)] stress responses were measured prior to initiation of exposure and at 8, 24, and 96 h during exposure. An ELISA revealed no alterations in plasma cortisol from any molybdenum treatment. Similarly, no changes in blood glucose, measured using a hand-held meter, or hematocrit that could be attributed to the stressor were found. Western blot analyses were used to measure the accumulation of HSPs in the liver and/or gills of fingerlings and in the liver, gills, heart, and erythrocytes of juveniles and MT in the liver of fingerlings and in the liver and gills of juveniles. HSP72 was not induced nor were there changes in HSP73, 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 molybdenum exposure. These experiments demonstrate, for the first time, that exposure to waterborne molybdenum of up to 1000 mg/L did not activate a physiological or cellular stress response in fish. These findings are consistent with previous studies suggesting that fish are resistant to molybdenum. Information from this study suggests that molybdenum water quality guidelines for the protection of aquatic life are highly protective of rainbow trout.

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ABC -- ATP-binding cassette
ACTH -- adrenocorticotropic hormone
AEBSF -- 4-(2-aminoethyl)benzenesulfonyl
ANOVA -- analysis of variance
AQUAMIN -- Assessment of the Aquatic Effects of Mining in Canada
ATP -- adenosine triphosphate
BCA -- bicinchoninic acid
BSA -- bovine serum albumin
cAMP -- cyclic adenosine monophosphate
CCME -- Canadian Council of Ministers of the Environment
cP450ssc -- cytochrome P450 side chain cleavage
cys -- cysteine
DNA -- deoxyribonucleic acid
ECL -- enhanced chemiluminescence
EDTA -- ethylemediaminetetraacetic acid
ELISA -- enzyme-linked immunosorbent assay
GAPDH -- glyceraldehyde 3-phosphate dehydrogenase
GRP-- glucose-regulated protein
HPI -- hypothalamic-pituitary interrenal
HPLC -- high performance liquid chromatography
HSD -- honestly significant differences
HSF -- heat shock transcription factor
HSP -- heat shock protein
ICP-MS -- inductively coupled plasma-mass spectrometry
LC_{50} -- lethal concentration that kills 50% of the test population
MEMPR -- Ministry of Energy, Mines and Petroleum Resources
Mo -- molybdenum
MOEE -- Ministry of Environment and Energy
mRNA -- messenger ribonucleic acid
MS-222 -- ethyl *m*-aminobenzoate methanesulfonate
MT -- metallothionein
MTF -- metal-responsive transcription factor
NRC -- Natural Resources Canada
NSERC -- Natural Sciences and Engineering Research Council
OMOEE -- Ontario Ministry of Environment and Energy
PAGE -- polyacrylamide gel electrophoresis
PKA -- protein kinase A
PNA -- peanut lectin agglutinin
PVDF -- polyvinylidene fluoride
SEM -- standard error mean
SDS -- sodium dodecyl sulphate
TRAP -- tumor necrosis factor receptor associated protein
TTBS -- Tween-Tris buffered saline
USGS -- United States Geological Survey

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DEDICATION

To

my family and friends

and

all those whom fuelled my passion for science

CHAPTER 1: INTRODUCTION

1.1 Molybdenum

1.1.1 Chemistry

Molybdenum is a second order transition metal that belongs to group VIb of the periodic table, along with chromium and tungsten. As a comparatively rare element, molybdenum comprises about 1 ppm or 0.0015% of the earth's crust (Chappell, 1975). In nature, this metal occurs in combination with sulphur, oxygen, tungsten, uranium, iron, magnesium, cobalt, vanadium, lead, bismuth, or calcium (Eisler, 1989). Of these, the most common mineral deposits are molybdenite (MoS_2), which predominates, powellite ($CaO \bullet Mo_3$), and wulfenite ($PbMoO_4$) (Friberg et al., 1975; Friberg and Lener, 1986; Goyer, 1986). In aquatic systems, molybdenum forms organometallic complexes (Cotton and Wilkinson, 1980). Although oxidation states of +2 to +6 exist for molybdenum, the most common forms in water are the tetravalent molybdenum sulphate (MoS_2) and the hexavalent anions, molybdate (MoO_4^{2-}) and biomolybdate ($HMoO_4^-$) (Jarrell et al., 1980). Most natural waters exhibit conditions under which molybdenum exists as the molybdate anion (Chappell et al., 1979). Thus, the readily soluble and stable molybdate is the only relevant source of molybdenum for biological systems.

1.1.2 Uses and Biological Function

Molybdenum is an economically important and versatile metal. Most of the molybdenum produced is used as an alloying agent to enhance hardenability, strength, toughness, and corrosion resistance (NRC, 2004). Molybdenum alloys are used in a variety of markets including electrical and electronic devices, glass manufacturing, high temperature furnaces and equipment, and aerospace and defense applications. Specific examples include X-ray tubes,

radio grids, and spark plugs (Stokinger, 1981). The remainder of molybdenum produced is used in pure metal form or in chemical compounds such as lubricants, dyeing agents, pigments, paints, glazes, enamels, electroplating compounds, fertilizers, and flame retardants (NRC, 2004). In human medicine, molybdenum-containing compounds may be used to treat dental caries and Wilson's disease, an autosomal recessive disorder characterized by the toxic accumulation of copper (Tanzi et al., 1993), as well as to lower both free fatty acids and blood glucose (Haywood et al., 1998). The glucose lowering effect of molybdate has only proven effective in diabetic animals (Ozcelikay et al., 1996; Panneerselvam and Govindaswamy, 2004).

Molybdenum is also an essential trace element to microorganisms, plants, and animals (Kisker et al., 1997) as it forms the catalytic center of over 50 enzymes (Ali et al., 2008). The most prominent molybdoenzymes in animals are xanthine oxidase, sulphite oxidase, and aldehyde oxidase. Xanthine oxidase catalyzes oxidative hydroxylation of purines and pyrimidines including conversion of hypoxanthine to uric acid (Kurosaki et al., 1995; Kisker et al., 1997). Sulphite oxidase catalyzes the oxidation of sulphite to sulphate, a process necessary for the degradation of sulphur containing amino acids (Karakas et al., 2005). Aldehyde oxidase oxidizes purines, pyrimidines, and pteridines and is involved in the metabolism of drugs and xenobiotics (Cates et al., 1980; Kisker et al., 1997).

Even though molybdenum is essential to life, the minimum dietary requirement for this metal for satisfactory growth and health has yet to be established for most species. According to Burguera and Burguera (2007) an accurate minimum requirement cannot be given because molybdenum deficiency has not been observed under natural conditions with any species. Watanabe et al. (1997) specifically point out that deficiency signs and requirements of molybdenum remain to be established in fish.

Molybdenum deficiency is rare in animals fed practical diets. In human adults, the actual daily intake of molybdenum is often three to eight times higher than the estimated minimum

requirement of 0.25 mg/day (Holzinger et al., 1998). Few cases of molybdenum deficiency have been reported to date and those were associated with disease conditions (Turnlund et al., 1995). In humans, a disorder known as molybdenum cofactor (molybdopterin) deficiency, the absence of molybdoenzymes, results in severe metabolic disorders or death (Johnson et al., 1980). Primary molybdenum deficiency has been reported in goats fed a diet low in molybdenum (Anke et al., 1985). These goats showed depressed growth, impaired reproduction, and poor fetal survival. Secondary molybdenum deficiency has been produced in chicks fed low molybdenum diets containing high levels of tungsten, a metal known to antagonize molybdenum transport, uptake, and utilization (Nell et al., 1980). Manifestations included growth retardation as well as decreases in tissue molybdenum levels and molybdoenzyme activities such as the conversion of hypoxanthine to uric acid.

1.1.3 Environmental Fate

Natural sources of molybdenum to the aquatic environment include weathering of ores from igneous and sedimentary rock and subsequent run-off to streams and lakes (CCME, 1999). Natural molybdenum concentrations in ground and surface waters rarely exceed 0.02 mg/L, unless contaminated by human activities (Eisler, 1989). Important anthropogenic sources of molybdenum into the aquatic environment include molybdenum mining and milling (Phillips and Russo, 1978), molybdenum smelting (Nriagu and Pacyna, 1988), combustion of fossil fuels (Goyer, 1986), uranium and copper mining and milling (Chappell et al., 1979), oil refining and shale oil production (Eisler, 1989), and the run-off of fertilizers containing molybdenum as a growth promoter (McNeely et al., 1979).

In Canadian freshwaters, concentrations of molybdenum range from below the detection limit (0.0001 mg/L) to 0.5 mg/L (OMOEE, 1995) and in British Columbia, the surface water concentrations range from below the detection limit to 0.06 mg/L (CCME, 1999). Near several

mining sites in British Columbia, Jones (1999) found total molybdenum from 0.003 to 0.22 mg/L in background water and 0.005 to 11.4 mg/L at sites downstream of mine discharges. Also in British Columbia, Whiting et al. (1994) reported mean and maximum molybdenum concentrations of 24.79 mg/L and 32.5 mg/L in a stream receiving discharge from molybdenum mining operations. Pyle et al. (2001) reported elevated levels of molybdenum in two lakes, Fox Lake (1.4 mg/L) and Unknown Lake (0.74 mg/L), receiving mill effluent from Key Lake Uranium mine in northern Saskatchewan. A reference lake upstream of the uranium mine only recorded molybdenum levels of 0.0004 mg/L. Levels of up to 100 mg/L have been reported in irrigation water from molybdenum mining and reclamation (Smith et al., 1987).

As a result of environmental exposure, molybdenum accumulates in animal tissues (Pandey et al., 2002). Molybdenum accumulation studies in freshwater fish are contradictory; however, the discrepancy appears to lie in the concentration of the metal that the organism is exposed to. Reid (2002) found that after a 3 day exposure to 5-250 mg/L both liver and gill tissue of kokanee salmon (*Oncorhynchus nerka* Kennerlyi) accumulated molybdenum in a dose-dependent manner. Goettl and Davies (1977) exposed rainbow trout (*O. mykiss*) to various concentrations of molybdenum for a maximum of 492 days and found that at the highest concentration of 18.7 mg/L fish accumulated significantly more molybdenum in the liver than the controls. Molybdenum concentrations in the liver and kidney of rainbow trout exposed for two weeks in a stream 1.6 km downstream from a molybdenum mine tailings outfall were significantly higher than in reference fish (Kienholz, 1977). At considerably lower molybdenum concentrations, rainbow trout tissues were only slightly affected by concentrations of molybdenum in the water. Tissue molybdenum concentrations ranged from 5-118 ug/kg wet weight in water containing trace concentrations (<0.006 mg/L), 10-146 ug/kg in water containing low concentrations (0.006 mg/L), and 13-322 ug/kg in water containing high concentrations (0.3 mg/L) of molybdenum (Ward, 1973). A similar pattern was reported in kokanee salmon (Ward,

1973). Saiki and Palawski (1990) measured whole fish molybdenum concentrations in striped

bass from the San Joaquin River and discovered that molybdenum was not elevated above

reference site concentrations. In summary, it appears that at environmentally relevant

concentrations freshwater fish do not significantly accumulate molybdenum.

1.1.4 Uptake

It has been demonstrated that in prokaryotes there are three modes of molybdenum uptake.

In the first mode, molybdenum is taken-up by high affinity molybdate transporters belonging to

the ATP-binding cassette (ABC) family (Grunden and Shanmugam, 1997). The two other

modes, a sulphate transport system and a non-specific anion transport system, are less preferred

(Grunden and Shanmugam, 1997). In contrast to the well-studied molybdate transport

mechanisms in bacteria, eukaryotic molybdate transport is still poorly understood. In the green

alga *Chlamydomonas reinhardtii* genetic evidence suggests the existence of a distinct molybdate

uptake system (Tejada-Jiménex et al., 2007). No molybdate transporter has been identified in

higher eukaryotes and thus little is known about the uptake of molybdenum in freshwater fish

and other animals. Cardin and Mason (1976) suggest that sulphate and molybdate are transferred

across the rat intestine by a common carrier system. Similarly, in rainbow trout, sulphate and

molybdate compete for a common gill transporter located in the PNA+ (peanut lectin agglutinin)

cells, a sub family of mitochondrial rich gill cells (Hoekstra and Reid, 2008). Thus, the rates of

uptake, retention, and excretion of molybdenum are inversely related to the level of inorganic

sulphate.

Although molybdenum is taken-up as molybdate, its speciation in body fluids and tissues

it not known. Since molybdenum exists as molybdate over a pH range corresponding to

physiological pH it is assumed that inside an organism molybdate would predominate over its

cationic and oxycationic forms. Recently, Matsuura et al. (2003) used a high performance liquid

chromatography (HPLC)/inductively coupled plasma-mass spectrometry (ICP-MS) hyphenated system to demonstrate that in salmon egg cytoplasm molybdenum (0.012 ug/g fresh weight) existed as molybdate. Once inside an animal molybdate is transported in the blood where it is distributed to various tissues for integration into enzyme systems or excreted in urine (Vyskocil and Viau, 1999).

1.1.5 Toxicity

Little is known about the effects of elevated molybdenum levels on animals. Most of the available information is based on gross morphological changes or readily visible symptoms in domestic animals. A summary of the findings indicates that the toxicological properties of molybdenum vary greatly from species to species, both in terms of the toxic dose and effect (Chappell et al., 1979). Cattle are the most susceptible followed by sheep, rats, rabbits, horses, and pigs (Chappell et al., 1979). In cattle, the lethal dose of molybdenum is on the order of 10 mg/kg body weight (Cook et al., 1966) whereas for rats it is about 100 to 150 mg/kg body weight (Maresh et al., 1940) and for guinea pigs it is 250 mg/kg body weight (Fairhall et al., 1945). The ruminal interaction between molybdenum, sulphur, and copper is responsible for the greater sensitivity of ruminants than non-ruminants (monogastrics) to molybdenum. In the reducing environment of the rumen compounds of sulphur and molybdenum called di-, tri-, and tetrathiomolybdates are formed. Tetrathiomolybdates bind irreversibly to solid phase of digesta and act as powerful chelators of copper. The retention of copper in the gut results in impaired copper absorption (Friberg and Lener, 1986; Strickland et al., 1987; Osman and Sykes, 1989). Trithiomolybdates can enter the circulation and interact cooperatively with albumin and copper resulting in further impairment of copper availability (Mason et al., 1986). The overall result is an ensuing copper deficiency.

Molybdenosis has been induced experimentally in many domestic animals including cattle, sheep, rabbits, guinea pigs, and chickens (Friberg et al., 1975). As previously mentioned, signs of molybdenum poisoning varies among species but typically include copper deficiency, reduced food intake and growth rate, diarrhea, anemia, dull, wiry, and depigmented hair (achromotrichia), liver and kidney pathology, joint and connective tissue lesions, bone abnormalities, loosening and loss of teeth, and reproductive impairment such as delayed puberty, female infertility, testicular degeneration, abnormal or delayed oestrus cycle, and decreased milk production (Ostrom et al., 1961; Underwood, 1971; Dollahite et al., 1972; Friberg et al., 1975; Erdman et al., 1978; Penumarthy and Oehme, 1978; Ward, 1978; Chappell et al., 1979; Mills and Bremner, 1980; Alary et al., 1981; Baldwin et al., 1981; Van Ryssen et al., 1982; Friberg and Lener, 1986). Biochemical alterations include decreases in plasma ceruloplasmin, increases in molybdenum-containing enzyme activities, and alterations of copper and molybdenum tissue concentrations (Kincaid, 1980; Ivan and Veira, 1985; Arthington et al., 1996; Ward and Spears, 1997). Eisler (1989) states that (1) early signs of molybdenosis are often irreversible, especially in young animals, (2) the severity of signs depends of the amount of molybdenum intake, relative to that of copper and inorganic sulphate, and (3) if afflicted animals are not removed from molybdenum contaminated diets, and given copper sulphate therapy, death may result.

It is likely that some of the above manifestations are a result of molybdate's inhibitory effects on various cellular proteins. Such inhibitions are also true of other transition metal oxyanions like vanadate and tungstate (Leach et al., 1979; Nishigori and Toft, 1980). Molybdate has been identified as a potent inhibitor of phosphatases, enzymes that remove phosphate groups (Nishigori and Toft, 1980). Specific targets include glucose-6-phosphatase (Nordlie and Arion, 1964), phosphoprotein phosphatase (Paigen, 1958), uteroferrin (Doi et al., 1988), and protein-tyrosine phosphatases PTP-1B and SHP-1 (Heo et al., 2002). The number of targets for molybdate is considerably huge since a vast majority of functionally important reactions are

controlled by phosphorylation-dephosphorylation mechanisms. In the process of exploring the potential involvement of widespread cellular control mechanisms of phosphorylation-dephosphorylation in the heat shock response (see section 1.2.2.2), Tiligada et al. (1999) discovered that molybdate induced thermotolerance during heat shock in yeast (*Saccharomyces cerevisiae*). The authors speculate that the inhibition of dephosphorylation by molybdate facilitated the induction of thermotolerance, possibly through the phosphorylation-dephosphorylation of heat shock proteins (HSPs; see section 1.2.2.1).

The metal oxyanion also inhibits the molecular chaperone HSP90 (see section 1.2.2.4). In doing so, molybdate induces structural changes in the C-terminal domain of HSP90 and alters its interactions with substrates (Hartson et al., 1999). Through the oxyanion's interaction with HSP90 it also has the ability to stabilize, by means of a direct and reversible interaction (Cardo et al., 1983), protein-HSP90 complexes (Hutchinson et al., 1992; Stancato et al., 1993). The most commonly stabilized complexes are steroid receptor-HSP90 complexes. Many papers have been published demonstrating the inhibition of glucocorticoid (Leach et al., 1979; Dahmer et al., 1981), progesterone (Nishigori and Toft, 1980; Yang et al., 1982), androgen (Wright et al., 1981), aldosterone (Marver, 1980), estrogen (Shayamala and Leonard, 1980; Krozowski and Murphy, 1981), and mineralocorticoid (Eisen and Harmon, 1986) receptors by molybdate whereby the oxyanion prevents receptor transformation into a DNA-binding state (Dahmer et al., 1984).

1.1.6 Toxicity Specific to Freshwater Fish

Short-term ($\leq$ 1 week) molybdenum exposure studies in freshwater fish indicate that the metal is relatively non-toxic. Exposure to molybdenum has lead to manifestations and mortality, but at high concentrations. Ninety-six hour LC_{50} (the concentration lethal to 50% of the population) toxicity tests in freshwater fish report concentrations ranging from 70 to >10000

mg/L of molybdenum depending on the test water conditions, fish species, and fish size (Table 1). In rainbow trout of similar size, the 96 h LC_{50} for molybdenum established by McConnell (1977) is approximately 60 000 than the 96 h LC_{50} for cadmium (0.022 mg/L; Hollis et al., 1999) and 86 times higher the 96 h LC_{50} for nickel (15.3 mg/L; Pane et al., 2003).

The following information is a summary of observations in response to short-term waterborne molybdenum exposure in freshwater fish. During a 96 h exposure, McConnell (1977) recorded manifestations such as fused gill lamellae, gut and pyloric caeca hemorrhaging, pale livers with hemorrhaging along liver margins, and pale kidneys in juvenile rainbow trout that died at 1500 mg/L. At similar concentrations, 1000-2000 mg/L, Reid (2002) observed a darker appearance, increased mucus production, and noticeably higher ventilatory frequencies in juvenile kokanee during a 96 h exposure. Reid (2002) further reported that prior exposure to 25 or 250 mg/L for 7 days resulted in post-exercise loss of equilibrium and exercised-induced delayed mortality. Yamaguchi et al. (2007) found that molybdenum had the ability to inhibit spermatogenesis in fish. Treatment of testes from the Japanese eel (*Anguilla japonica*) with 0.096-9.6 mg/L of molybdenum in combination with 11-ketotestosterone for 6 days decreased germ cell proliferation. In fathead minnow (*Pimephales promelas*) eggs a 96 h exposure to concentrations of up to 100 mg/L of molybdenum had no impact on egg hatchability, or time-to-hatch (Pyle, 2000). Additionally, fathead minnow larval growth was not affected by a 7 day exposure to 100 mg/L (Pyle, 2000).

Long-term (> 1 week) molybdenum exposure studies are consistent with the short-term studies in that fish are relatively resistant to molybdenum. Rainbow trout exposed for a year to molybdenum concentrations ranging from 0-17 mg/L showed no significant biological differences in mortality, growth, or hematocrit. Neither eyed eggs, sac-fry, nor fingerling life-stages showed any toxicological differences when compared to controls (McConnell, 1977). Pyle (2000) exposed fertilized white sucker (*Catostomus commersoni*) eggs to concentrations as

high as 1.7 mg/L of molybdenum and observed no impacts to hatchability or time-to-hatch after 12 days of exposure and no impacts to larval growth after 22 days of exposure. In a non-lethal field study of juvenile rainbow trout residing in a lake, recording levels of 6.0 mg/L of molybdenum, downstream of the Endako molybdenum mine in British Columbia it was determined that effluent from the mine was not causing adverse effects on the survival, condition, growth, or reproductive performance of the fish (Galloway et al., 2006). In contrast to the above observations, Yamaguchi et al. (2007) correlated increasing molybdenum (0-1000 ng/g dry weight liver), rubidium, arsenic, and lead concentrations with decreasing gonadosomatic index (gonad weight/body weight x 100) in male catfish (*Pangasianodon hypophthalmus*) residing in the Mekong Delta in South Viet Nam, an aquatic environment polluted by a variety of organochlorines and metals.

It was previously believed that molybdenum was more toxic to younger fish than older fish. The studies by Birge (1978) and Birge et al. (1980) demonstrated a 28 day LC_{50} of 0.73 mg/L and 0.79 mg/L, respectively, in rainbow trout exposed to molybdenum immediately after fertilization to 4 days post hatch. However, Davies et al. (2005) repeated the study and could not replicate the high molybdenum toxicity. Instead they determined the 32 day LC_{50} to be >400 mg/L, a result comparable to the other studies (see Table 1) reporting low toxicity of molybdenum to fish.

1.1.7 Water Quality Guidelines for the Protection of Aquatic Life

Toxicity studies have been utilized to develop interim molybdenum water quality guidelines for the protection of aquatic life. These values are interim due to the fact that the guidelines are based on a limited data set. In Canada, the lowest chronic toxicity value, 28 day LC_{50} of 0.73 mg/L for rainbow trout (Birge, 1978), was multiplied by a safety factor of 0.1 to obtain a guideline of 0.073 mg/L (CCME, 1999). Some Canadian provinces have developed

their own guidelines. The limit in Ontario, 0.04 mg/L, was obtained by dividing 0.73 mg/L (Birge, 1978) by an uncertainty factor of 16 (MOEE, 1994). The current guideline in British Columbia is 2 mg/L (Swain, 1986). In the United States, molybdenum is not an Environmental Protection Agency priority pollutant and no national water quality criteria for the protection of freshwater aquatic life has been developed. The lack of a guideline is shocking since the United States is the world's largest producer of molybdenum (USGS, 2009).

1.2 The Stress Response in Fish

The environment in which an organism lives does not remain constant but changes over time and space. Environmental, physical, and biological changes put stress on an organism. As a mechanism to avoid or cope with these challenges to homeostasis a stress response is initiated. Organisms that are not able adapt to a change in their environment may succumb to the potentially detrimental effects of the stressor at hand (Feder and Hofmann, 1999). Fish, like other vertebrates, exhibit a stress response at both the physiological and cellular level.

1.2.1 The Physiological Stress Response

The stress response in fish is broadly categorized into three stages: primary, secondary, and tertiary (Mazeaud et al., 1977). The primary response, or the neuroendocrine response, is characterized by the rapid release of stress hormones such as catecholamines and corticosteroids into the circulation (Wendelaar Bonga, 1997; Mommsen et al., 1999). These stress hormones mediate the secondary response, or the metabolic response, by activating a number of metabolic pathways that result in changes in blood chemistry and hematology such as increased glucose and hematocrit (Barton and Iwama, 1991; Mommsen et al., 1999). The tertiary response encompasses whole animal and population level changes associated with stress. These changes can be a result of energy repartitioning required to cope with the increased energy demand.

Therefore, long-term exposure to a stressor can potentially lead to decreases in growth, disease resistance, reproductive success, smolting, and swimming performance (Iwama et al., 1999).

1.2.1.1 The Hypothalamic-Pituitary Interrenal Axis

The hypothalamic-pituitary interrenal (HPI) axis is responsible for releasing corticosteroids in response to a stressor. Cortisol, a glucocorticoid, is the predominant circulating corticosteroid in fish (Donaldson, 1981; Sathiyaa et al., 2001). When a fish perceives a stimulus as a stressor the hypothalamus releases corticotropin-releasing hormone in order to stimulate the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH enters the blood stream and stimulates the steroidogenic interrenal cells in the anterior head kidney to synthesize cortisol (Hontela, 1997; Mommsen et al., 1999). In brief, ACTH binds to its receptor leading to the activation of protein kinase A (PKA) by cyclic adenosine monophosphate (cAMP). PKA activates cholesterol ester hydrolase releasing free cholesterol from cholesterol esters within the cell matrix (Stocco, 2000). Free cholesterol is transported into the mitochondria where a cytochrome P450 side chain cleavage ($cP450_{scc}$) converts cholesterol to pregnenolone (Mommsen et al., 1999; Stocco, 2000). Pregnenolone then moves to the cytoplasm where cytochrome $P450_{17!}$ and $P450_{c21}$ create 11-deoxycortisol. This precursor is transported back to the mitochondria where it is transformed into cortisol by cytochrome $P45_{11}$ (Hontela, 1997; Mommsen et al., 1999).

Cortisol affects a variety of systems that regulate homeostasis. It can increase mobilization of liver glycogen reserves, increase plasma glucose, increase heart rate, increase blood flow to the gills, alter plasma electrolytes (Hontela, 1998), stimulate protein degradation (Freeman and Idler, 1973), increase Na^+/K^+ATPase activity (Shrimpton and McCormick, 1999), induce metallothioneins to sequester metals (Hyllner et al., 1989), reduce stress-induced levels of

heat shock proteins (Basu et al., 2001), suppress the immune system, and suppress maturation (Carragher and Sumpter, 1990).

Elevated plasma cortisol levels indicate that an organism is under acute or sub-chronic stress, while low levels indicate no stress, interrenal exhaustion, or impairment of the HPI axis (Wedemeyer and McLeay, 1981; Girard et al., 1998; Hontela, 1998). Elevation of cortisol in response to stressors has made it the most commonly used indicator of stress in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Mommsen et al., 1999). The approximate resting and stressed levels of cortisol in the plasma of salmonids are <10 ng/mL and 30-300 ng/mL, respectively (Barton and Iwama, 1991; Iwama et al., 1998). It is important to note that these values serve only as general guidelines as species differences (Vijayan and Moon, 1994), seasonal and diel changes (Pickering and Pottinger, 1983; McLeese et al., 1994), photoperiod (Audet et al., 1986), nutritional conditions (Vijayan et al., 1993), sexual maturity (Pickering et al., 1987), genetic characteristics, prior rearing history, and the local environment (Iwama et al., 1999) are known to modify the plasma cortisol values for control and stressed states.

A variety of biotic and abiotic stressors activate a cortisol response in fish. Examples include handling and confinement (Benfey and Biron, 2000), background color (Rotllant et al., 2003), sea lice challenge (Bowers et al., 2000), density/crowding (Ruane et al., 2002), social stress (Sloman et al., 2002), fluctuating flow regimes (Flodmark et al., 2002), pesticides (Wood et al., 1996), and metals (Bleau et al., 1996; Miller et al., 2007).

1.2.2 The Cellular Stress Response

Stress also causes modifications in the pattern of gene expression, specifically resulting in the increased expression of selected genes. These genes include those that encode stress inducible proteins such as the heat shock protein (HSP) and metallothionein (MT) families.

Recently, these two protein families have received much interest for use as diagnostic probes for monitoring the condition of fish and their environment.

1.2.2.1 Heat Shock Proteins

Heat shock proteins are molecular chaperones that function by regulating cellular homeostasis and promoting survival through the successful folding, assembly, intracellular localization, secretion, regulation, and degradation of other proteins (Feder and Hofmann, 1999). These highly conserved and ubiquitous proteins have been found in all organisms from bacteria to plants and animals (Lindquist and Craig, 1988), including fish (Iwama et al., 1998). Heat shock proteins are categorized into families based on their molecular mass, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Iwama et al., 1998; Zarate and Bradley, 2003). The major families are low molecular weight HSPs (16-30 kDa), HSP60, HSP70, HSP90, and HSP100. There are three functional groups of HSPs: (1) HSPs that are constitutively expressed and respond little to stress, (2) HSPs that possess developmental functions and are upregulated after stress, and (3) HSPs having little or no function in the absence of stress that are intensely upregulated after stress (Krebs and Bettencourt, 1999).

1.2.2.2 Heat Shock Response

The term 'heat shock response' arose from early observations on *Drosophila* exposed to severe heat stress (Ritossa, 1962). Today, this term is misleading since the expression of HSPs has been documented in response to a number of different stressors other than heat. In fish, these proteins are induced in cell lines, primary cell cultures, and whole organisms by a variety of stressors including industrial effluents (Janz et al., 1997; Vijayan et al., 1998; Hori et al., 2008), pesticides (Sanders, 1993; Hassanein et al., 1999), pollutants (Dyer et al., 1993; Grôsvik and Goksôyr, 1996), pathogens (Cho et al., 1997; Ackerman and Iwama, 2001), and metals (Heikkila

et al., 1982; Misra et al., 1989; Sanders et al., 1995; Williams et al., 1996; Duffy et al., 1999; Boone and Vijayan, 2002). 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.

The mechanism by which a cell detects stress is unclear; however, it is thought that the presence of misfolded proteins triggers the heat shock response. Ananthan et al. (1986) demonstrated that *Hsp* genes were activated after the injection of denatured proteins into frog oocytes. Heat shock protein induction results mainly from the binding of an activated heat shock transcription factor (HSF) to a palindromic promoter region known as the heat shock element located upstream of the *Hsp* genes (Bienz and Pelham, 1987). Since most of the inducible *Hsp* genes do not contain introns the mRNA is rapidly translated into new protein within minutes following exposure to a stressor (Iwama et al., 1998). Although production of HSP proteins depends on the synthesis and accumulation of *Hsp* mRNA, increases in the mRNA do not always correspond to an increase in protein production (Bruce et al., 1993). The degree to which HSPs are induced and repressed depends on several factors such as tissue type (Råbergh et al., 2000), distinct HSP family (Smith et al., 1999), type, magnitude, and duration of stressor (Airaksinen et al., 2003), season (Fader et al., 1994), species (Basu et al., 2001), sex (Afonso et al., 2003), and developmental stage (Lele et al., 1997; Rendell et al., 2006).

The overexpression of HSPs is often sufficient enough to protect an organism against otherwise lethal stresses. In the natural environment where there are a multitude of stressors, increased levels of HSPs have the ability to induce stress tolerance in that they can transiently increase the resistance of an organism to a subsequent stressor (Basu et al., 2002). Brown et al. (1992) found that exposure of winter flounder (*Pleuronectes americanus)* renal epithelium to a heat stress protected cells against the detrimental effects of subsequent extreme temperature or chemical challenge. In rainbow trout, Bradley et al. (1985) showed that pre-exposure to a lethal

concentration of zinc increased the tolerance of the metal by almost three fold. There are obvious benefits to increased levels of HSPs; however, at extremely high concentrations HSPs may become toxic by consuming excess amounts of a cell's or organism's nutrient and energy stores and by occupying a huge percentage of the catabolic machinery (Calow, 1991; Hoffmann, 1995).

Not all stressors induce a heat shock response. Those of importance are common husbandry practices and experimental procedures. Sanders (1993) established that a heat shock response is not induced as a result of handling the organism during collection. In rainbow trout, handling stress did not alter levels of hepatic HSP70 (Vijayan et al., 1997). In concordance, Washburn et al. (2002) showed that transport, handling, and anesthesia stress did not increase HSP70 and HSP60 levels in muscle, gill, or heart tissue of rainbow trout. Common forms of hatchery related stress such as anesthesia, formalin exposure, hypoxia, hyperoxia, capture stress, crowding, feed deprivation, and cold stress did not alter levels of gill HSP30, HSP70, or HSP90 in Atlantic salmon (*Salmo salar*) (Zarate and Bradley, 2003).

1.2.2.3 HSP70

Of all the HSP families, the HSP70 family is the most prominent and best characterized as its members play important roles as molecular chaperones in unstressed and stressed cells (Tavaria et al., 1996; Boone and Vijayan, 2002). The HSP70 family has several members of similar size such as HSP72 (cytosol/nucleus) and HSP73 (cytosol/nucleus) and the glucose-regulated proteins GRP75 (mitochondria) and GRP78 (endoplasmic reticulum) (Tavaria et al., 1996). HSP72 and HSP73 are prominent members of the HSP70 family. HSP73 is constitutively synthesized in unstressed cells and after stress there is only a minimal increase. On the contrary, HSP72 is detectable to a small extent in the unstressed cell but is synthesized in cells that are experiencing stress (Kregel, 2002). HSP72 is the most extensively studied protein

(Iwama et al., 1999) and is the major stress inducible protein in fish (Kothary and Candido, 1982).

1.2.2.3.1 Structure

Members of the HSP70 family share remarkable sequence and structural similarity. The HSP72 and HSP73 proteins have over 90% sequence identity (Welch and Feramisco, 1984). The monomeric HSP70 protein consists of a highly conserved ~45 kDa ATP-binding domain at the N-terminal, plus a region with protease sensitive sites (Bukau and Horwich, 1998; Goloubinoff and De Los Rios, 2007) and a variable ~25 kDa substrate recognition domain at the C-terminal. The C-terminal contains a 15 kDa conserved peptide-binding domain with an EEVD motif (Freeman et al., 1995; Bukau and Horwich, 1998). This motif enables HSP70 to bind to co-chaperones and other HSPs.

1.2.2.3.2 Function

Members of the HSP70 family assist in folding newly synthesized proteins, refolding damaged proteins, translocating proteins across organellar membranes, disassembling aggregated protein structures, and facilitating proteolytic degradation of unstable proteins (Bukau and Horwich, 1998). HSP70 proteins promiscuously recognize short hydrophobic peptide segments such as those exposed in nascent polypeptide chains (Young et al., 2001). Because chaperone proteins often cooperate in folding any polypeptide bound by HSP70 at an early stage of folding can be transferred to other co-chaperones such as HSP90 to reach its final native state (Hartl, 1996).

HSP70 chaperones also control the biological activity of folded regulatory proteins. Examples include nuclear receptors (steroid hormone receptors), kinases (Raf, cyclinB1/cdk1), and transcription factors (HSF, c-Myc, pRb) (Mayer and Bukau, 2005). Thus, HSP70 family

members are involved in signal transduction, cell cycle regulation, differentiation, and programmed cell death. It is not surprising then that HSP70 chaperones are important in oncogenesis (Jolly and Morimoto, 2000), aging (Shpund and Gershon, 1997), neurodegenerative (Barral et al., 2004) and autoimmune (Millar et al., 2003) diseases, and viral infections (Mayer, 2005).

1.2.2.4 HSP90

HSP90 is one of the most abundant proteins in the unstressed cell and makes up 1-2 % of total cellular protein (Lai et al., 1984). This family consists of four isoforms: HSP90! (inducible/major form) and HSP90 (constitutive/minor form) are found predominately in the cytosol, Grp94 is localized in the endoplasmic reticulum, and tumor necrosis factor receptor associated protein 1 (TRAP-1) resides in the mitochondria (Maloney and Workman, 2002).

1.2.2.4.1 Structure

HSP90 proteins share a common structural plan. The HSP90 protein contains a highly conserved 25 kDa N-terminal ATP binding domain, a ~10 kDa middle domain that regulates the ATPase activity of the N-terminal domain, a flexible 35 kDa middle domain, and a 12 kDa C-terminal dimerization domain (Stebbins et al., 1997; Maruya et al., 1999; Bracher and Hartl, 2006). The protein also contains an EEVD sequence at the C-terminus that is an anchor point for co-chaperones (Bracher and Hartl, 2006).

1.2.2.4.2 Function

Unlike HSP70, HSP90 does not act generally in nascent protein folding (Nathan et al., 1997). HSP90, on the other hand, interacts with specific client signal transduction proteins and maintains their activity. Just over 100 HSP90 substrates have been identified and include steroid

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hormone receptors (Leach et al., 1979), kinases (Stancato et al., 1993), transcription factors (Blagosklonny et al., 1996), polymerases (Crevel et al., 2001), actin (Koyasu et al., 1986), myosin (Kellermayer and Csermely, 1995), and tubulin (Sanchez et al., 1988). When these client proteins interact with HSP90 they are in a near native state ready for activation by ligand binding or interaction with other factors (Jakob et al., 1995).

Disruption of HSP90 function leads to multiple physiological defects. Some known disruptors of HSP90 function include radicicol (Schulte et al., 1998), novobiocin (Marcu et al., 2000), geldanamycin (Whitesell et al., 1994), molybdate (Hartson, et al. 1999), and stress. Stress is a disruptor of HSP90 because as more and more proteins become denatured, HSP90 becomes overwhelmed and gets diverted from its normal function in signal transduction through its affinity for denatured proteins (Sangster et al., 2004). When HSP90 is taxed HSP90-dependent pathways become sensitive to the effects of cryptic genetic variation. In the fruit fly *D. melanogaster* (Rutherford and Lindquist, 1998) and the plant *Arabidopsis thaliana* (Queitsch et al., 2002) an array of morphological phenotypes were observed when HSP90 was disrupted. Different individuals had different defects collectively affecting almost every visible structure. These new phenotypes can become fixed by selection thereby adjusting the evolvability of an organism (Rutherford, 2003).

1.2.2.5 Metallothionein

Metallothioneins (MTs) make up a family of metal-binding proteins that are conserved both in nature and function in a wide range of organisms from prokaryotes to eukaryotes (Van Campenhout et al., 2004). All MTs can be characterized by the following properties: (1) cytosolic location in the cell, (2) low molecular weight, (3) high metal content (4-12 metals atoms per mole of protein), (4) high cysteine content (~30%), (5) absence of both histidine and aromatic amino acids, (6) unique amino acid sequence (location of cysteines), (7) metal-thiolate

clusters, and (8) heat stability (60°C for 5 min). These criteria are based upon horse renal cadmium-binding proteins, which were the first MTs discovered (Vallee and Maret, 1993; Viarengo et al., 1997). Proteins that do not meet one or more of these qualities are known as metallothionein-like proteins.

Metallothioneins are classified based on the locations of cysteine residues and the mode of synthesis (Roesijadi, 1992). Class I MTs are polypeptides with cysteine locations similar to those of the horse kidney and are found in vertebrates and some invertebrates. Class II MTs are polypeptides that have cysteine locations only distantly related to those of horse kidney that are found in some invertebrates, cyanobacteria, yeasts, and plants. Class III MTs are nontranslationally synthesized metal-thiolate polypeptides that contain #-glutamyl cysteinyl units. This latter class is found in the plant kingdom (Rauser, 1999). Each class of MT is further separated into several isoforms (i.e. MT-Ia, MT-Ib, etc.; Hamer, 1986).

1.2.2.5.1 Structure and Metal Binding Properties

Metallothioneins from higher eukaryotes are straight-chained proteins, 60 to 63 amino acids in length. The amino acid sequence is characterized by the abundance and location of cysteine (cys) residues that form cys-cys, cys-X-cys, and cys-X-Y-cys motifs, where X and Y represent another amino acid. The fact that each sequence is flanked by either argenine or lysine implies that these two amino acids play a part in the metal binding role of MT (Hamer, 1986). Placement of these short sequences in MT is highly conserved among species (Hamer, 1986; Kägi and Schaffer, 1988). All fish species have cysteines conserved in number and position (Vallee and Maret, 1993). Eight different MT amino acid sequences, all being 60 amino acids in length except for rainbow trout MT-1a (61 amino acids), have been obtained from fish (Vallee and Maret, 1993).

Apo-MT (or thionein), MT without any metals bound to it, is a randomly coiled protein (Andrews, 1990). Upon metals binding, which occurs cooperatively to the sulphydryl groups of the cysteines, the backbone of MT folds into two metal binding domains (Newman and Unger, 2003). These domains are the N-terminal beta-domain and the C-terminal alpha-domain (Olsson, 1996). The binding of metals to these domains results in the formation of two metal-thiolate clusters: cluster A on the alpha-domain and cluster B on the beta-domain (Vallee and Maret, 1993). In cluster A, four metal atoms form an adamantane-like coordination with five bridging and six terminal cysteine ligands (Nielson and Winge, 1983; Vallee and Maret, 1993). In cluster B, three metal atoms form a chair arrangement with three bridging and six terminal cysteine ligands. Thus, MT is a reservoir for seven metal atoms.

Metal binding to apo-MT is an ordered and reversible process (Olsson, 1996). Cluster A preferentially binds zinc and cadmium while cluster B shows a preferential incorporation of copper (Olsson, 1996). Metals are more easily displaced from cluster B than cluster A, probably due to the greater number of cys-metal-cys cross-links in cluster A (Andrews, 1990). Metallothionein has a high binding affinity for group Ib and IIb transition metals (Durnam and Palmiter, 1981); therefore, in addition to zinc, copper, and cadmium, MT can also bind with mercury, silver, and gold.

1.2.2.5.2 Expression and Function

Although the physiological functions of MTs remain obscure, it is accepted that MTs regulate essential trace metals such as zinc and copper and detoxify metals such as cadmium and mercury (Kägi and Schaffer, 1988). Metallothioneins are present at low basal levels in association with zinc; therefore, MT functions as a zinc chaperone. Zinc is a constituent of the active site of more than 300 enzymes and indispensable to the function of at least as many DNA-binding proteins (Vallee and Maret, 1993). The binding of zinc to MT is thermodynamically

stable, making MT an ideal zinc reservoir *in vivo* (Kang, 2006). Under conditions of metal overload zinc can be readily displaced (Kang, 2006). The multiple cysteine residues of MT can be oxidized releasing bound metal in the process. Clusters of sulfurs that bind zinc create an oxidoreductive environment for zinc at a redox potential so low that MT can be readily oxidized by mild cellular oxidants with the release of zinc (Palmiter, 1998; Kang, 2006). The binding affinity for metals to MT is highest for mercury followed by copper, cadmium, and zinc in decreasing order.

Several types of intracellular interactions can be envisioned for the role of MT in protection against metal toxicity. These include binding of newly taken-up metal ions, metal abstraction from structures that have bound toxic metal ions, and metal exchange with metal ions that have replaced zinc in zinc-binding sites (Newman and Unger, 2003). Several studies have shown that MT levels in organisms exposed to metals increase with increasing concentrations of metals (Canli et al., 1997; Dallinger et al., 1997). In fish, this increase is readily detectable in the liver, gills, and kidney, tissues known to have the highest abundance of metallothionein (Wu et al., 2006). If MT expression is not sufficient to keep up with exposure the above interactions can be compromised resulting in metal toxicity.

In addition to direct potent inducers of MT such as zinc, cadmium, and copper other metals that do not belong to group Ib or IIb such as chromium, iron, cobalt, manganese, and nickel can also induce MT, but to a lesser extent (Fleet et al., 1990). These weak inducers of MT do not bind to the metalloprotein, but induce it indirectly through an inflammatory response. Inflammatory mediators such as cytokines are known inducers of MT (Thirumoorthy et al., 2007).

Considering the extensive knowledge of the induction and regulation of MTs in fish and the availability of sensitive and reliable assay methods for MTs, these proteins have good potential as tools in monitoring exposure to toxic metals (Newman and Unger, 2003). When

using MTs as biomarkers one must be careful as normal physiological processes such as growth, molting, reproduction, development, and cellular differentiation require increased concentrations of essential metals (Roesijadi, 1992). This increased demand for metals during physiological processes, may induce MT and thus reduce its effectiveness as a biomarker.

1.3 Rationale for This Study

1.3.1 Why Study Molybdenum?

Molybdenum is a valued natural resource and the use of the metal in a variety of applications, primarily as an alloying agent, has resulted in an increase in its world production from 127 000 tons in 2003 (USGS, 2004) to 212 000 tons in 2008 (USGS, 2009). Due to the increased demand for the metal there is renewed interest in exploration activity in molybdenum-bearing deposits. Canada has five operating mines producing molybdenum, all located in British Columbia. Two mines are primary producers and the other three mines produce molybdenum as a by-product of copper mining. British Columbia hosts over 1350 known molybdenum-containing ore deposits, 430 of which contain molybdenum as the primary commodity (NRC, 2004). As a result of the province's molybdenum resources, several new mines are currently under development and there are over 25 major exploration projects (MEMPR, 2008). Molybdenum's rapidly growing production (whether it be from molybdenum, copper, or uranium operations) and use represents a potential for increased release and distribution into the aquatic environment. Therefore, it is necessary to determine the physiological impacts, harmful or beneficial, of molybdenum on organisms.

Toxicity of metals to fish has received much research attention, especially in the last decade. However, the focus has been on metals such as silver, cadmium, copper, zinc, and lead. Molybdenum has received very little attention, which is surprising since there are numerous

different anthropogenic activities that introduce molybdenum into natural waters. The need for more studies investigating molybdenum toxicity has been addressed by the Atomic Energy and Control Board (Golder Associates Ltd., 1996). Investigations into molybdenum toxicity to biota inhabiting aquatic systems have both significant socioeconomic and academic relevance, especially in Canada. The majority of Canada's economy is based on its natural resources, of which mining is a significant portion. Worldwide, Canada is the fifth largest producer of molybdenum (USGS, 2009), the top producer of uranium (The Uranium Institute, 1999), and one of the top copper producers (USGS, 2008). All of these mining operations elevate molybdenum concentrations in the aquatic environment.

1.3.2 Why Use Fish in Toxicity Studies?

The most important source of metal pollution in the aquatic environment is wastewaters arising from mining activities (Lloyd, 1992). Fish represent the resident biota of mine receiving waters and have an intrinsic importance, both ecologically and socioeconomically.

Every species in an ecosystem has a specific and important niche, all together forming a complex and finely balanced ecosystem. Fish, both predator and prey, interact directly with predators, prey, competitors, parasites, and symbionts and even indirectly with even more species in food webs and community matrices (Helfman, 2007). Fish, in addition to benthic invertebrates, have been identified by AQUAMIN (Assessment of the Aquatic Effects of Mining in Canada) as key ecosystem components (AQUAMIN Working Groups 7 and 8, 1996). Removal of a single fish species will alter the basic ecosystem properties and decrease the integrity of the ecosystem (Chapin et al., 2000). Owing to their importance in aquatic communities, fish are currently used to characterize and monitor environmental perturbations such as in mine-polluted waters. Water bodies that change from supporting healthy fish populations to ones with few or no fish or that exhibit changes in fish population structure are a

warning of environmental modification. The Index of Biotic Integrity, a habitat assessment protocol, employs fish numbers and diversity as indicators of system health (Helfman, 2007). Fish are used in this method because they are sensitive to water quality, long-lived, and relatively sedentary (Moyle, 1994; Simon and Lyons, 1995). Fish are also important socioeconomically as they provide for human need both directly and indirectly. They provide food, medicine, entertainment, and employment. When the public considers the impact of mining on aquatic systems, it rarely considers the effects on benthic invertebrates; instead, it considers the effects on fish.

1.3.2.1 Rainbow Trout as a Test Species

The wide occurrence, availability, and ease of culture have made rainbow trout one of the most intensively studied fish species in biological research (Øverli et al., 2005). Rainbow trout are excellent toxicity test fish species because they are sensitive to a wide variety of toxicants.

The natural distribution of rainbow trout is in the eastern Pacific Ocean and freshwater west of the Rocky Mountains from Alaska to Mexico (Page and Burr, 1991). Today, this species of fish is thriving all around the world (Lee et al., 1980) and rainbow trout farming has become important in every country that can provide a suitable environment (Sedgwick, 1982). In Canada, rainbow trout can be found from Newfoundland to British Columbia. This species is widely abundant in Canada mainly due to its popularity as a sport-fish species and its economic viability as an aquaculture species (Gall and Crandell, 1992; Currie et al., 2000). Most important to this study is that rainbow trout inhabit waters surrounding molybdenum mines and thus are prone to living in molybdenum-enriched waters. For example, in British Columbia, Wilkie Creek near the MAX Mine contains resident rainbow trout (FortyTwo Metals Inc., 2005) and the Nithi River near the Endako Mine is a prime rainbow trout spawning habitat (Bustard, 1989).

In British Columbia, freshwater resident rainbow trout are not a major conservation issue (McPhail, 2007); however, the anadromous rainbow trout (steelhead) are. Data from a 1996 assessment of steelhead stocks indicate that of 867 stocks, 9 were listed as extirpates, 8 were listed as at high risk of extirpation, and 143 stocks were of special concern (Slaney et al., 1996). Other closely related species such as the cutthroat trout (*O. clarkii clarkii* and *O. clarkii lewisi*) are also at risk of extinction in British Columbia (British Columbia Conservation Data Centre, 2008).

1.4 Objectives

Molybdenum is an element whereby both the lack of and the excess of can impact animals. Knowledge gaps exist in understanding the action of molybdenum on living systems, especially fish. Most of the molybdenum research on fish has focused on growth, mortality, and bioaccumulation studies. Although valuable, these types of studies do not fully assess the mechanism of toxic action. More research is needed to determine how acute molybdenum exposures influence other indicators of fish health such as the stress response. Based on knowledge that exposure to metals such as copper, zinc, and cadmium triggers a stress response in fish it is possible that molybdenum may also have adverse effects on the stress response at the physiological and cellular levels. The link between molybdate and components of the stress response (namely thermotolerance and HSP90) further supports this idea.

The purpose of this study was to investigate the effects of acute sub-lethal waterborne molybdenum exposure on the physiological and cellular stress responses in both fingerling and juvenile rainbow trout. The specific objectives for this study were as follows:

1) To determine what effect molybdenum exposure has on aspects of the physiological stress response, namely plasma cortisol, blood glucose, and % hematocrit.

2) To determine what effect molybdenum exposure has on aspects of the cellular stress response.

    a) To determine if molybdenum induces HSP72, HSP73, and HSP90 levels in the liver, heart, gills, and erythrocytes.

    b) To determine if MT can be induced in the liver and gills upon exposure to molybdenum.

3) Provide a better basis for the development of appropriate regulations for molybdenum in the freshwater aquatic environment.

Table 1. Summary of 96 h LC$_{50}$ molybdenum toxicity studies for various freshwater fish species.

Freshwater Fish Species	Molybdenum Species	Developmental Stage	Water Hardness (mg/L as CaCO$_3$)	Water pH	96 h LC$_{50}$ (mg/L)	Reference
Bluegill (*Lepomis macrochirus*)	(NH$_4$)$_2$Mo$_2$O$_7$	-	-	-	157	Bentley, 1973
	MoO$_3$	-	-	-	87	Bentley, 1975
	Na$_2$MoO$_4$	-	-	-	1320	Easterday and Miller, 1963
	Na$_2$MoO$_4$	-	148	7.1	6790	Bentley, 1973
Fathead minnow (*Pimephales promelas*)	MoO$_3$	-	20	7.4	70	Tarzwell and Henderson, 1960
	MoO$_3$	-	400	8.2	370	Tarzwell and Henderson, 1960
	MoO$_3$	8-10 mm	Hard	8.6	628	Kimball, 1978
	Na$_2$MoO$_4$	-	148	7.1	7630	Bentley, 1973
	Na$_2$MoO$_4$	<24 h post fertilization	112	7.9	>100	Pyle et al., 2001
Kokanee trout (*Oncorhynchus nerka*)	Na$_2$MoO$_4$	1-2 g	78	7.4-7.6	>2000	Reid, 2002
Rainbow trout (*O. mykiss*)	Na$_2$MoO$_4$	55 mm	25	6.9	1320	McConnell, 1977
	Na$_2$MoO$_4$	20 mm	25	6.9	800	McConnell, 1977
	Na$_2$MoO$_4$	-	148	7.1	7340	Bentley, 1973
	Na$_2$MoO$_4$	Alevin, 2.1 g	112	7.9	>1000	Pyle et al., 2001
Chinook salmon (*O. tshawytscha*)	Na$_2$MoO$_4$	Eyed egg, alevin, 0.31, 0.5, 0.7, 1.6 g fry	42	7.6	>1000	Hamilton and Buhl, 1990
Coho salmon (*O. kisutch*)	Na$_2$MoO$_4$	0.5 g	42	7.6	>1000	Hamilton and Buhl, 1990
Stone loach (*Nemacheilus botia*)	(NH$_4$)$_6$Mo$_7$O$_{24}$ •4H$_2$O	5.8 cm	60	- -	211	Pundir, 1989
Silverside (*Basilichthys australis*)	Na$_2$MoO$_4$	-	-	-	>50	Trucco et al., 1990
Northern pike (*Esox lucius*)	Na$_2$MoO$_4$	<24 h post fertilization	112	7.9	>127	Pyle et al., 2001
White sucker (*Catostomus commersoni*)	Na$_2$MoO$_4$	<24 h post fertilization	112	7.9	>2000	Pyle et al., 2001
Flannelmouth sucker (*C. latipinnis*)	Na$_2$MoO$_4$	16 mm	144	7.9	1940	Hamilton and Buhl, 1997
Channel catfish (*Ictalurus puntatus*)	Na$_2$MoO$_4$	-	148	7.1	>10000	Bentley, 1973

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Animals

Juvenile and fingerling rainbow trout (*O. mykiss*) of either sex were purchased from Campbell Lake Trout Hatchery (Little Fort, BC, Canada) and transported to the University of British Columbia Okanagan (Kelowna, BC, Canada). Fish were held indoors in 600 L circular polyethylene tanks (juveniles) or 70 L square polyethylene tanks (fingerlings) supplied with continuously flowing, aerated, and dechlorinated City of Kelowna (Glenmore-Ellison Improvement District) tap water [see Reid (2002) for water ionic composition]. Water temperature varied between 10 and 13°C. Photoperiod was set to a 12:12 h light/dark cycle. Fish were acclimated to these conditions for at least four weeks prior to experimentation. Trout were fed 2% of their body weight with a diet of commercial fish pellets every second day. Food was withheld 48 h prior to experimentation. Experiments reported in this study were undertaken in January, July, and August of 2008.

Experimental procedures were carried out in accordance with requirements outlined by the Canadian Council of Animal Care and were approved by the University of British Columbia Animal Care Committee.

2.2 Animal Preparations and Experimental Set-up

2.2.1 Cannulation of the Dorsal Aorta

Juvenile fish were anesthetized in a 0.1 g/L solution of ethyl *m*-aminobenzoate methanesulfonate (MS-222; Sigma-Aldrich) adjusted to pH 7.5 with $NaHCO_3$ (Fisher Scientific). Fish were then placed onto an operating table to allow continuous retrograde irrigation of the gills with anesthetic solution. To permit periodic blood sampling, an indwelling cannula was

implanted into the dorsal aorta according to standard techniques (Soivio et al., 1975) using flexible polyethylene tubing (Clay-Adams PE-50). Fish were revived on the operating table by irrigation of the gills with aerated water and then transferred to individual exposure chambers. 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 ammonium heparin, Sigma-Aldrich) Cortland saline [7.25 g/L NaCl, 0.38 g/L KCl, 0.16 g/L CaCl$_2$, 0.47 g/L MgSO$_4$•7H$_2$0, 0.40 g/L Na$_2$H$_2$PO$_4$•H$_2$O, 1.0 g/L NaHCO$_3$, pH 7.8; Fisher Scientific] to prevent clotting (Wolf, 1963).

2.2.2 Exposure Chambers

Upon exposure initiation fish were randomly assigned to black plastic experimental boxes (3.0-3.5 L) supplied with continuously flowing, aerated, and dechlorinated water. The boxes were placed on a wet table receiving a constant flow of water for temperature control. Juvenile fish were placed one per box and fingerling fish were placed eight per box. Replicates were arranged on the wet table in random order to reduce systematic bias of results. Fish were allowed to acclimate to the exposure boxes for 48 h prior to experimentation.

2.3 Waterborne Exposure to Molybdenum

Fish were exposed for 96 h in a static system to 0, 2, 20, or 1000 mg/L of molybdenum. Two mg/L was chosen as the minimum concentration as it is the molybdenum water quality limit for the protection of freshwater aquatic life set by the province of British Columbia (Swain, 1986).

Molybdenum was delivered as sodium molybdate dihydrate (Na$_2$MoO$_4$•2H$_2$O; Fisher Scientific) in the same water used for fish husbandry. Existing molybdenum in the water was less than 0.0010 mg/L (Caro Analytical Services, 2008). At time 0, 100% of the water was

replaced with the appropriate molybdenum treatment. To ensure that water quality and molybdenum levels were adequately maintained water was replaced every 8 h. The following water quality parameters were monitored throughout the experiments: temperature (10-13°C) general hardness (140 ± 12 mg/L $CaCO_3$), pH (8.0 ± 0.2), and ammonia. The latter three parameters were measured using a freshwater master test kit (Aquarium Pharmaceuticals). Fish were not fed throughout the 96 h exposure in order to ensure that the source of molybdenum was waterborne and not dietary. Human activity within the environmental room was kept to an absolute minimum.

2.3.1 Experiment I: Time Course Molybdenum Exposure to Cannulated Juvenile Fish

Cannulated juvenile fish (363.3 ± 18.1 g, 31.8 ± 1.7 cm, n = 12) exposed to 0, 2, or 20 mg/L had their dorsal aortic cannulas sampled immediately prior to initiation of exposure and at 8, 24, and 96 h during exposure for analysis of plasma cortisol, blood glucose, and hematocrit. Fish were processed randomly across all treatments to minimize systematic measurement error. Blood (0.5 mL) was withdrawn using a syringe previously rinsed with heparinized Cortland saline. Approximately 0.3 mL of Cortland saline was reinjected into the cannula after each blood collection to partially maintain blood volume. Immediately upon collection, whole blood was analyzed for blood glucose concentration and % hematocrit before being centrifuged at 10000 rpm for 2 min on an Eppendorf mini spin plus (Cole-Parmer) to separate the plasma from the erythrocytes. All plasma samples were stored at -80°C until the determination of cortisol levels. Twenty-four hours after the end of the exposure period fish were sacrificed from a cephalic blow. Weight and fork length (from the tip of the snout to the tip of the compressed tail) were recorded.

2.3.2 Experiment II: 96 h Endpoint Molybdenum Exposure to Juvenile Fish

Non-cannulated juvenile fish (320.3 ± 21.8 g, 29.5 ± 0.6 cm, n = 12, four fish per treatment) exposed to 0, 2, or 20 mg/L were sampled at 96 h. Fish were processed randomly across all treatments to minimize systematic measurement error. Fish were sacrificed from a sharp blow to the head. Weight and fork length were recorded. Sampling of each fish was done on ice and took no more than 5 min. Blood (0.5 mL) was sampled by caudal puncture using a syringe previously rinsed with heparinized Cortland saline. Upon collection, blood was analyzed for glucose concentration and % hematocrit before being centrifuged at 10000 rpm for 2 min on an Eppendorf mini spin plus (Cole-Parmer) to separate the plasma from the erythrocytes. Plasma and erythrocyte samples were stored at -80°C until the determination of cortisol and HSP levels. Following blood collection, the gills were perfused with Cortland saline and excised from both the fish and the gill arch. Excess blood and blood clots were removed from the external surface of the gill by rinsing the gills in the same water used for fish husbandry. 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 for later analysis of HSPs and/or MTs.

2.3.3 Experiment III: Time Course Molybdenum Exposure to Fingerling Fish

Fingerling fish (11.3 ± 1.1 g, 9.7 ± 0.1 cm, n = 64, sixteen fish per treatment) exposed to 0, 2, 20, or 1000 mg/L were sampled prior to initiation of exposure and at 8, 24, and 96 h during exposure. Fish were processed randomly across all treatments to minimize systematic measurement error. Fish were sacrificed from a cephalic blow and measured for weight and fork length. Sampling of each fish was done on ice and took no more than 3 min. Blood was collected into a heparinized hematocrit tube by means of capillary action after severing the caudal peduncle with a razor blade and immediately analyzed for glucose concentration and % hematocrit. The liver and gills were excised. Gill tissue was removed from the gill arch and

rinsed in the same water used for fish husbandry. All tissues were flash frozen in liquid nitrogen
and stored at -80°C for later analysis of HSPs and/or MTs.

2.4 Analysis of Physiological Stress Response Parameters

2.4.1 Hematocrit

Hematocrit (% erythrocytes) values were measured in duplicate by means of micro
hematocrit tubes (Fisher Scientific). Tubes were spun at 2510 rpm for 5 min on a clinical
centrifuge fitted with a hematocrit head (International Equipment Company).

2.4.2 Blood Glucose

Glucose (mM) was measured from whole blood using a hand-held, battery-operated
blood glucose meter designed for clinical use. The Precision Xtra™ (Abbott Laboratories) blood
glucose system records in the range of 1.1-27.8 mM. In this method, a drop of blood (0.6 uL)
placed onto the disposable test strip employs a glucose dehydrogenase reaction. This reaction
converts glucose to gluconic acid, which generates an amperometric reaction through two silver
bars. The electrons produced from the reaction form a current that is then calibrated to measure
glucose concentration (Wang, 2000).

Other brands of glucose meters such as One Touch II™ (Page et al., 1999), Advantage™
(Wells and Pankhurst, 1999), and Glucotrend™ (Glencross et al., 2004) have been used for
glucose analysis in rainbow trout. Page et al. (1999) previously validated the use of glucose
meters by comparing glucose values measured on the meter against the same samples measured
with a hexokinase enzymatic glucose kit. The comparison revealed a coefficient of
determination of 95.4%.

2.4.3 Plasma Cortisol

Plasma cortisol levels (ng/mL) were determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Neogen Corporation). This test kit has an assay range of 0.04-10 ng/mL, a coefficient of variation $\leq 10\%$ for both an inter- and intra-assay, and displays cross-reactivity with corticosterone (4.8%), 11-deoxycortisol (15.0%), and cortisone (15.8%). This particular kit has previously been used to measure plasma cortisol levels in various fish species such as rainbow trout (Basu et al., 2003), chinook salmon (*O. tshawytscha*; Afonso et al., 2003), haddock (*Melanogrammus aeglefinus* L.; Afonso et al., 2008), cod (*Gadus morhua* L.; Pérez-Casanova et al., 2008), Atlantic salmon (*Salmo salar* L.; Fast et al., 2007), and killifish (*Fundulus heteroclitus*; Scott et al., 2004).

All chemicals and protocols relevant for the extraction and incubation of cortisol were supplied with the kit. Cortisol was extracted from the plasma prior to the assay. Previously frozen plasma samples were allowed to defrost on ice. A 100 uL plasma aliquot was added to 1 mL of ethyl acetate in a glass test tube. The solution was mixed for 30 sec and the two phases allowed to separate. The organic phase was then transferred to a 1.5 mL Eppendorf tube and the solvent evaporated with a stream of air. The residue was dissolved in 100 uL of diluted extraction buffer and then further diluted 1:100 in extraction buffer prior to assaying. The assay was conducted by incubating 50 uL of standards or diluted cortisol extracts with 50 uL of enzyme conjugate (cortisol labeled with horseradish peroxidase) in wells of the cortisol antibody coated microtiter plate for 1 h at room temperature. The wells were then washed three times and incubated with 150 uL of substrate (3'3'5'5' tetramethylbenzidine and hydrogen peroxide) for 30 min at room temperature. The plate was read at 630 nm on an OpsysMR™ microplate reader (Dynex Technologies). Absorbance values were used to calculate the percentage of maximal binding. A standard curve was generated and values were linearized using a logit

transformation. Unknown values were extrapolated and retransformed. These retransformed values were corrected for the 1:100 dilution performed after the extraction.

Estradiol (Sigma-Aldrich), a steroid similar in structure to cortisol, was used as a negative control. Cortisol recovery was determined by spiking separate parallel plasma samples with 91 ng/mL of cortisol and comparing the calculated amount with the corresponding unspiked sample. Cortisol recoveries from the samples were approximately 87%.

2.5 Analysis of Cellular Stress Response Parameters

2.5.1 Preparation of HSP70 and MT Positive Controls

In order to obtain a positive control for HSP70 analysis, a juvenile fish (433.8 g, 33.1 cm) was placed in a 48 L tank connected to a heater and allowed to recover from transfer for 1 h prior to experimentation. The fish was heat shocked according to Rendell et al. (2006) by raising the water temperature 3°C/h from 13°C to 25°C and then maintaining this heat shock temperature for 1 h. Following heat shock, the water temperature was slowly returned to 13°C over a 2 h period. The fish remained at this temperature for 20 h. The fish was sacrificed and the liver, gills, heart, and erythrocytes were collected, immersed in liquid nitrogen, and transferred to -80°C for later protein analysis.

The positive control for metallothionein, copper exposed hepatocytes (prepared according to Weil, 2005), was a gift from M.M. Vijayan (University of Waterloo). As there was not enough sample to run numerous gels, the copper exposed hepatocytes were only used to test the specificity and efficacy of the MT primary antibody. For MT analysis, fingerling fish $(7.0 \pm 0.8$ g, 7.9 ± 0.2 cm) were exposed to 33 uM $CuSO_4 \cdot 5H_2O$ (Fisher Scientific) and after 24 h of exposure the liver was excised, immersed in liquid nitrogen, and transferred to -80°C for later protein analysis.

2.5.2 Protein Isolation

Whole liver, gill, and heart tissue and erythrocytes were added 1:1 (w/v) to ice-cold homogenization buffer [100 mM Tris (Bio-Rad)-HCl pH 7.5, 0.1% sodium dodecyl sulfate (SDS; Bio-Rad), and a SigmaFAST™ protease inhibitor tablet containing 2 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl), 1 mM EDTA (ethylenediaminetetraacetic acid), 130 uM bestatin, 14 uM E-64, 1 uM leupeptin, and 0.3 uM aprotinin (Sigma-Aldrich)]. Samples were sonicated on ice using a Model 100 Ultrasonic Dismembrator (Fisher Scientific). Sonication (level/time) was as follows: liver (3/3 x 6 sec), gills (5/3 x 6 sec), heart (3/3 x 6 sec), and erythrocytes (3/1 x 10 sec). After sonication, samples were cleared by centrifugation at 10000 g for 3 min at room temperature (Afonso et al., 2003; Hori et al., 2008). The supernatant containing the protein was isolated and kept at -80°C until use.

2.5.3 Protein Quantification

Protein concentrations (mg/mL) were determined by means of the bicinchoninic acid (BCA) protein assay, according to the manufacturers protocol (Sigma-Aldrich), with bovine serum albumin (BSA; Sigma-Aldrich) as the protein standard. Standards and samples were loaded in triplicate onto a polystyrene 96 well assay plate in 10 uL aliquots with the addition of 200 uL of BCA solution (reagent A; 50 parts) and 4% copper (II) sulfate pentahydrate (reagent B; 1 part). Samples were mixed and the plates were incubated for 30 min at 37°C. Reactions were analyzed at 490 nm using an OpsysMR™ microplate reader (Dynex Technologies). BSA standards were used to construct a standard curve in order to determine the protein concentration of each sample. This concentration in mg/mL was used to calculate mg protein/g tissue wet weight.

The remaining supernatant was mixed 1:1 (v/v) with 2X SDS-sample dilution buffer [12 mL 0.5 mM Tris-HCl pH 6.8, 9.6 mL glycerol (Sigma-Aldrich), 19.2 mL 10% SDS, 2.4 mL

0.05% bromophenol blue (Sigma-Aldrich), and 4.8 mL -mercaptoethanol (Sigma-Aldrich);

Laemmli, 1970], boiled at 95°C for 3 min, cooled, and then stored at -80°C. Prior to analysis

and in order to equalize total protein concentration all of the samples were further diluted in 1X

SDS-sample dilution buffer (Hori et al., 2008).

2.5.4 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Sample protein (10 ug total protein for HSP and 25 ug liver/27.5 ug gill total protein for

MT analysis) was separated using a discontinuous polyacrylamide gel consisting of a 4%

stacking gel and a 12% separating gel for HSPs or 15% separating gel for MTs on a Mini-

Protean III electrophoresis cell (Bio-Rad). Each gel contained a Dual Color Precision Plus

Protein™ Standard (Bio-Rad) and a MagicMark™ XP Western Protein Standard (Invitrogen) in

the first and last lane, respectively. A positive control, either 10 ug of protein from heat shocked

fish samples, 25 or 27.5 ug of copper exposed fish samples, 50 ng of purified bovine brain

HSP70 (Sigma-Aldrich, H-9776), or 100 ng of purified human HSP90 standard (Assay Designs,

SPP-770) was loaded in at least duplicate. Positive controls were used to calibrate the bands on

each blot to enable comparisons between blots. Gels were electrophoresed with 1X electrode

running buffer [25 mM Tris, 0.2 M glycine (Bio-Rad), and 1 mM SDS] at 75 V until samples

reached the separating gel at which time the voltage was increased to 150 V until the dye front

reached the bottom of the gel.

2.5.5 Western Blotting

Following electrophoretic separation the gel and membrane were equilibrated in Towbin

transfer buffer [25 mM Tris, 192 mM glycine, and 20% MeOH (Fisher Scientific), pH 8.3] for 25

min. The Hybond-P polyvinylidene fluoride (PVDF; GE Healthcare) membrane was prepared

according to the manufacturer's protocol. The proteins were transferred from the gel onto the

membrane using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) containing Towbin transfer buffer at 100 V for 1 h for HSPs or at 80 V for 1 h for MTs. After transfer, the blot was stained with Ponceau-S (Sigma-Aldrich) stain [0.5% (w/v) Ponceau-S red and 1% (v/v) acetic acid (Fisher Scientific)] for 5 min to determine the success of the transfer and equal loading. The membrane was briefly destained with distilled water. Successfully transferred proteins appeared as bright red bands on the membrane. Images were scanned using an AGFA SnapScan e50 scanner. 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 (Assay Designs, CSA-335).

2.5.6 Primary and Secondary Antibodies

The primary antibodies used in this study are either fish specific or have previously been used to detect fish proteins. The polyclonal rabbit anti-HSP70 primary antibody (Agrisera, AS05061) detects only the inducible form (HSP72) and not the constitutive form (HSP73) of HSP70. The peptide sequence is specific to the C-terminal portion of salmonid HSP70. The polyclonal rabbit anti-HSP90 primary antibody (Agrisera, AS05063) detects both the alpha and beta form of the protein. In fish, a cross-reactive band is detected at approximately 40kDa. Rendell et al. (2006) previously tested the specificity of the above two primary antibodies by premixing the antibodies with excess free peptide. Their study found that the treatment efficiently blocked detection of the proteins on the immunoblot. The monoclonal mouse anti-bovine HSP70 (BRM-22) primary antibody (Sigma-Aldrich, H-5147), which recognizes both HSP72 and HSP73, has been shown to cross-react with rainbow trout (Weber et al., 2002) and the polyclonal rabbit anti-cod (KH-1) MT primary antibody (Biosense Laboratories, M04406210-500) has been shown to cross-react with salmonid fish species (Hylland et al., 1995).

All mouse host primary antibodies were detected using goat anti-mouse (Assay Designs, SAB 100) and all rabbit host primary antibodies were detected with goat anti-rabbit (Assay Designs, SAB 300) secondary antibodies. Both secondary antibodies were conjugated with horseradish peroxidase.

2.5.7 Immunodetection of Proteins

Following Western transfer, the PVDF membranes were incubated in 5% skim milk powder (EMD Chemicals) in Tween-Tris buffered saline [TTBS; 20 mM Tris, 500 mM NaCl, pH 7.6, 0.1% Tween-20 (Bio-Rad)] for 1 h to prevent non-specific binding. The membranes were then briefly rinsed using two changes of TTBS and probed for 1 h with primary antibody diluted in TTBS. Primary antibody concentrations used for the detection of proteins in each tissues sampled are listed in Table 2. Excess unbound antibody was removed by washing the membranes two times for 10 min in TTBS. The membranes were then incubated for 1 h with secondary antibody diluted in TTBS. Appropriate secondary antibody concentrations used in this study are listed in Table 2. Membranes were washed in TTBS three times for 10 min. All incubations were done at room temperature on an orbital shaker (Cole-Parmer) at 40 rpm.

2.5.8 Enhanced Chemiluminescence and Densitometry

Protein detection was performed using the enhanced chemiluminescence system (ECL; GE Healthcare) according to the manufacturer's instructions. The chemiluminescence was captured using autoradiography Hyperfilm[TM] (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.

The band intensities for all the proteins were quantified using Quantity One software (Version 4.6.3; Bio-Rad) (Clarkson et al., 2005; Afonso et al., 2008; Hori et al., 2008). SDS-

PAGE and Western blotting steps were repeated in duplicate and the average densitometric

values were expressed as a percentage of positive controls to give relative band density.

2.6 Statistics and Data Analyses

All data are presented as means ± SEM (standard error mean). Data were analyzed using

JMP IN statistical software (Version 7.0.1; SAS). P values of less than 0.05 were considered

significant for all statistical tests. 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 Honestly Significant Differences (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.

Table 2. List of primary antibodies and their respective secondary antibody used for Western blotting on various tissues from fingerling and juvenile rainbow trout.

Antibody	Tissue Type							
	Liver		Gill		Heart		Erythrocyte	
	Fingerling	Juvenile	Fingerling	Juvenile	Fingerling	Juvenile	Fingerling	Juvenile
Anti-HSP72 (Agrisera, AS05061)	1:5000	1:5000	1:20000	1:20000	1:5000	1:5000	1:5000	1:5000
Anti-rabbit 2° antibody (Assay Designs, SAB-300)	1:10000	1:10000	1:40000	1:40000	1:10000	1:10000	1:10000	1:10000
Anti-HSP70 (Sigma, H-5147)	1:5000	1:5000	1:5000	1:5000	1:5000	1:5000	1:5000	1:5000
Anti-mouse 2° antibody (Assay Designs, SAB-100)	1:10000	1:10000	1:10000	1:10000	1:10000	1:10000	1:10000	1:10000
Anti-HSP90 (Agrisera, AS05063)	1:5000	1:5000	-	1:20000	1:5000	1:5000	1:5000	1:5000
Anti-rabbit 2° antibody (Assay Designs, SAB-300)	1:10000	1:10000	-	1:40000	1:10000	1:10000	1:10000	1:10000
Anti-MT (Biosence, M04406210-500)	1:1000	1:1000	-	1:500	-	-	-	-
Anti-rabbit 2° antibody (Assay Designs, SAB-300)	1:5000	1:5000	-	1:6000	-	-	-	-
Anti-GAPDH (Assay Designs CSA-335)	-	-	-	-	-	-	-	1:3000
Anti-rabbit 2° antibody (Assay Designs, SAB-300)	-	-	-	-	-	-	-	1:4000

CHAPTER 3: RESULTS

3.1 Survival

No mortalities occurred during the course of exposure.

3.2 Physiological Stress Response Parameters

3.2.1 Plasma Cortisol

Pre-exposure levels of plasma cortisol ranged from 3.3-82.5 ng/mL, with a mean of 25.1 ± 7.2 ng/mL, in all treatments of cannulated juvenile fish (Figure 1A). Cortisol levels were not affected by sampling time (pre-exposure, 8, 24, and 96 h) or molybdenum treatment (0, 2, or 20 mg/L) and no interaction between these two main effects was detected. The large variation in data observed prior to exposure and at 8 h decreased by 24 h and by 96 h the individual treatment means were within a 4 ng/mL range. Exposure to the same molybdenum concentrations had no detectable effect on plasma cortisol levels in non-cannulated juvenile fish sampled at 96 h (Figure 2A). The 96 h pooled treatment means in non-cannulated (33.6 ± 7.7 ng/mL) and cannulated (26.1 ± 5.6 ng/mL) fish were not significantly different.

3.2.2 Blood Glucose

In cannulated juvenile fish, blood glucose levels were significantly affected by sampling time (pre-exposure, 8, 24, and 96 h; p = 0.0397) but not by molybdenum treatment (0, 2, or 20 mg/L). No interaction between these two main effects was detected. Prior to molybdenum exposure blood glucose levels ranged from 2.6-18.4 mM, with a mean of 7.1 ± 1.3 mM, in all treatments of cannulated fish (Figure 1B). When treatment data was pooled to obtain a mean for each sampling time there was an observable decrease in blood glucose overtime. Compared to the pre-exposure pooled treatment mean the pooled treatment mean at 96 h had decreased by

34%. The high glucose levels and variation in data observed in the control fish, at all time

points, were the result of one fish. Removing this particular fish lowered the pre-exposure

pooled treatment mean to 6.1 ± 0.8 mM (identical to the pre-exposure pooled treatment mean in

fingerling fish) and the pooled treatment means at 8, 24, and 96 h to 5.7 ± 1.1, 5.2 ± 1.0, and 3.8

± 0.6 mM, respectively. These new control values are similar to those measured in the 2 and 20

mg/L treatment groups of cannulated juvenile fish. In non-cannulated juvenile fish exposed to

the same conditions the control and molybdenum exposure groups demonstrated similar blood

glucose levels when sampled at 96 h (Figure 2B). The 96 h pooled treatment mean in non-

cannulated juvenile fish (5.3 ± 0.5 mM) was similar to the 96 h pooled treatment mean in

cannulated juvenile fish (4.5 ± 1.0 mM).

Prior to initiation of exposure, there was no difference in blood glucose levels in

fingerling fish sampled from each of the different exposure chambers (0, 2, 20, and 1000 mg/L).

Pre-exposure blood glucose levels in all fingerling fish ranged from 1.8-14.0 mM, with a mean

of 6.1 ± 0.8 mM (Figure 3A). Molybdenum treatment did not alter blood glucose levels in

fingerling fish, even at concentrations of 1000 mg/L whereas sampling time did (8, 24, and 96 h;

$p = 0.0252$) (Figure 3A). There was no detectable interaction between these two main effects.

The 8 h (3.4 ± 0.4 mM) and 24 h (4.6 ± 0.4 mM) pooled treatment means, although significantly

different from each other, did not differ from the 96 h pooled treatment mean (3.6 ± 0.2 mM).

Despite the lack of a declining trend in the data, the pooled treatment mean at all three time

points during exposure were lower than the pre-exposure pooled treatment mean. The 96 h

pooled treatment mean in fingerling fish was not different from the 96 h pooled treatment mean

in cannulated or non-cannulated juvenile fish.

3.2.3 Hematocrit

Pre-exposure hematocrit values for all cannulated juvenile fish ranged from 10.5-32.1%, with an average of 23.4 ± 1.9% (Figure 1C). There were no significant differences in hematocrit levels through time within each group (0, 2, 20, or 1000 mg/L) or between groups at a specific time (pre-exposure, 8, 24, and 96 h). A significant main effect of time (p = 0.0009) but not of treatment was observed. At 8 and 24 h, the pooled treatment mean decreased 4.1% and 7.5%, respectively, relative to the pre-exposure pooled treatment mean. Over the next 48 h when no sampling occurred the pooled treatment mean increased and was only 6.5% lower than the pre-exposure pooled treatment mean. Only the pooled treatment mean at both 8 and 24 h was significantly different from the pre-exposure pooled treatment mean. Non-cannulated juvenile fish subjected to the same conditions did not express differences in hematocrit when sampled at 96 h (Figure 2C). The 96 h pooled treatment mean in non-cannulated juvenile fish (30.3 ± 2.9%) was approximately two fold higher than the 96 h pooled treatment mean in cannulated juvenile fish (16.0 ±1.4%).

Hematocrit levels in fingerling fish taken from each of the different exposure chambers (0, 2, 20, and 1000 mg/L) were not significantly different prior to initiation of exposure (Figure 3B). Pre-exposure hematocrit levels in all fingerling fish ranged from 20.5-50.4%, with a mean of 33.9 ± 2.0%. Over the 96 h exposure period to 0, 2, 20, or 1000 mg/L of molybdenum all groups responded in a similar manner (Figure 3B). Molybdenum exposure had no effect on hematocrit whereas sampling time did (8, 24, and 96 h; p = 0.0011). Comparing pooled treatment means at 8, 24, and 96 h revealed a decrease in hematocrit overtime. At 8 h the pooled treatment mean was 44.2 ± 1.9% and by 96 h the pooled treatment mean had decreased by 10% to the same level as the pre-exposure pooled treatment mean. The 96 h pooled treatment mean in fingerling fish (33.9 ± 1.9%) was comparable to the 96 h pooled treatment mean in non-cannulated juvenile fish.

3.3 Cellular Stress Response Parameters

3.3.1 Total Protein Concentration

Total liver, gill, heart, and erythrocyte protein concentrations from juvenile fish sampled after 96 h of exposure to 2 or 20 mg/L of molybdenum showed no statistically significant differences when compared to controls (Figure 4). When all data were pooled, ignoring treatment, erythrocytes had the highest concentration of protein (322 mg/g wet weight) followed by the liver (139 mg/g tissue wet weight), heart (97 mg/g tissue wet weight), and gills (80 mg/g tissue wet weight).

Similar results were observed in fingerling fish. Those fingerling fish sampled from each of the different exposure chambers (0, 2, 20, and 1000 mg/L) showed no significant difference in liver or gill total protein concentration prior to exposure (Figure 5). Pre-exposure total protein levels in the liver and gills were 198.9 ± 2.7 and 67.4 ± 0.5 mg/g tissue wet weight, respectively. During the exposure period all fish responded in a similar manner in both liver and gill tissue (Figure 5). There was also no treatment (0, 2, 20, or 20 mg/L) or time (8, 24, and 96 h) effect in either tissue.

3.3.2 Equal Protein Loading

Figure 6 shows a representative PVDF membrane containing a protein band from the 30-50 kDa region visualized with Ponceau-S in liver, gill, and heart tissue as well as representative Western blot of erythrocyte GAPDH. Juvenile fish were exposed to 0, 2, or 20 mg/L for 96 h. Equal loading was apparent in all experiments thus any significant differences/similarities seen in HSP72, HSP73, HSP90, or MT protein levels are a result of molybdenum exposure.

3.3.3 HSP72

The liver, gills, heart, and erythrocytes from juvenile trout were analyzed for HSP72 induction after a 96 h molybdenum exposure to 2 or 20 mg/L. There was no detectable induction of HSP72 under control conditions or after 96 h of metal exposure in the gills or heart. In liver tissue, two of the 12 fish expressed HSP72, but only at 58% (control fish) and 31% (2 mg/L exposed fish) of the heat shocked positive control. Overall, there was no significant difference between control fish and molybdenum exposed fish. In erythrocytes, all 12 fish expressed HSP72 at about 35-50% of the heat shocked positive control. Despite detecting low basal levels of this stress protein in the erythrocytes there was no significant difference between molybdenum exposed groups and controls (Figure 7).

Fingerling fish exposed to molybdenum of up to 1000 mg/L did not express HSP72 prior to exposure or at 8, 24, and 96 h during exposure in the gills. In the liver, however, 13 of the 64 fish expressed HSP72. Those fish expressing HSP72 had levels that were, in all cases, less than 20% of the positive control (heat shocked fish). Still, there were no significant differences in liver HSP72 levels in fish sampled from the different exposure chambers (0, 2, 20, and 1000 mg/L) prior to exposure. During exposure, there was also no significant main effect of treatment or time nor was there an interaction between these two main effects.

3.3.4 Total HSP70

Figure 8 shows that the expression of total HSP70 (HSP72 and HSP73) in the liver, gills, heart, and erythrocytes of juvenile rainbow trout remained at control levels after the 96 h exposure period to 2 or 20 mg/L.

Fingerling fish sampled from each of the exposure chambers (0, 2, 20, and 1000 mg/L) prior to initiation of molybdenum exposure did not significantly differ in their liver and gill total HSP70 levels (Figure 9). During the 96 h exposure, total HSP70 levels in the liver and gills were

not affected by sampling time (8, 24, and 96 h) or molybdenum treatment (0, 2, 20, or 1000 mg/L) and no detectable effect of the interaction between these two factors was observed (Figure 9).

3.3.5 HSP90

Levels of HSP90 in the liver, gills, heart, and erythrocytes of juvenile fish did not change relative to controls after 96 h exposure to 2 or 20 mg/L (Figure 10).

Figure 11 shows that fingerling fish sampled from each of the exposure chambers (0, 2, 20, and 1000 mg/L) prior to addition of molybdenum did not differ in liver HSP90 levels. Upon addition of the metal, a 96 h time course analysis of HSP90 expression in the liver did not reveal significant differences within or between groups of fish exposed to levels of up to 1000 mg/L (Figure 11). There were no significant main effects of sampling time (8, 24, and 96 h) or treatment (0, 2, 20, or 1000 mg/L).

3.3.6 MT

Analysis of MT band density in liver and gill tissue of juvenile fish did not reveal any significant differences between control fish and molybdenum exposed (2 or 20 mg/L) fish after 96 h of exposure (Figure 12).

Fingerling fish that were sampled from each of the different exposure chambers (0, 2, 20, and 1000 mg/L) prior to molybdenum exposure expressed similar MT levels in the liver. During molybdenum exposure, liver MT levels were not affected by sampling time (8, 24 and 96 h) or treatment (0, 2, 20, or 1000 mg/L) and no interaction between these two main effects was detected.
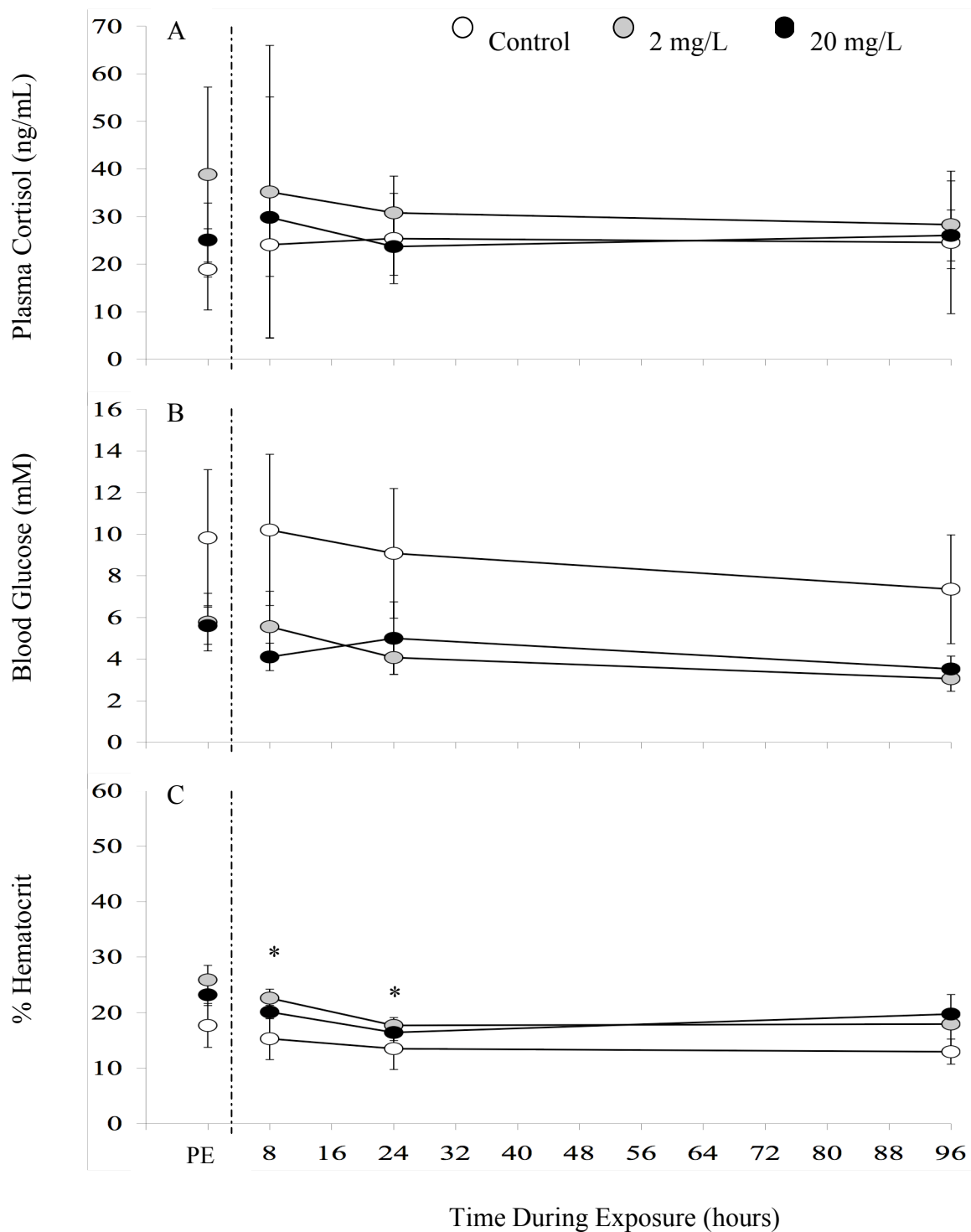
Figure 1. Plasma cortisol (ng/mL) (A), blood glucose (mM) (B), and hematocrit (%) (C) levels in cannulated juvenile rainbow trout exposed to 0, 2, or 20 mg/L molybdenum for 96 h. Fish were sampled prior to metal exposure (PE = pre-exposure) and at 8, 24, and 96 h during exposure. Values are expressed as means ± SEM, where n = 4. There were no significant differences ($p > 0.05$) through time within each group or between groups at a specific time. Asterix (*) represent a significant ($p < 0.05$) time contrast between pre-exposure and a specific time during exposure. Not all data points or error bars may be visible where they overlap.
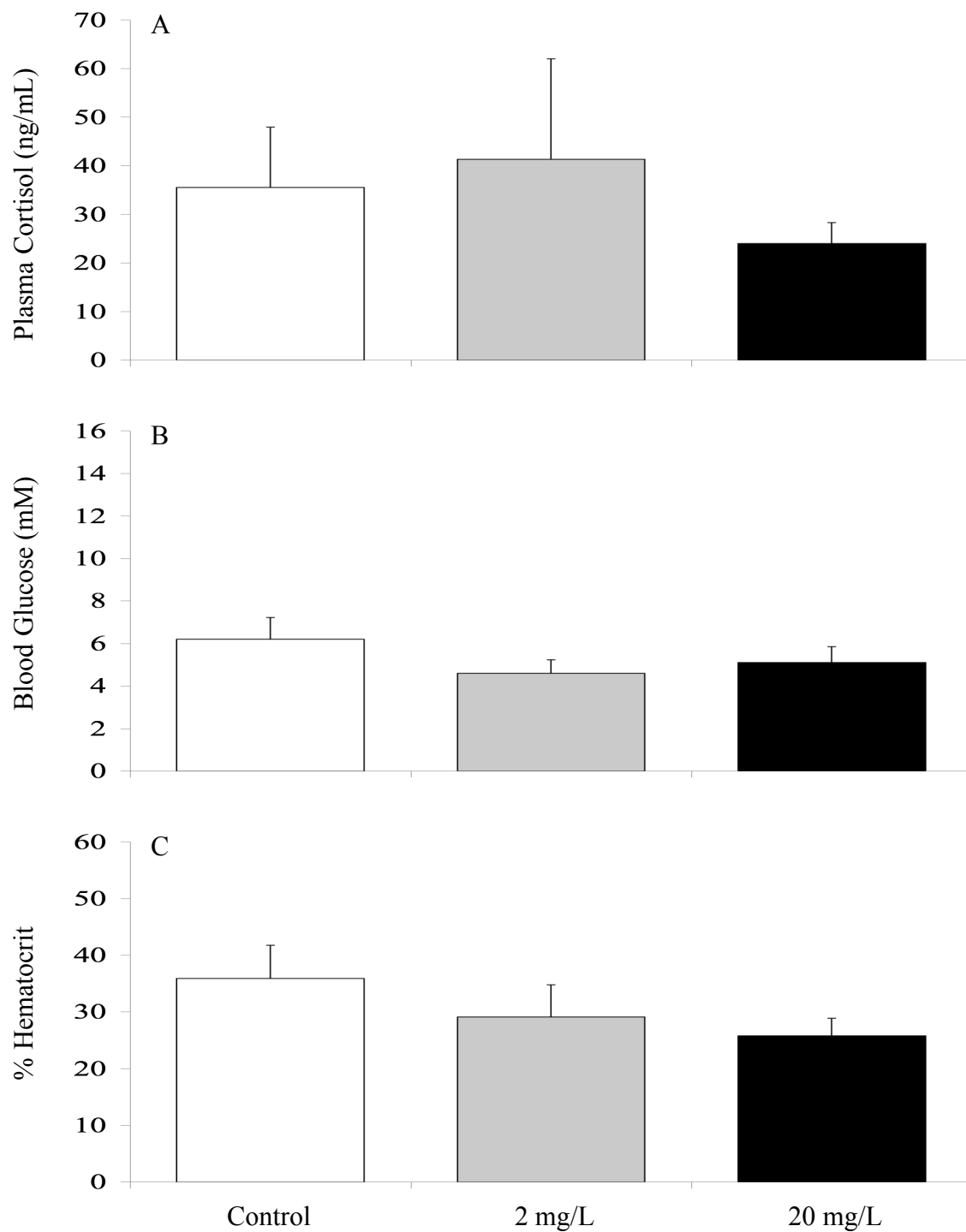
Figure 2. Plasma cortisol (ng/mL) (A), blood glucose (mM) (B), and hematocrit (%) (C) levels in non-cannulated juvenile rainbow trout exposed to 0, 2, or 20 mg/L molybdenum for 96 h. Fish were sampled at 96 h. Values are expressed as means ± SEM, where n = 4. There were no significant differences (p > 0.05) between groups.
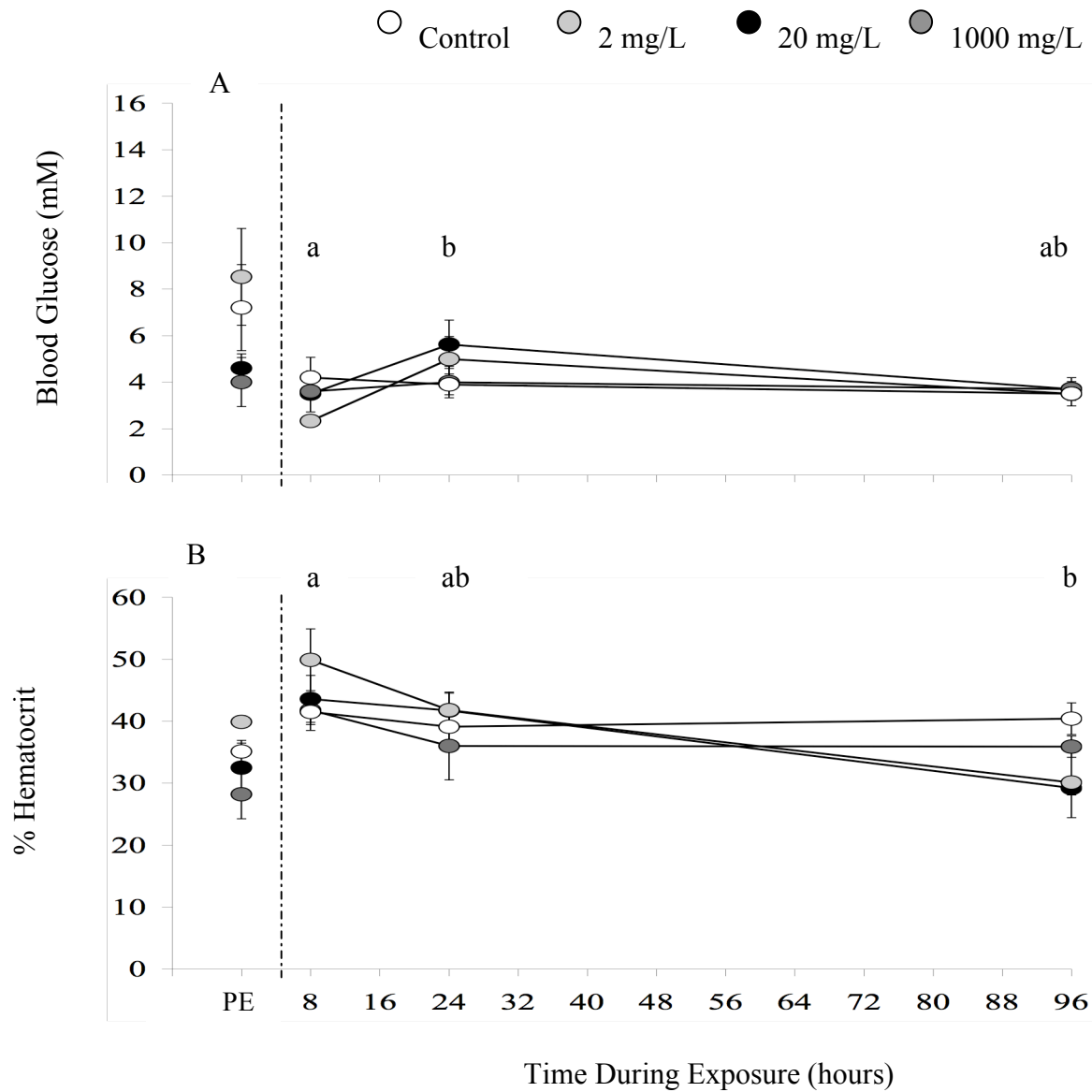
Figure 3. Blood glucose (ng/mL) (A) and hematocrit (%) (B) levels in fingerling rainbow trout exposed to 0, 2, 20, or 1000 mg/L molybdenum for 96 h. Fish were sampled prior to exposure (PE = pre-exposure) and at 8, 24, and 96 h during exposure. Values are expressed as means ± SEM, where n = 4. There were no significant differences (p > 0.05) between groups prior to exposure. During exposure, there were no significant differences (p > 0.05) through time within each group or between groups at a specific time during exposure. Significant (p < 0.05) time effects are identified by lower case letters. Not all data points or error bars may be visible where they overlap.
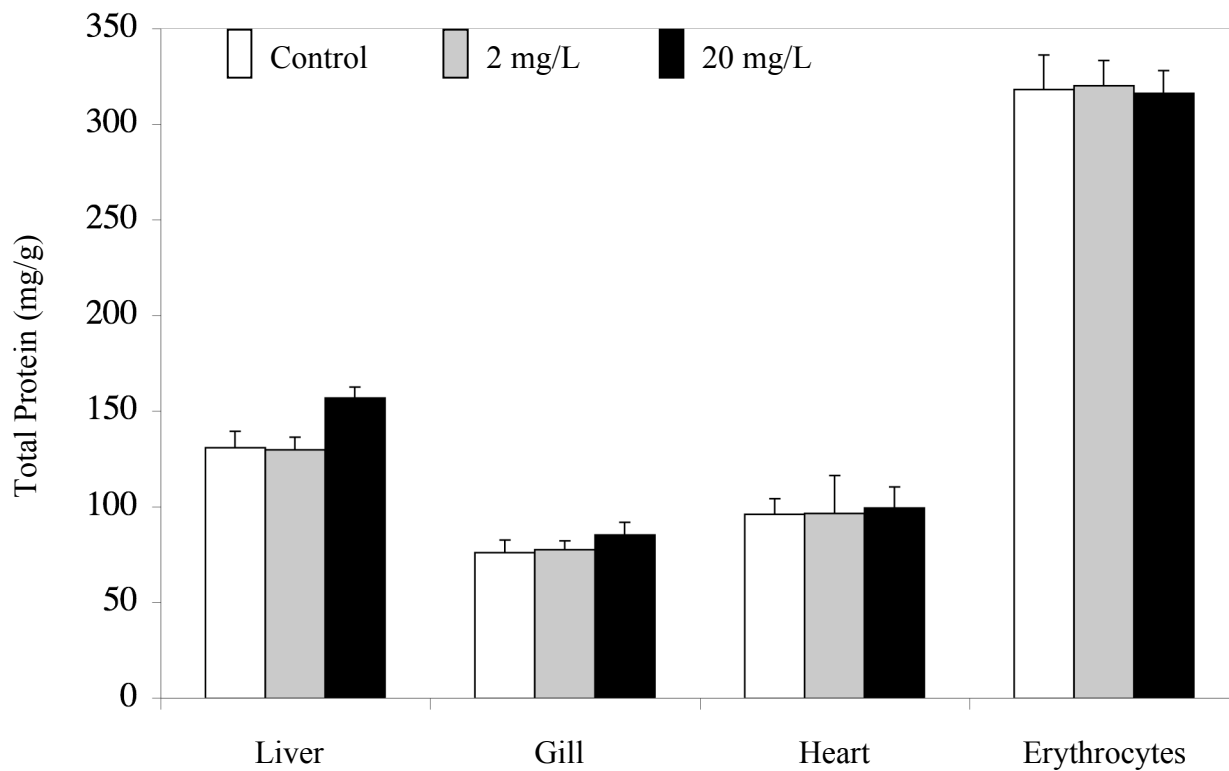
Figure 4. Mean liver, gill, heart, and erythrocyte total protein concentration (mg/g tissue wet weight) in juvenile rainbow trout after a 96 h molybdenum exposure to 0, 2, or 20 mg/L. Fish were sampled at 96 h. Values are expressed as means ± SEM, where n = 4. There were no significant differences (p > 0.05) between groups.
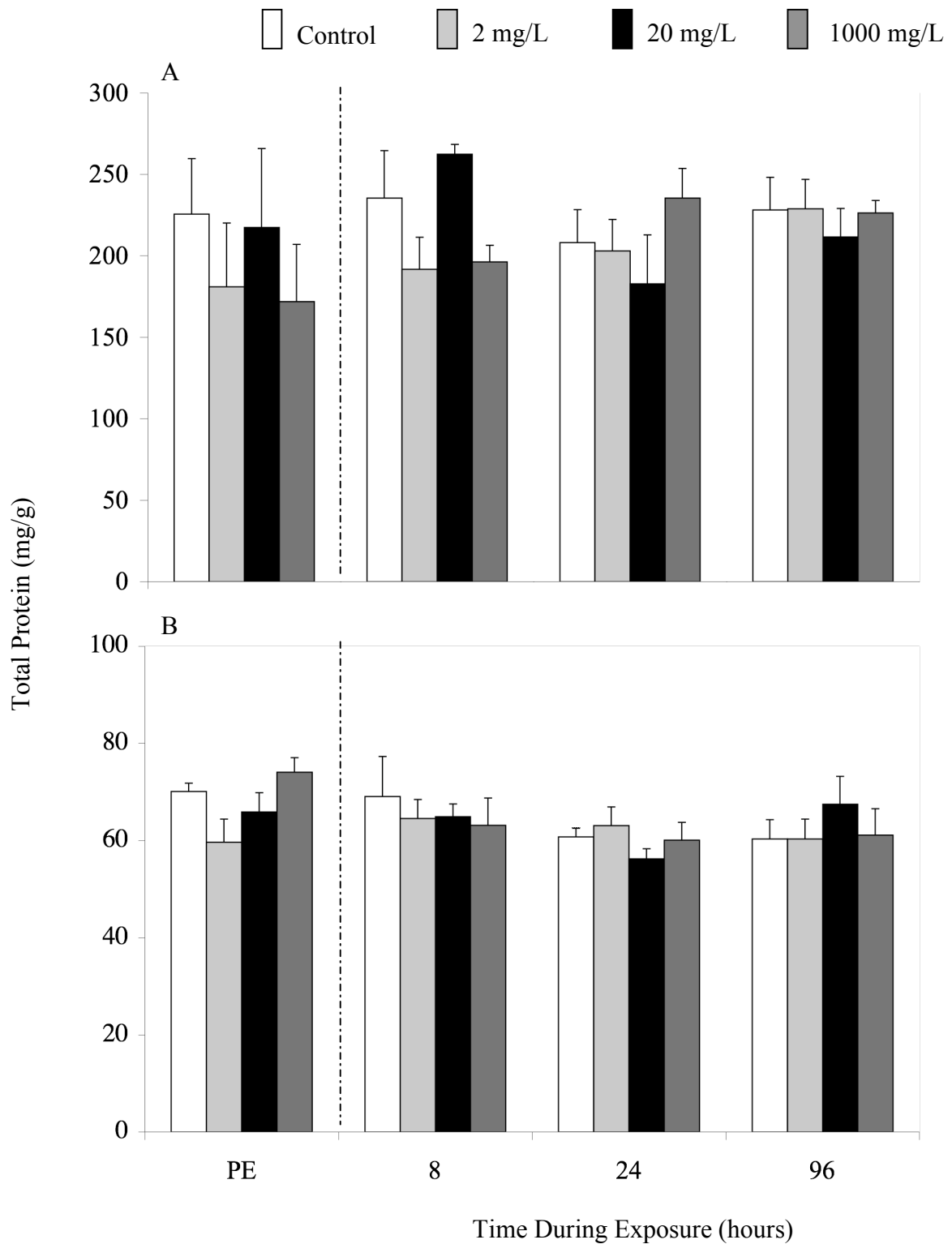
Figure 5. Mean liver (A) and gill (B) total protein concentration (mg/g tissue wet weight) in fingerling rainbow trout after a 96 h molybdenum exposure (0, 2, 20, or 1000 mg/L). Fish were sampled prior to exposure (PE = pre-exposure) and at 8, 24, and 96 h during exposure. Values are expressed as means ± SEM, where n = 4. No significant differences (p > 0.05) were found.
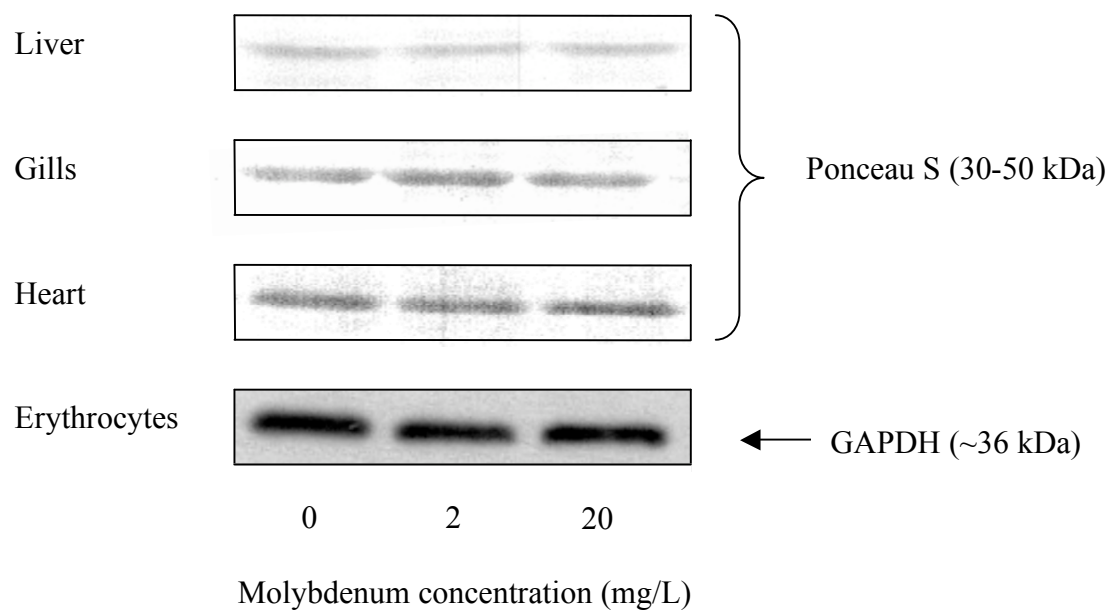
Figure 6. Verification of equal protein loading. Representative PVDF membrane containing liver, gill, or heart protein in the range of 30-50 kDa stained with Ponceau-S and representative Western blot containing erythrocyte GAPDH. Tissues were excised from juvenile fish exposed to 0, 2, or 20 mg/L of molybdenum for 96 h.
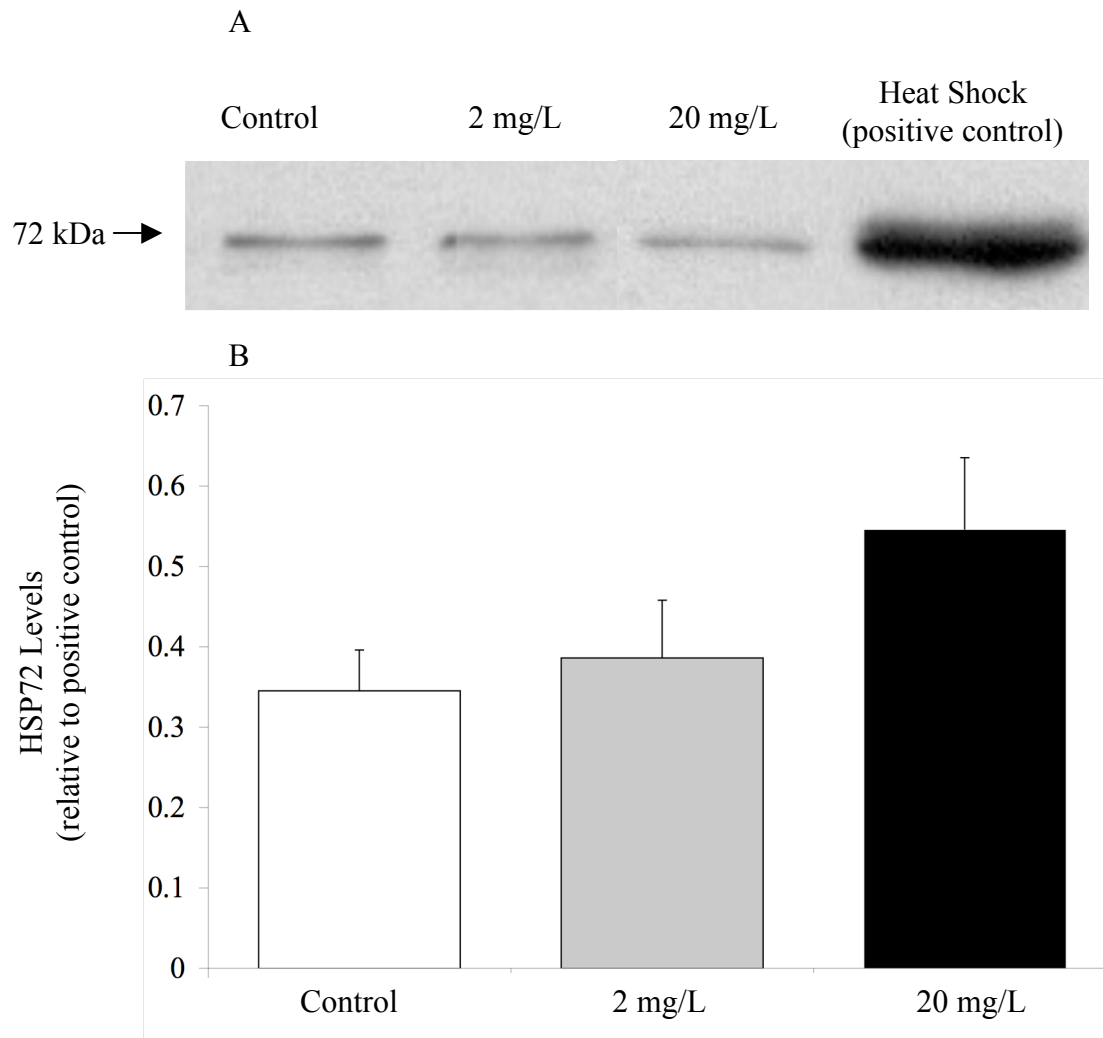
Figure 7. HSP72 levels in juvenile rainbow trout erythrocytes exposed to molybdenum (0, 2, or 20 mg/L) for 96 h. Fish were sampled at 96 h. (A) Representative Western blot of HSP72 expression in juvenile rainbow trout erythrocytes. The heat shock (positive control) sample is included for comparison. (B) Erythrocyte HSP72 levels (relative to positive control). Values are expressed as means ± SEM, where n = 4. There were no significant differences (p > 0.05) between groups.
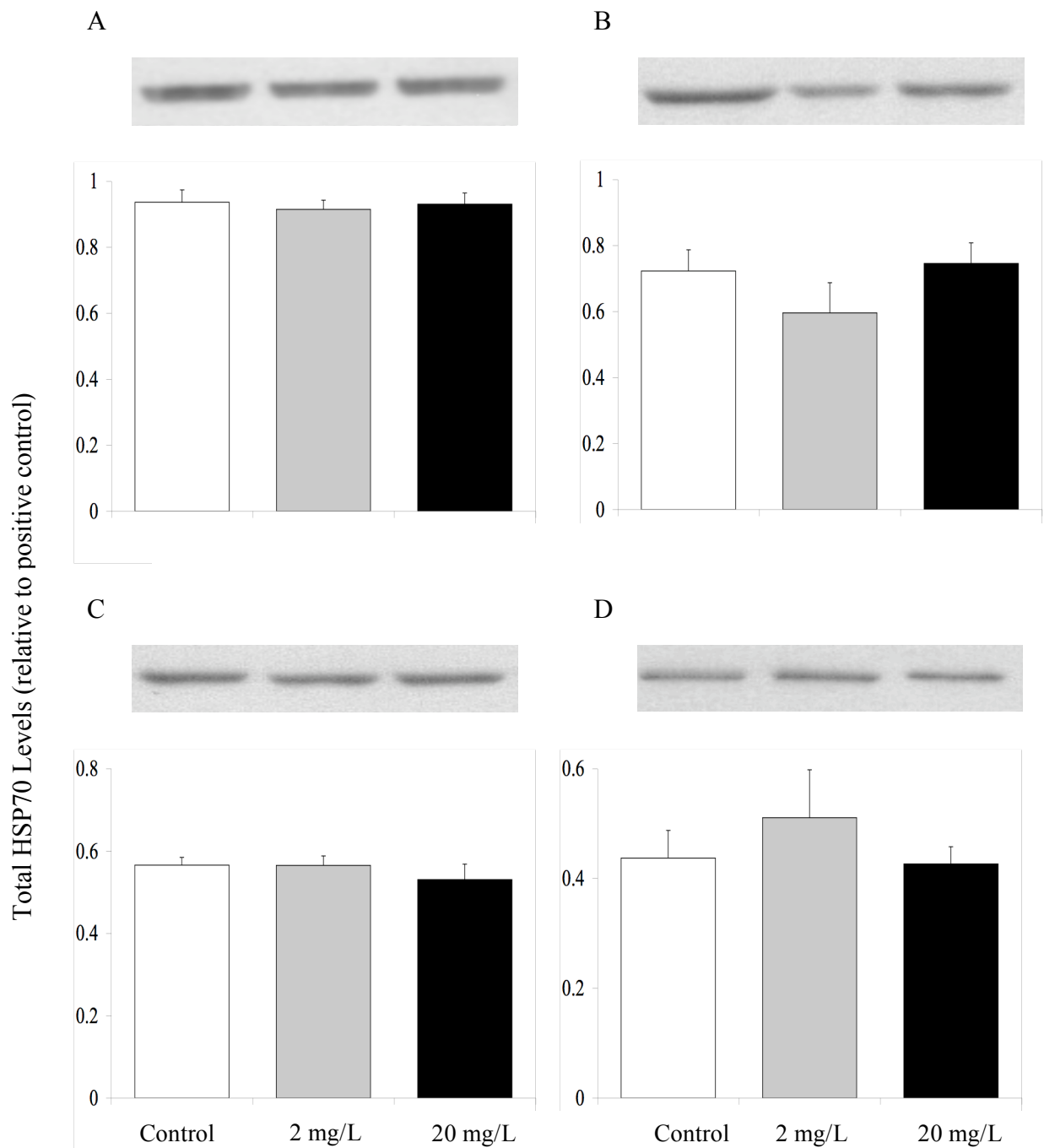
Figure 8. Liver (A), gill (B), heart (C), and erythrocyte (D) total HSP70 levels (relative to positive control) in juvenile rainbow trout exposed to molybdenum (0, 2, or 20 mg/L) for 96 h. Fish were sampled at 96 h. Values are expressed as means ± SEM, where n = 4. There were no significant differences ($p > 0.05$) between groups. Representative Western blots for total HSP70 in the various tissues are shown above their respective bar graph.
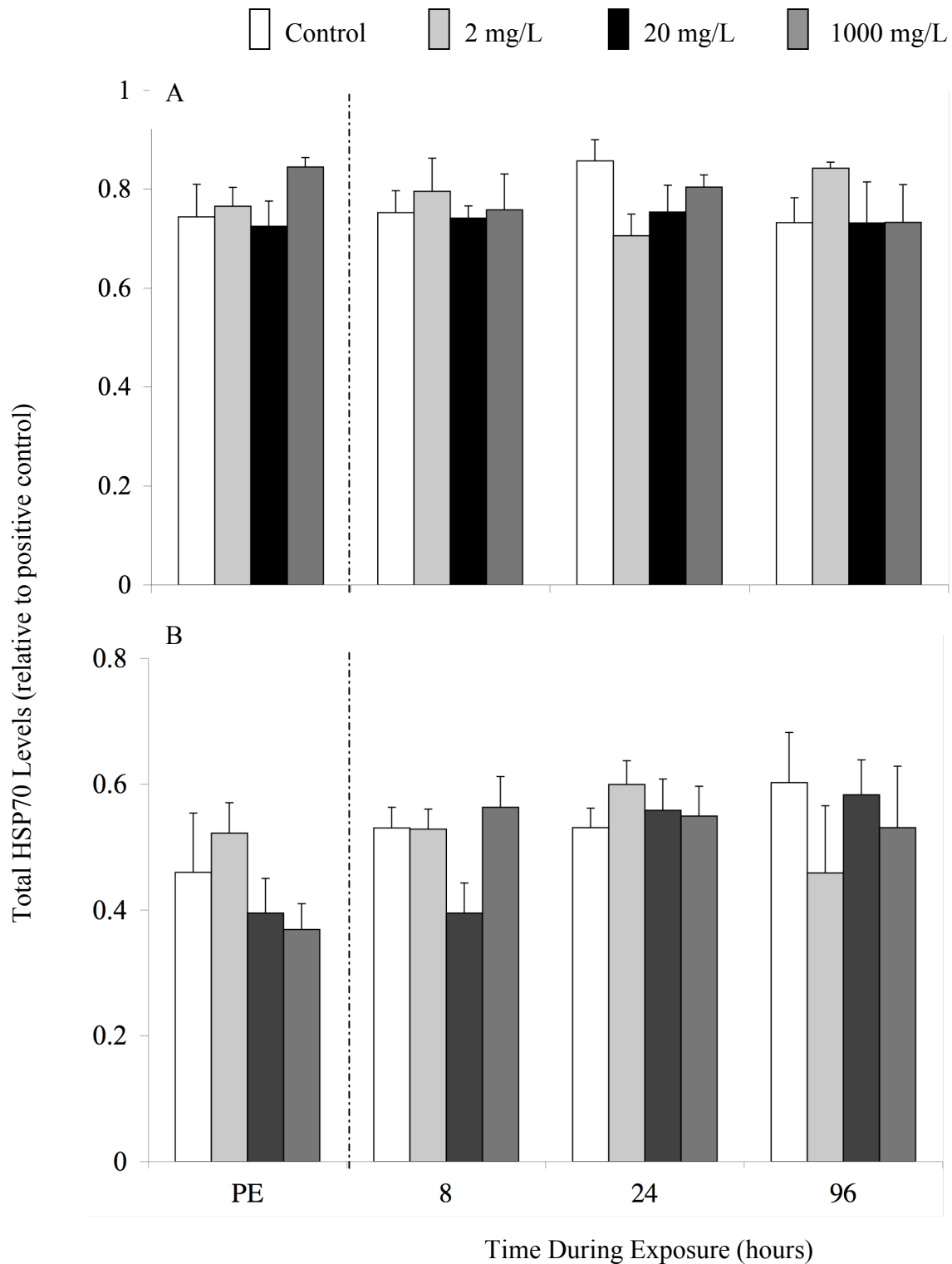
Figure 9. Liver (A) and gill (B) total HSP70 levels in fingerling rainbow trout after a 96 h molybdenum exposure to 0, 2, 20, or 1000 mg/L. Fish were sampled prior to exposure (PE = pre-exposure) and at 8, 24, and 96 h during exposure. Values are expressed as means ± SEM, where n = 4. No significant differences ($p > 0.05$) were found.
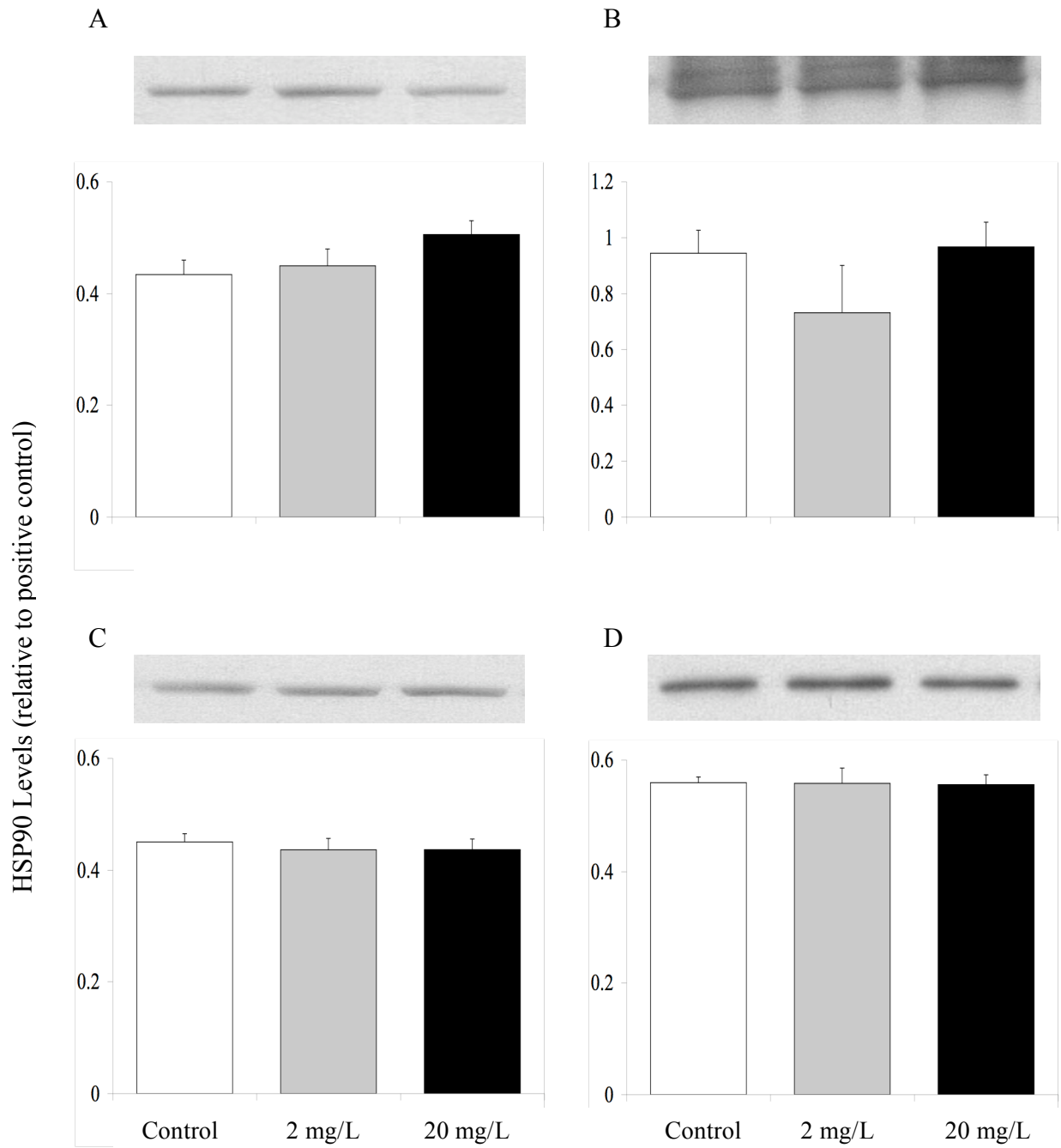
Figure 10. Liver (A), gill (B), heart (C), and erythrocyte (D) HSP90 levels (relative to positive control) in juvenile rainbow trout exposed to molybdenum (0, 2, or 20 mg/L) for 96 h. Fish were sampled at 96 h. Values are expressed as means ± SEM, where n = 4. There were no significant differences ($p > 0.05$) between groups. Representative Western blots for HSP90 in the various tissues are shown above their respective bar graph.
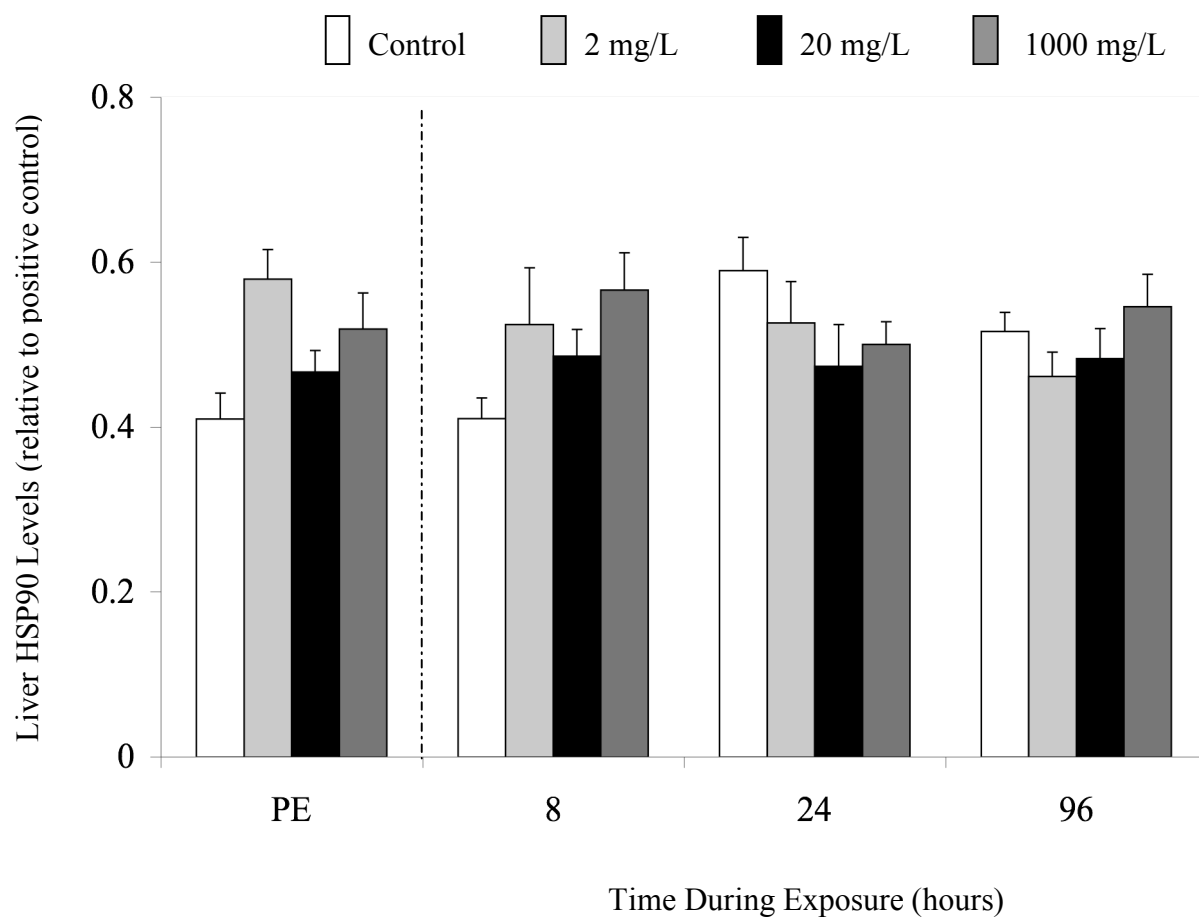
Figure 11. Liver HSP90 levels (relative to positive control) in fingerling rainbow trout exposed to 0, 2, 20, or 1000 mg/L of molybdenum prior to exposure (PE = pre-exposure) and during exposure (8, 24, and 96 h). Values are expressed as means ± SEM, where n = 4. No significant differences ($p > 0.05$) were found.
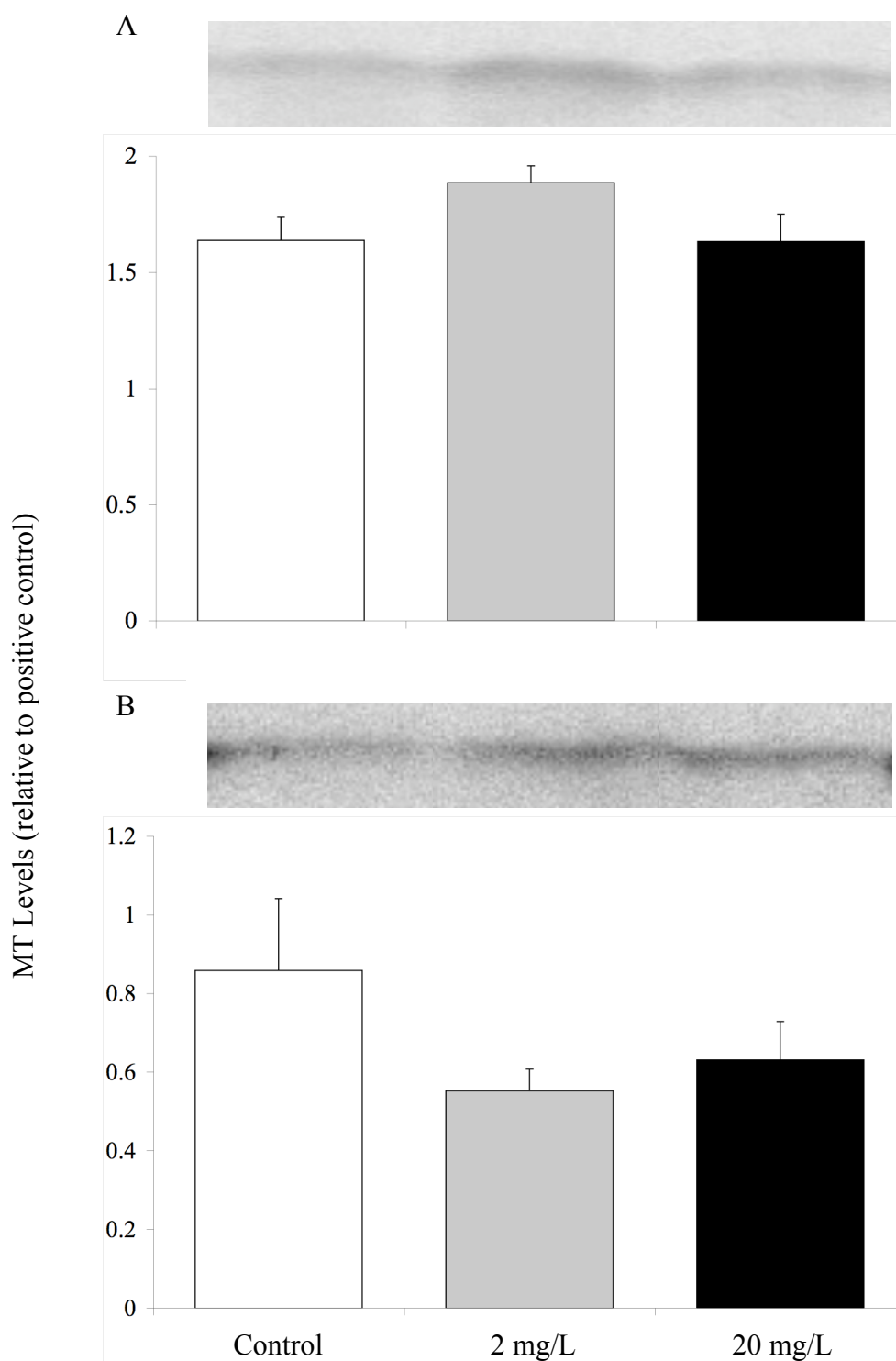
Figure 12. Liver (A) and gill (B) MT levels (relative to positive control) in juvenile rainbow trout exposed to molybdenum (0, 2, or 20 mg/L) for 96 h. Fish were sampled at 96 h. Values are expressed as means ± SEM, where n = 4. There were no significant differences ($p > 0.05$) between groups. Representative Western blots for MT in the liver and gills are shown above their respective bar graph.
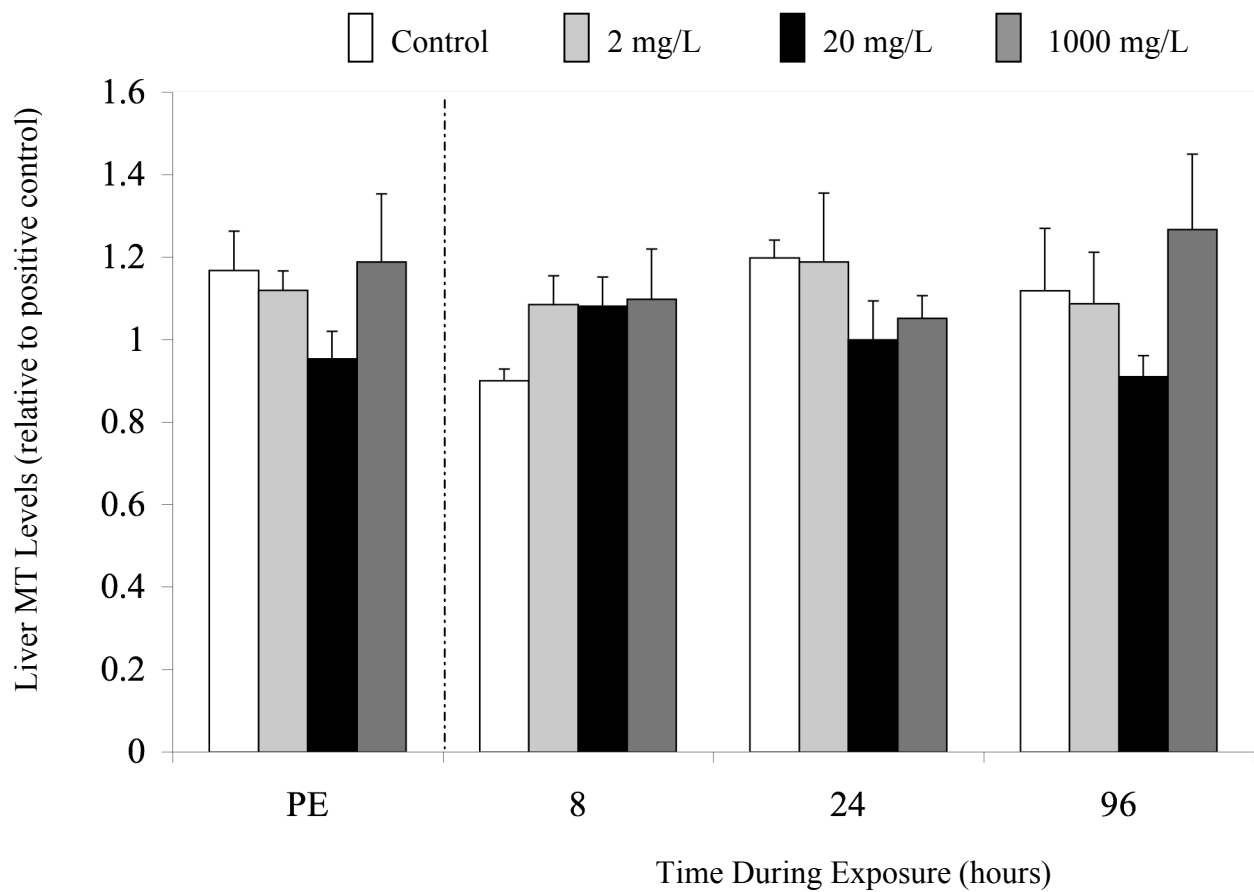
Figure 13. Liver MT levels (relative to positive control) in fingerling rainbow trout after a 96 h exposure to 0, 2, 20, or 1000 mg/L of molybdenum. Fish were sampled prior to exposure (PE = pre-exposure) and at 8, 24, and 96 h during exposure. Values are expressed as means ± SEM, where n = 4. No significant differences (p > 0.05) were found.

CHAPTER 4: DISCUSSION

Fish in nature are exposed to a variety of biotic and abiotic stressors that can adversely affect their health. In order to cope with stress, fish can respond to it by eliciting a physiological and/or 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, copper, mercury, and zinc, information concerning molybdenum is extremely limited.

This study investigated the effects of an acute 96 h molybdenum exposure on the stress response of rainbow trout (*O. mykiss*). Neither fingerling nor juvenile fish elicited a neuroendocrine (cortisol), metabolic (glucose and hematocrit), or cellular (HSP72, HSP73, HSP90, and MT) aspect of the stress response when exposed to acute sub-lethal molybdenum concentrations of up to 1000 mg/L and 20 mg/L, respectively.

4.1 Acute Waterborne Molybdenum Exposure Did Not Cause Mortality

Since there were no mortalities, it is proposed that the 96 LC_{50} for molybdenum in this study is greater than 1000 mg/L. This value, although an estimate as this study was not designed to test for mortality, is in agreement with previous toxicity studies for fish of the genra *Oncorhynchus*. Hamilton and Buhl (1990) reported a 96 LC_{50} of >1000 mg/L for coho and chinook salmon. Comparable values were also reported in rainbow trout (McConnell, 1977; Pyle et al., 2001).

4.2 Cannulation Did Not Appear to Interfere With the Investigation of Cortisol, Glucose, and Hematocrit Responses

Dorsal aorta cannulation has been used to monitor blood/plasma parameters in many species of fish, including rainbow trout, with minimum stress (Reid et al., 1993; Ron et al., 1995; Lo et al., 2003). The surgery of inserting the cannula, however, is known to induce a stress response, as determined by increased levels of cortisol, glucose, and hematocrit. As a result, a recovery time is required before the fish can be considered a valid tool for the investigation of physiological responses. In this study, the 48 h recovery period (also used by Reid et al., 1993; Pane et al., 2003) allotted to the fish prior to molybdenum exposure was long enough to allow the stress response to dissipate. In tilapia (*Oreochromis mossambicus*), plasma cortisol and hematocrit returned to normal levels 48-72 h after dorsal aorta cannulation (Ron et al., 1995). Shorter recovery periods were observed in the Adriatic sturgeon (*Acipenser naccarii*) in that cortisol and glucose levels stabilized by 24 h post surgery (Di Marco et al., 1999) and in trout whereby plasma cortisol levels returned to near control levels 24 h post surgery (Iwama and Ishimatsu, 1994). In a similar technique known as hepatic portal vein cannulation, Eliason et al. (2007) reported that cortisol, glucose, and hematocrit levels returned to baseline 24, 72, and 1 h post-surgery, respectively, in the Atlantic salmon. Although the stress of surgery did not interfere with the results of this study, it may account for some of the variation in cortisol and glucose levels measured at 8 and 24 h during molybdenum exposure, times corresponding to 56 and 72 h post-surgery.

4.3 Exposure Chamber Effects Did Not Appear to Interfere With the Investigation of Blood Glucose, Hematocrit, Total Protein, HSP72, HSP73, HSP90, and MT

Analysis of pre-exposure data from fingerling fish did not detect any differences in blood glucose, hematocrit, total protein, HSP72, HSP73, HSP90, and MT in fish sampled from the different exposure chambers (0, 2, 20, and 1000 mg/L). This information suggests that factors

such as the location of the exposure chambers on the wet table, temperature, oxygen supply, ammonia, and social stress did not interfere with analysis of the data. Therefore, any significant differences or similarities seen in the exposure data (8, 24, and 96 h) are a result of molybdenum exposure.

4.4 Acute Waterborne Molybdenum Exposure Did Not Activate the HPI Axis

Control plasma cortisol concentrations were within the range of values reported for unstressed rainbow trout (Milligan and Wood, 1987; Carballo et al., 1995; Dethloff et al., 1999; Gregory and Wood, 1999; Benfey and Biron, 2000; Ackerman and Iwama, 2001; Basu et al., 2003). Due to the extreme sensitivity of the HPI axis, many researchers use an increase in cortisol as an indicator of acute stress in fish. An acute molybdenum exposure of up to 20 mg/L did not activate a plasma cortisol response when measured at 8, 24, and 96 h during exposure in juvenile rainbow trout. If a cortisol response was elicited, levels characteristic of fishes responding to acute stressors, which range from 30-300 ng/mL (Wedemeyer et al., 1990; Barton and Iwama, 1991), would have been observed. The lack of cortisol response is very surprising given the high sensitivity of both the HPI axis and fish to environmental perturbations.

Results from this study are consistent with data from a 168 h endpoint molybdenum exposure to 25 or 250 mg/L in kokanee salmon reporting no difference in plasma cortisol levels between molybdenum exposed and control fish (Reid, 2002). As far as the author is aware, there are no other studies that have assessed the effect of molybdenum toxicity on cortisol levels. The inability of molybdenum to activate the HPI axis is probably not due to its speciation as an oxyanion. Anions such as cyanide (Carballo et al., 1995) and oxyanions such as nitrite (Carballo et al., 1995), selenite (Miller et al., 2007), and chromate (Roche and Bogé, 1996) are known to cause increases in plasma cortisol in fish.

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) caused plasma cortisol levels to increase by ~280 ng/mL after 24 h of exposure (Carballo et al., 1995). Similarly, plasma cortisol levels increased ~140 ng/mL at 72 h during exposure to cadmium (0.01 mg/L, 50% of the 96 h LC_{50} determined by Hollis et al., 1999; Chowdhury et al., 2004) and increased ~65 ng/mL after 96 h of exposure to selenite (2.52 mg/L, 35% of the 96 h LC_{50} determined by Hodson et al., 1980; Miller et al., 2007). In contrast, but similar to the results of this study, waterborne exposure to lower doses of copper (0.016 mg/L; 6.4% of the 96 h LC_{50} determined by Syvokiene et al., 2006) for 72 h (Dethloff et al., 1999) and selenite (0.72 mg/L, 10% of the 96 h LC_{50} determined by Hodson et al., 1980) for 96 h (Miller et al., 2007) did not elevate plasma cortisol levels. All of the aforementioned exposures studies used rainbow trout as the test species.

It is possible that in this study a cortisol response was elicited but not during the times chosen for sampling or that the exposure concentrations were not high enough to elicit a response, if molybdenum can even activate the HPI axis. In studies involving acute physical stressors (i.e. capture, handling, temperature), cortisol levels spiked rapidly following application of the stressor (Biron and Benfey, 1994; Ryan, 1995; Flodmark et al., 2002; Clement et al., 2005; Barreto and Volpato, 2006). Most fish species show the highest increase in cortisol within 0.5-1 h after a stressful physical disturbance (Barton and Iwama, 1991). In contrast, cortisol elevation in response to acute metal stressors is much more delayed, often detectable around 24-96 h during exposure (Carballo et al., 1995; Chowdhury et al., 2004; Miller et al., 2007). The sampling times chosen in this study (8, 24, and 96 h) are comparable to other studies that detected increases in cortisol levels at these times. Pratap and Wendelaar Bonga (1990) conducted a 35 day time course study and observed increased cortisol levels at 24 h up to 14

days in fish exposed to cadmium. Therefore, it seems more likely that 20 mg/L was not a high

enough concentration to elicit a cortisol response. Although ten times the molybdenum water

quality guideline for the protection of aquatic life in British Columbia, the concentration of

20 mg/L was only 0.27% of the 96 h LC_{50} reported by Bentley (1973), whose study used

rainbow trout of similar size to rainbow trout in this study. As previously mentioned, copper and

selenite both lost their ability to induce a cortisol response as they became less concentrated.

The only way to determine if this is also true of molybdenum would be to expose fish to

concentrations closer to and above the 96 h LC_{50}. Although testing at such high concentrations

is plausible, it is not environmentally relevant.

4.5 Glucose and Hematocrit Levels Were Not Affected by Acute Waterborne Molybdenum Exposure

Control glucose and hematocrit levels are within the range previously reported in

unstressed rainbow trout (McCarthy et al., 1973; Ricard et al., 1998; Benfey and Biron, 2000;

Ruane et al., 2000; Pane et al., 2003; Chowdhury et al., 2004). The lower hematocrit values

observed in the cannulated fish versus the non-cannulated fish are characteristic of cannulated

fish (Wells and Weber, 1991; Ron et al., 1995; Sohlberg et al., 1996; Mugnier et al., 1998). One

explanation may be blood loss due to the cannulation procedure. The slow decline in hematocrit

of the cannulated fish suggests mild hemodilution, caused by repeated sampling of the blood

(500 uL per sample) and injection of 200-300 uL of saline after each sampling. Previous studies

have shown that 10% of the total blood volume can be withdrawn without adverse effects on the

fish, particularly when the blood is replaced with an equivalent amount of saline (McLean and

Ash, 1989; Gallaugher et al., 1995). In this study, 5.5% of the total blood volume was

withdrawn.

Changes in blood glucose are a useful general indicator of stress in fish. Nemcsók and Boross (1982) stated that blood glucose appeared to be a sensitive indicator of environmental stress in fish. The stress-related hyperglycemia reported in fish is mediated mainly by catecholamines and to some extent cortisol. Catecholamines induce rapid, short-term elevations in blood glucose through the glycogenolytic pathway (Vijayan and Moon, 1992; Vijayan et al., 1997) whereas cortisol is involved, either directly and/or indirectly, in the long-term mobilization of non-carbohydrate energy stores (Vijayan and Moon, 1992; Wendelaar Bonga, 1997). Hyperglycemia ensures that fish have adequate circulating energy substrate to supply the increased energy demand required to cope with the stress (Barton and Schreck, 1987; Mommsen et al., 1999). 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 in both juvenile and fingerling fish. The decline in glucose is most likely a result of withholding feed during acclimation and experimentation (a total of 8 days) and the exhaustion of energy stores. Brown et al. (1986) reported a similar alteration in plasma glucose in catheterized rainbow trout and attributed it to withholding feed.

In response to stress animals increase the oxygen-carrying capacity of their blood in order to prepare them for the metabolic cost of coping with the stress (Caldwell and Hinshaw, 1994). This increase in oxygen-carrying capacity is detected by determining the percent of packed cell volume, or hematocrit, of the blood. According to Vosyliene (1996), an increase in hematocrit in metal-exposed fish is an alarm reaction and the subsequent decrease indicates adaptation. In this study, there was no effect of waterborne molybdenum exposure on percent hematocrit. Although there was no effect of molybdenum exposure on hematocrit, there was an increase in hematocrit at 8 h in all groups of fingerling fish. This increase is likely an artifact of the stress experienced as a result of sampling prior to exposure. The act of sampling caused the free-swimming fish to

increase their activity (fight or flight response) in order to evade capture thus increasing their hematocrit. Stress induced elevations in hematocrit can occur as a result of (1) increased muscle activity and the concomitant movement of water from plasma to muscle (Jones and Randall, 1978), (2) induction of splenic contractions and subsequent mobilization of stored erythrocytes, and (3) erythrocytic swelling caused by intracellular accumulation of ions and the movement of water from plasma into the intracellular compartment (Yamamoto et al., 1980). Since there are several methods used by an organism to elevate hematocrit the exact cause in this study is hard to explain, especially since other hematological parameters such as hemoglobin and ion levels were not measured. It is noteworthy to mention that there was no hyperglycemia around 8 h. This could be due to the fact that the stress, maximum 5 sec in duration, was not severe enough to cause substantial activation of the HPI axis. As a result, there would either be no change in glucose concentration or a small increase in glucose that returned to basal levels by 8 h. Unfortunately, this idea cannot be confirmed as cortisol was not measured in fingerling fish due to blood volume constraints.

The results of this hematocrit study are in concordance with data from a chronic (1 year) waterborne exposure of up to 17 mg/L reporting no change in hematocrit in various life stages of rainbow trout (McConnell, 1977). Findings outlined by McConnell (1977) regarding observations of fused gill lamellae in rainbow trout and by Reid (2002) regarding increased ventilation and mucus production in kokanee during molybdenum exposure, however, would preclude one to think that these manifestations would have an effect on hematocrit. According to Heath (1995), any pollutant that results in gill damage and subsequent internal hypoxia can be expected to increase hematocrit. This indicates that waterborne molybdenum, despite affecting/irritating the gills, does not induce internal hypoxia. This is also true of the metal lead. When Hodson et al. (1978) and Martinez et al. (2004) exposed rainbow trout and *Prochilodus*

lineatus to waterborne lead they observed that although the metal caused changes in gill morphology hematocrit remained unaffected.

Studies in ruminants fed diets high in molybdenum have reported decreased hematocrits, a measurement indicative of anemia (Britton and Goss, 1946; Barshad, 1948; Cook et al., 1966). The anemia of molybdenum toxicosis is related to a deficiency in copper caused by the formation of copper binding thiomolybdates in the rumen. The decrease in copper availability reduces the amount of the copper containing enzyme ceruloplasmin, which is required for the incorporation of iron into the heme molecule (Lindh, 2005). Contrasting observations have been made in non-ruminants. In rabbits fed high molybdenum diets, Arrington and Davis (1953) reported decreased hematocrit, as measured by decreases in hemoglobin concentration and number of erythrocytes, whereas Cook et al. (1966) did not observe any changes in hematocrit. In chickens fed excess molybdenum, Davies et al. (1960) and Underwood (1971) reported a decrease in hematocrit whereas Kratzer (1952) and Arthur et al. (1958) reported no change in hematocrit. In rats administered molybdenum, those that developed mandibular exostoses had lower hematocrits than both the controls and those that did not develop exostoses (Ostrom et al., 1961). In the above studies observing anemia in non-ruminants the mechanism for which it occurs is unclear.

From studies measuring decreased hematocrits in non-ruminants, the question arises as to whether dietary molybdenum uptake is a potential source of toxicity to fish. There is evidence that uptake of metals such as cadmium, copper, lead, and zinc from food is the predominant pathway in fish (Dallinger et al., 1987). There is no existing data on molybdenum testing its dietary toxicity in fish. If molybdenum turns out to be toxic when ingested by fish, this could be the first known example whereby only the dietary form is toxic. Clearly, research is required to determine if dietary molybdenum is toxic to fish.

4.6 Acute Waterborne Molybdenum Exposure Did Not Alter Total Protein Levels in the Liver, Gills, Heart, and Erythrocytes

The absence of a physiological stress response in response to molybdenum exposure suggests that fish must be utilizing parameters of their cellular stress response to remain homeostatic. Unexpectedly, acute exposure to 2-1000 mg/L of molybdenum did not cause changes in protein concentration in the liver, gills, heart, or erythrocytes. The total protein concentrations in mg/mL obtained for each tissue were almost identical to those values obtained by Trenzado et al. (2006), except for in the erythrocytes which were three times lower in this study. The difference is probably due to residual plasma that was not completely removed after centrifugation. Total protein concentrations in mg/g tissue wet weight calculated in this study are also consistent with previous studies (Smith, 1981; Sephton and Driedzic, 1999; Reid et al., 1997; Peragón et al., 1998)

These results are consistent with the findings from this study of unaltered HSP72, HSP73, HSP90, and MT protein levels in molybdenum exposed fish. The lack of change in protein concentration suggests that there was also no detectable effect of molybdenum on the induction or inhibition of synthesis of proteins not analyzed in this study. This is just a general assumption and each protein of interested should be analyzed on its own, especially since it may be difficult to see a change in proteins that have low basal concentrations.

The findings from this acute study are opposite to data from chronic studies. At low concentrations molybdenum is beneficial to animal growth and protein synthesis. Chicks grew more rapidly and had higher hemoglobin count when molybdenum was added to the diet (Norris, 1969; Humphries, 1970) and trout raised on supplemental ammonia molybdate were not only larger and better developed but also had higher protein content of the blood serum than control fish (Jurca and Matei, 1967). At high concentrations molybdenum has a negative effect of protein synthesis. Rats fed high levels of molybdenum eventually excreted alpha amino nitrogen

(Johnson and Miller, 1963) and Peive (1968) showed that molybdenum inhibited incorporations of leucine into protein in rat liver preparations. The differences in effects between results from this acute study and the aforementioned chronic studies highlights the necessity of conducting chronic molybdenum exposure studies in fish.

4.7 Expression Patterns of HSP72, HSP73, and HSP90 Were Not Affected by Acute Waterborne Molybdenum Exposure

In the present study, exposure of rainbow trout to molybdenum failed to upregulate expression of HSP72, HSP73, and HSP90. There was no response in the liver, gills, heart, or erythrocytes of juveniles exposed to a maximum of 20 mg/L or in the liver or gills of fingerlings exposed to a maximum of 1000 mg/L. As a result, there appears to be no utility of these proteins as measures of molybdenum exposure.

There is confidence that the lack of induction in response to acute molybdenum exposure in trout does not reflect a reduced capacity of fish to activate a heat shock response. In this study, heat shocked fish responded 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 (Rendell et al., 2006; Currie et al., 2008). Heat shock in rainbow trout has also lead to increases in HSP70 mRNA in the liver, gill, heart, and blood (Currie et al., 2000). The lack of HSP induction by molybdenum is also not due to metal load sequestering by MT because, as discussed later, there was no induction of MT in response to molybdenum exposure.

HSP72 is the most stress-inducible protein in fish (Kothary and Candido, 1982) in that it is required to help refold proteins that become denatured as a result of exposure to a stressor. Prior to molybdenum exposure HSP72 was not apparent in any of the tissues with the exception of erythrocytes. The detection of low basal levels of HSP72 in erythrocytes in this present study

is consistent with previous findings. Currie et al. (2000) and Currie et al. (2008) detected low basal levels of HSP72 mRNA and HSP72 protein, respectively, under control conditions in rainbow trout erythrocytes. The low basal levels of HSP72 are most likely a mechanism used by the fish to rapidly respond to a stress thus giving them a better chance to minimize cellular damage and survive. For example, heat shocked Atlantic salmon that had artificially elevated levels of branchial and hepatic HSP72 were better able to tolerate a subsequent stress relative to controls (DuBeau et al., 1998). Erythrocytes may also require inert protection as they circulate throughout the body coming into contact with various xenobiotics. Overall, acute exposure to molybdenum did not alter/stimulate HSP72 synthesis in any of the tissues. Some fish from all exposure groups did express low levels of HSP72, but only in the liver. This is not surprising since the liver is a center of protein biosynthesis and likely has a large pool of cytoplasmic proteins in need of chaperoning. The exact cause of induction is difficult to pinpoint since there is a wealth of abiotic and biotic stressors known to trigger HSP72 synthesis in fish (see Basu et al., 2002).

This study did not detect any changes in total HSP70, a combination of both HSP72 and HSP73. Total HSP70 is a relatively insensitive measure of the heat shock response; however, because there was no observed change in HSP72 expression it was assumed that there was also no change in HSP73 expression. Since no inducible HSP70 was detected these results are what would be expected for constitutive HSP70, a molecular chaperone that functions primarily in unstressed cells. This finding supports those from previous studies that also failed to observe changes in HSP73 accumulation upon exposure of rainbow trout hepatocytes to heat shock, copper, cadmium, or arsenic (Boone and Vijayan, 2002). Similarly, there was no change in HSP73 mRNA levels upon exposure to fish CHSE cells to cadmium or zinc (Zafarullah et al., 1992) or exposure to rainbow trout erythrocytes to azide, hypoxia, or zinc (Currie et al., 1999). Taken together, these results suggest that sub-lethal acute stressors in fish do not modulate

HSP73 expression. One reason for this observation is that HSP73 levels might already be high enough to overcome potentially harmful effects of stressors.

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 (Washburn et al., 2002). Furthermore, various forms of husbandry stress such as anesthesia, hypoxia, capture, crowding, feed deprivation, and cold stress had no affect on HSP70 mRNA levels in the gills of Atlantic salmon (Zarate and Bradley, 2003). The commonality of these stressors is that none of them have been demonstrated to denature proteins. Therefore, it can be concluded that molybdenum, at concentrations tested in this study, does not cause detectable proteotoxicity.

HSP90 supports the structure and function of a variety of signal transduction proteins important in all aspects of normal growth and development. Molybdate is known to inhibit HSP90-mediated protein folding by stabilizing HSP90-protein complexes. For example, molybdate stabilizes the HSP90-glucocorticoid receptor complex maintaining the receptor in an inactive, non DNA-binding state (Cardo et al., 1983). Therefore, one would expect that with an overdose of molybdenum, HSP90-protein complexes would be maintained in an inactive form resulting in the reduction and/or activity of client proteins.

Disruption of HSP90 function has lead to morphological mutations, predominately in developing organisms (Rutherford and Lindquist, 1998). Bishop et al. (2002) exposed ascidian embryos to an HSP90 inhibitor and observed arrested morphogenesis and Rutherford and Lindquist (1998) found abnormalities affecting nearly every adult structure in HSP90 mutant *Drosophila*. No observable morphological changes were observed in fingerling or juvenile fish most likely due to the fact that when molybdenum was administered the fish were not undergoing important developmental processes. Thus, research is needed to elucidate the affects of molybdate exposure on the fertilization process all the way through development to fry.

Molybdenum exposure studies have been done on eggs and early life stages (McConnell, 1977; Birge, 1978; Pyle, 2000); however, molybdenum concentrations did not exceed 100 mg/L and may not have been high enough to induce a response. Significantly increasing the concentration may lead to some interesting developmental questions.

In this study the levels of HSP90 were tested based on the idea that if molybdenum acted as a potent stressor it could (1) divert HSP90 from its function in signal transduction to help fold proteins or (2) reduce the amount of free HSP90. Both scenarios could potentially cause an increase HSP90 levels. It was very surprising then that no change in HSP90 was detected. As for the first scenario, since there was no HSP72 response, HSP90 would not be needed to help fold denatured proteins, thereby being maintained at basal levels. In the second scenario, the duration of exposure and dose of stressor may not have been long enough or strong enough to invoke a response. The absence of a response was not anticipated, as it is known the molybdate interacts with HSP90 and that the metal can cross the plasma membrane and cause extreme effects in animals.

4.8 Acute Waterborne Molybdenum Exposure was Unable to Directly or Indirectly Induce MT Synthesis

Metallothioneins are intracellular, low molecular weight, cysteine-rich proteins. Ubiquitous in eukaryotes, MTs have unique structural characteristics to give potent metal binding and redox capabilities. Synthesis of MTs is induced to a greatest degree by exposure to metals and to a lesser degree by hormones, cytokines, and organic contaminants (Kägi, 1993). Although a number of metals induce MT, there is a general assumption that molybdenum does not have this ability. Jakobsen et al. (2007) 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

molybdenum. Furthermore, Pyle (2000) observed MT induction in rainbow trout exposed to molybdenum, but he attributed the induction to an increase in cadmium concentration. Koizumi et al. (1984) demonstrated that molybdenum 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 molybdenum does not stimulate MT protein expression. Waterborne molybdenum exposure of concentrations up to 1000 mg/L did not cause an up-regulation of MT in the liver or gills of rainbow trout, tissues that are known to possess high levels of MT (Wu et al., 2006) and accumulate molybdenum (Reid, 2002). The lack of MT induction suggests that molybdenum neither induces MT directly through binding to MT nor indirectly through activation of an inflammatory response.

Metal binding to MT is dependent on the metal binding properties of the metal in question. All metals known to bind to MT such as zinc, copper, cadmium, mercury, silver, and bismuth can be classified as covalent class B (nitrogen/sulphur seeking) metals or borderline metals with strong class B character (Nieboer and Richardson, 1980). Borderline refers to metals that have both class B and ionic class A (oxygen-seeking) character. MT contains a richness of cysteine residues and cysteine is a sulphur containing amino acid group. Therefore, it is apparent why metals having a high affinity for sulphur donor ligands bind readily to MT. Metals that significantly bind to MT also have an electronic configuration of d(10), which is suited for the complex formation with multiple cysteines of MT (Vasák, 1998).

Molybdenum is a borderline, d(5) metal (Nieboer and Richardson, 1980). As such, the metal has significant oxide and sulphide chemistries as demonstrated by its formation of molybdenite (MoS_2) and wulfenite ($PbMoO_4$). As a result, molybdenum can be expected to bind to the negatively charged thiolate groups of MT if it exists as a cation. Other borderline metals such as chromium [d(5)] and manganese [d(5)] can bind to MT but with low affinity (Suzuki and Yoshikawa, 1976). Lead, for example, has a high affinity for MT *in vitro* (Waalkes et al., 1984)

but binds sparingly *in vivo* (Ulmer and Vallee, 1969). The lack of MT induction, however, suggests that molybdenum did not bind to MT. Due to the fact that anions are not able to interact with the vast number of negatively charged thiolate clusters of MT, this research proposes that molybdate is the speciation of molybdenum inside fish. Therefore, molybdenum, which exists as molybdate in natural aquatic environments is taken-up as molybdate (Hoekstra and Reid, 2008) and is distributed internally as molybdate. This is exciting information since the speciation of molybdenum in body fluids and tissues is not fully known. This finding is consistent with the study conducted by Matsuura et al. (2003) that demonstrates that molybdenum exists as molybdate inside salmon egg cytoplasm. Further, this finding suggests that MT levels cannot be used as an indicator of previous environmental exposure to molybdenum.

Exposure to chromium, iron, cobalt, nickel, arsenic (Fleet et al., 1990; Albores et al., 1992), cerium (Kobayashi et al., 2005), and vanadate (Kobayashi et al., 2006), 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 (De et al., 1990). The fact that molybdenum 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 (1977) is probably not true tissue damage but rather a response to ionoregulatory disruption, as suggested by Pyle (2000), by excess sodium as a result of adding sodium molybdate.

4.9 Conclusions and Recommendations

Acute exposure to waterborne molybdenum of up to 1000 mg/L did not trigger a physiological (cortisol, glucose, or hematocrit) or cellular (HSP72, HSP73, HSP90, or MT) stress response in rainbow trout. In addition, there were no detectable differences in sensitivity

between fingerling and juvenile life stages. These findings are consistent with previous studies demonstrating that an elevation in molybdenum is not perceived as a toxic threat by fish (McConnell, 1977; Hamilton and Buhl, 1990; Pyle et al., 2000; Reid, 2002). It is possible that an acute molybdenum exposure at concentrations above 1000 mg/L may have an effect on the stress response; however, testing at such concentrations is not environmentally relevant.

Data from this study bring into question the molybdenum water quality guidelines for the protection of freshwater aquatic life of 0.073, 0.04, and 2 mg/L set by Canada (CCME, 1999), Ontario (MOEE, 1994), and British Columbia (Swain, 1986), respectively. The criteria used by Canada and Ontario to develop their guideline comes from a study by Birge (1978) on rainbow trout eggs. Not only did Birge's 1978 study fail to include control data but also the low 28 day LC_{50} of 0.073 mg/L was unable to be replicated by Davies et al. (2005). The authors attributed the high mortality to the way Birge (1978) handled the trout eggs, which exposed them to additional stress. Furthermore, the guideline in British Columbia is based on a 96 h LC_{50} of 70 mg/L reported by Tarzwell and Henderson (1960) that was misreported as 40 mg/L by Swain (1986). In addition, Tarzwell and Henderson (1960) exposed fathead minnows to molybdenum trioxide, which is not representative of the speciation of molybdenum found in natural aquatic environments. Although molybdenum is not a major threat to ecological systems it is relatively common and naturally occurs in Canadian waters. Therefore, there is a need for a guideline that is appropriate, scientifically defensible, and based on up to date toxicological studies.

Results from this study propose that the current limits are overly protective of rainbow trout and should be re-examined. Last year a report prepared by Tetra Tech Inc. (2008) suggested that the State of Nevada change the aquatic life water quality criteria for molybdenum from 0.019 mg/L to 6.16 mg/L for acute exposure and 1.65 mg/L for chronic exposure. Having a higher guideline could actually prove beneficial, as molybdenum has been shown to alleviate the toxicity of some metals. Morgan et al. (1986) demonstrated that molybdenum reduced the

toxicity of manganese in *Mytilus edulis* mussel larvae and in rats, molybdenum pretreatment protected against the toxic effects of mercury (Koizumi and Yamane, 1984) and cadmium (Koizumi at al., 1991). There are also studies demonstrating that molybdenum has anticarcinogenic properties (Ogata et al., 2008).

Although findings from this study suggest that the current guidelines are too stringent, it does not suggest that the limit should be set to 1000 mg/L. This study examined the acute effect of molybdenum alone and it does not accurately estimate the effects of molybdenum on fish in an environmental context. Hamilton and Buhl (1990) demonstrated that molybdenum added to test mixtures of boron, selenite, and selenate increased the acute toxicity of these metals to chinook and coho salmon. Similarly, Naddy et al. (1995) reported that molybdenum enhanced selenium toxicity and selenium, at high concentrations, enhanced molybdenum toxicity in *Ceriodaphnia dubia.* Reid (2002) reported that pre-exposure of kokanee to molybdenum resulted in post-exercise loss of equilibrium and exercised-induced delayed mortality. Furthermore, California sea lions (*Zalophus califonianus*) that died from infectious diseases had significantly higher levels of hepatic zinc and molybdenum (Harper et al., 2007). These studies provide evidence that molybdenum in combination with additional environmental stressors could contribute to increased mortality in fish.

More research is obviously needed to successfully assess the effects of molybdenum on fish. Future experiments should focus on the toxic effects of dietary molybdenum as well as on chronic studies such as those that begin at fertilization and follow the development of the exposed fish. Of key importance is to study the effects of molybdenum in combination with additional environmental stressors on fish. All of these types of studies are more realistic to the natural environment fish inhabit.

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APPENDIX

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-1420

Investigator or Course Director: Scott D. Reid

Department: UBCO Admin Unit 2 Arts & Sci

Animals:

Trout Rainbow trout (Oncorhynchus mykiss); juveniles. 200

Trout Rainbow trout (Oncorhynchus mykiss); fingerlings 800

Fish Zebrafish (Danio rerio) 100

Start Date:	**September 25, 2006**	**Approval Date:**	**October 16, 2006**

Funding Sources:

Funding Agency:	Natural Science Engineering Research Council
Funding Title:	Molybdenum metabolism in freshwater fish

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.