LEAD-INDUCED IONOREGULATORY DISRUPTION IN THE RAINBOW TROUT

A MECHANISTIC APPROACH TO ACUTE LEAD TOXICITY IN THE RAINBOW TROUT: INVESTIGATIONS OF LEAD-INDUCED IONOREGULATORY DISRUPTION

By

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ABSTRACT

Relative to other metals, little is known about lead toxicity in fish. The use of predictive models such as the biotic ligand model (BLM) has been limited, a situation that is at least partially due to the lack of understanding of lead's acute toxic mechanism and characterization of key binding sites involved in this toxicity.

Using the rainbow trout as a model species, the acute toxic mechanism for lead was found to be ionoregulatory disruption. While having no apparent respiratory or acid/base effects, Pb exposure resulted in significant ionoregulatory impacts that affected Ca^{2+} homeostasis, as well as Na^{+} and Cl^{-} balance

Active Ca^{2+} uptake by the gills obeyed typical Michaelis-Menten kinetics, and Pb interacted in a competitive fashion with the uptake process. Exposure to increasing waterborne Pb concentrations resulted in significant increases in K_m value while J_{max} showed little or no change. A slower, non-competitive interaction occurred after prolonged Pb-exposure, evidenced by a significant reduction of high-affinity Ca^{2+}-ATPase activity that correlated well with branchial Pb accumulation. Conversely, calcium had a protective effect against branchial Pb accumulation, this relationship being predominately competitive in nature. Voltage-independent calcium channel blockers La^{3+}, Cd, and Zn significantly reduced gill Pb burden while the voltage-dependent, L-type calcium channel blockers, nifedipine and verapamil, did not, suggesting Pb enters fish by a similar mechanism to that of Ca^{2+}. Stimulated stanniocalcin release by $CaCl_2$ injection also significantly reduced branchial Pb accumulation.

Based on the evidence presented in this thesis, it is apparent that acute Pb toxicity occurs by ionoregulatory disruption. It is likely that Pb shares a similar uptake pathway as that for Ca^{2+} and that resulting accumulation results in disruption of Ca^{2+} influx as well as Na^{+} and Cl^{-} balance. This study has provided data essential to the characterization of key binding sites involved in Pb toxicity, and ultimately, validates the development and application of predictive models such as the BLM.

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Lastly, a thank you to my family, a pillar of strength –you have my love and admiration always.

THESIS ORGANIZATION AND FORMAT

This thesis is organized into four chapters. Chapter 1 provides a general introduction and an outline of objectives set for this project. Chapters 2 and 3 are manuscripts, published or submitted for publication in peer-reviewed journals. Chapter 4 provides a summary of results and conclusions from Chapters 2 and 3.

Chapter 1: **Introduction and Project Objectives**

Chapter 2: **Ionoregulatory Disruption as the Acute Toxic Mechanism for Lead in the Rainbow Trout (*Oncorhynchus mykiss*).**

Authors: Joseph T. Rogers, Jefferey G. Richards, and Chris M. Wood

Comments: This study was conducted by JTR under the supervision of CMW and with the aid of JGR. This paper has been published in *Aquatic Toxicology* 64: 215-234.

Chapter 3: **Characterization of Branchial Lead-Calcium Interaction in the Freshwater Rainbow Trout (*Oncorhynchus mykiss*).**

Authors: Joseph T. Rogers and Chris M. Wood

Comments: This study was conducted by JTR under the supervision of CMW. This paper has been submitted for publication to the *Journal of Experimental Biology.*

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF TABLES

CHAPTER 3

CHAPTER 1

INTRODUCTION AND PROJECT OBJECTIVES

Introduction

Ionoregulation in the freshwater teleost fish

In coping with a fluctuating aquatic environment, the teleost fish utilizes a number of regulatory processes to balance their internal environment and maintain ionic, osmotic, and acid-base homeostasis. Ionoregulation is especially important due to flux rates that vary greatly based on the ionic and osmotic properties of the water medium. For the freshwater teleost, the surrounding hypo-osmotic environment (approximately 10 mosmol kg^{-1} compared to plasma osmolality of 300 mosmol kg^{-1}) presents a challenge, as the potential loss of ions by diffusion down a concentration gradient must be countered by active uptake of electrolytes from the water to maintain a homeostatic state.

The primary site for active uptake of ions from the water column is the gill, more specifically, at specialized cells called mitochondrial-rich (MR) cells or chloride cells that represent approximately 10 percent of the branchial epithelial surface. As the name implies, these cells possess a large number of mitochondria, as well as an extensive tubular network associated with the baso-lateral membrane that is rich in enzymes involved in the active transport of ions into the plasma. Mechanisms of ion regulation at the chloride cell, some of which remain controversial, are outlined in Figure 1-1. For the purpose of this introduction, the uptake of Ca^{2+}, Na^{+}, and Cl^{-} will be discussed.

Calcium, an ion under intense homeostatic control, is absorbed in a series of steps that include uptake across the apical membrane, intracellular transport, and finally, active extrusion into the plasma via a high-affinity ATP-driven Ca^{2+} transporter on the basolateral membrane (Flik et al., 1985a; Verbost et al., 1994). Because environmental Ca^{2+} concentrations outside the cell are in the millimolar range compared to intracellular, sub-micromolar concentrations, movement across the apical surface of the gill cell is primarily by passive diffusion through voltage-insensitive channels (Flik et al., 1993). These channels are lanthanum-sensitive, and under the tight control of the stanniocalcin-mediated pathway. Once entering the chloride cell, Ca^{2+}-binding proteins, such as calmodulin, serve to transport Ca^{2+} to the baso-lateral membrane. The final step in Ca^{2+} uptake involves extrusion to the plasma by high-affinity Ca^{2+}-ATPase (Flik et al., 1985a; Verbost et al., 1994; Marshall, 2002). Contributions may also be made by a Na^{+}/Ca^{2+} exchange mechanism (Flik et al., 1994; Flik et al., 1997, Verbost et al., 1997).

Transepithelial absorption of Na^{+} and Cl^{-} begins with transport across the apical membrane of the branchial cell. In the case of Na^{+}, movement is thought to be generated by an apical H^{+}-ATPase that actively extrudes protons, creating a favourable electrochemical gradient by which Na^{+} can enter the cell through a coupled Na^{+} channel (Perry and Fryer, 1997; Clarke and Potts, 1998, Fenwick et al., 1999; Wilson et al., 2000). Additionally, Na^{+} uptake may also be coupled to ammonia excretion (Wilkie, 1997). Though this mechanism of Na^{+}/NH_4^{+} exchange remains controversial, experiments using Na^{+} transport blockers show that a portion of ammonia excretion is inhibited simultaneously (Kerstetter and Keeler, 1976; Payan, 1978); Wright and Wood, 1985; Wilson et al., 1994). The route for Cl^{-} uptake

is thought to be by a 1:1 exchange with bicarbonate through the action of an apical Cl^-/HCO_3^- exchanger (Wood and Goss, 1990; Perry, 1997). The movement of Na^+ and Cl^- across the apical gill cell membrane is driven indirectly by the action of the baso-lateral enzyme Na^+/K^+-ATPase, which contributes to a favourable electrochemical gradient for ion movement, and by hydration of intracellular CO_2 by carbonic anhydrase, providing H^+ and HCO_3^- for apical Na^+ and Cl^- exchange (Figure 1-1).

The action of Na^+/K^+-ATPase not only powers apical Na^+ transport, but also works to pump Na^+ ions across the baso-lateral membrane of epithelial cells into circulation. The mechanism of Cl^- extrusion from the epithelium is presently unknown.

Lead in the environment

Lead is a naturally occurring metal that is present in the earth's crust, rock, soil, and water. Entrance into the aquatic environment can occur through natural processes such as geologic weathering or volcanic action, however, most waterborne lead derives from human activities that include mining, smelting, coal burning, cement manufacturing, and use in gasoline, batteries and paint (World Health Organization, 1995). Background waterborne lead concentrations normally fall within the range of 0.6 to 120 $\mu g\ Pb\ L^{-1}$ (Demayo et al., 1982); however, under circumstances of contamination concentrations reaching 890 $\mu g\ L^{-1}$ have been reported (Research Triangle Institute, 1999).

Assessment of a metal toxicant requires the understanding of its relative persistence in the water column. Persistence is defined as the characteristic of a metal that is indicative of the constancy and duration of exposure of the available metal forms in a particular medium (DiToro et al., 2001). Examples of metal characteristics

that influence persistence are complexation, precipitation, mineralization, and transformation. These factors influence how abundant a metal remains in the water column, and the availability for uptake by an aquatic organism. It has been well documented that lead toxicity in an aquatic ecosystem is dependent upon speciation, which ultimately dictates bioavailability. In theory, it is the free ionic form of lead, Pb^{2+}, which is most readily available for uptake by an organism (Tao et al., 2000), and therefore, most toxic (Davies et al., 1976; Hodson et al., 1978). The presence of divalent lead is a function of water chemistry, specifically, water hardness/alkalinity which is normally expressed as the concentration of $CaCO_3$. It is the carbonate component that will readily bind lead, creating an insoluble $PbCO_3$ precipitate that is not readily transported across fish gills. This is believed to be protective against Pb toxicity (Davies et al., 1976; Holcombe et al., 1976); however, mechanisms for branchial uptake of particulate Pb have been proposed (Tao et al., 1999).

Calcium is another important component of hardness/alkalinity and is thought to compete with Pb for uptake, contributing to the protective effects of water hardness (Sayer et al., 1989; Sorensen, 1991). Water pH has also been correlated with Pb toxicity where the rate of uptake is accelerated with decreasing pH levels, presumably because all the Pb present is converted to its ionic form Pb^{2+} (Hodson et al., 1978).

Physiological effects of lead exposure

The primary site for Pb uptake in the freshwater teleost is the gill (Varanasi et al., 1978); however, uptake may also occur through the intestinal epithelium (Sorensen, 1991) and minimally via the skin (Varanasi and Markey, 1977). An abundance of circumstantial evidence suggests that Pb enters fish via a pathway

similar to that of Ca^{2+}, though there has been no research on the actual mechanism(s) involved. Once in the organism, Pb shares other handling pathways with Ca^{2+}. For example, Pb has been characterized as a bone-seeking element (Sorensen, 1991), accumulating in significant amounts in opercular bone (Hodson et al., 1977). Most often, deposition of Pb into the skeletal system will lead to developmental difficulties in juvenile fish, most notably, lordoscoliosis, which is a spinal deformity observed in juvenile trout exposed chronically to waterborne Pb (Davies et al., 1975, 1976; Holcombe et al., 1976). These deformities increase mortality rates and prevent successful reproduction in fish. As a calcium antagonist, Pb also interferes with neurological processes, inducing a series of behavioural abnormalities affecting feeding, reproduction, movement, and aggression. Weber et al. (1991, 1993) noted significant alterations in feeding and reproductive behaviours in Pb-exposed fathead minnows.

Lead is readily bound by sulfhydryl and carboxyl groups, contributing to a strong interaction with proteins and enzymes. For example, Pb interacts with Na^+/K^+-ATPase, an enzyme essential to the active uptake of Na^+ and Cl^- in freshwater teleosts and crustaceans. In chronically exposed crayfish, inhibition of Na^+/K^+-ATPase occurs causing disruptions in Na^+ balance and osmoregulation (Ahern and Morris, 1998). Sorensen (1991) links lead poisoning to the inhibition of a number of other enzymes. Glutamic oxalacetic transaminase (GOT), lactate dehydrogenase (LDH), alkaline phosphatase, acid phosphatase, RNase, and catalase are among a few of the important enzymes inhibited by lead. One of the signature enzymes disrupted is delta-aminolevulinic acid dehydratase (ALAD) (Hodson et al., 1976). This enzyme catalyses the formation of porphobilinogen (PGB), a precursor of heme synthesis,

from aminolevulinic acid (ALA). Essential sulfhydryl groups in the enzyme are bound up by lead, resulting in inhibition of heme synthesis and decreased formation of hemoglobin. A direct relationship exists between ALAD activity and the log of blood lead concentrations (Hodson et al., 1977). This action is likely an important contributor to the chronic toxic action of lead, but not to its acute toxicity.

Lead toxicity and the use of predictive modelling

Fundamental to our present understanding of the toxic responses of the gill to metal contaminants in the environment are the ideas originally put forth by Pagenkopf (1983). Here, the gill surface is recognized as containing numerous binding sites where metals in the environment could potentially interact. Metal binding and its resultant toxic effect, however, is dependent upon water quality, specifically competing cations such as H^+, Ca^{2+} and Mg^{2+} and complexing anions such as carbonate or dissolved organic matter. Interactions of a metal with the biological ligand and other components of the water column are characterized by the application of conditional equilibrium stability constants, or log K values. In general, the larger the log K value, the stronger the binding. To date, major breakthroughs in predictive modelling have been made, including computer-based geochemical modeling programs such as MINEQL+ (Schecher and McAvoy, 1992) that allow for organization of common log K values and entry of gill-metal K values for computation. Additionally, the development of physiological experiments that measure metal deposition on gills in the presence of known concentrations of competing and complexing agents, has allowed for successful development of binding models for metals like copper, cadmium, silver, and cobalt (Wood, 2001).

Characterization and understanding of the toxic mechanism of a waterborne metal is essential to the process of predictive modelling using a metal-gill interaction approach (Playle, 1998). Models, such as the biotic ligand model (BLM; McGeer et al., 2000; Paquin et al., 2000, DiToro et al., 2001), are used to predict metal toxicity on a water chemistry basis, and as outlined above, assess the affinity of a metal for a biological membrane such as the fish gill in the presence of competing ions and complexing agents in the water column. While an accepted BLM for Pb does not yet exist, lead-gill binding models are currently being developed (MacDonald et al., 2002) (Figure 1-2), representing a link between physiological toxic effect and water chemistry. Further development of binding models for Pb, and ultimately, construction of an acceptable BLM similar to that for Ag (McGeer et al., 2000), depends upon an intense understanding of the acute toxic mechanism for Pb and of the key binding sites involved in this toxicity.

Project Objectives

The main goal of this thesis was to both mechanistically and physiologically investigate Pb toxicity in the rainbow trout. In working towards this goal, the following objectives were set forth:

1. To determine the acute toxic mechanism for waterborne Pb in the rainbow trout; establishing whether toxicity is based on respiratory and acid/base disturbance, or alternatively, ionoregulatory disruption (Chapter 2).
2. To characterize key binding sites involved in acute Pb toxicity. Based on work in Chapter 2, characterization includes investigation into the branchial interaction of Pb and Ca^{2+} in the rainbow trout using both physiological and pharmacological techniques. Specifically, whether Pb shares the same uptake pathway as Ca^{2+}, including competition at apical Ca^{2+} channels on the fish gill, and interaction at high-affinity Ca^{2+}-ATPase at the baso-lateral membrane (Chapter 3).

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Fig. 1-1

A schematic representation of ionoregulatory mechanisms at the apical (freshwater) and baso-lateral (plasma) sides of the teleost chloride cell. Dotted lines represent diffusion. Solid lines associated with ATP (adenosine 5' triphosphate) labelled carriers represent active transport. Other solid lines associated with carriers indicate electroneutral exchange (from Wood (2001)).

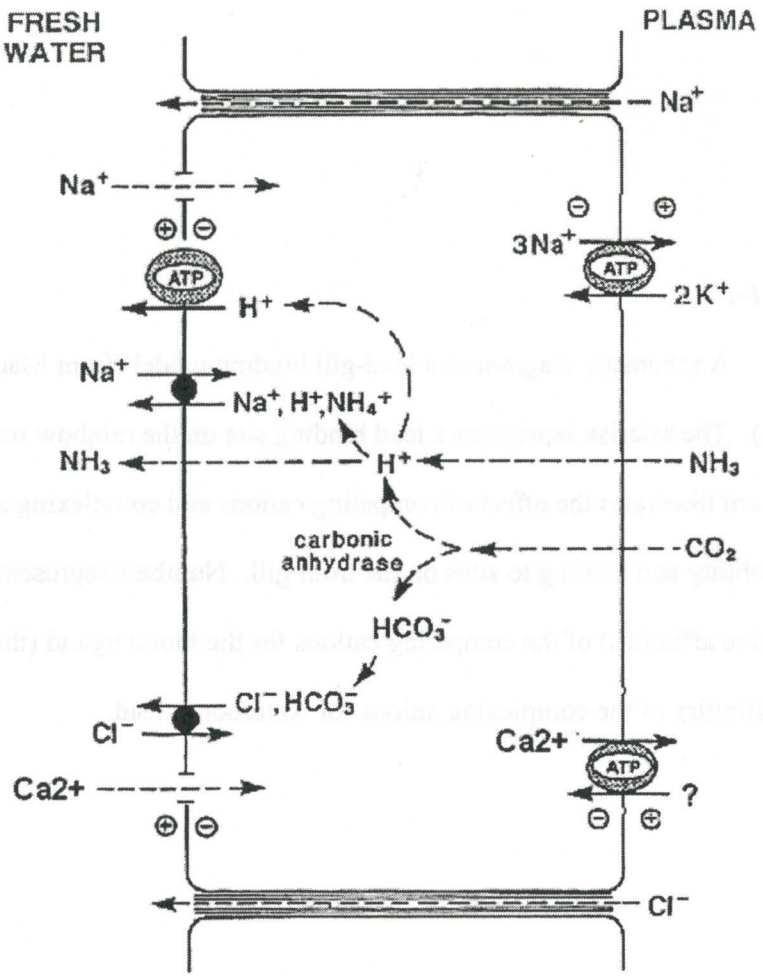
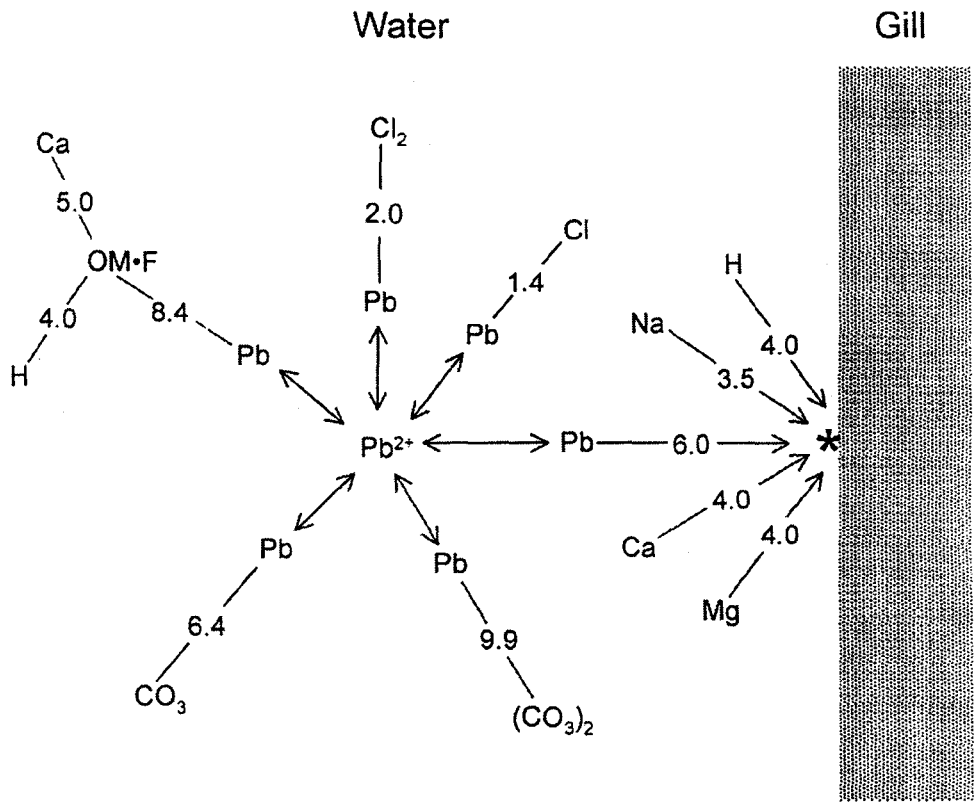

FRESH WATER
PLASMA
Na+
Na+
3Na+
ATP
2K+
H+
Na+
Na+, H+, NH4+
NH3
H+
NH3
carbonic anhydrase
CO2
HCO3-
Cl-, HCO3-
Cl-
Ca2+
Ca2+
ATP
?
Cl-

Fig. 1-2

A schematic diagram of a lead-gill binding model (from Macdonald et al., 2002). The asterisk represents a lead binding site on the rainbow trout gill. The diagram illustrates the effect of competing cations and complexing agents on lead availability and binding to sites on the trout gill. Numbers represent the log K values (relative affinities) of the competing cations for the biotic ligand (the trout gill) and the affinities of the complexing anions for waterborne lead.


Water
Gill
Ca
5.0
OM•F
4.0
H
8.4
Pb
Cl2
2.0
Pb
Cl
1.4
Pb
H
Na
4.0
3.5
Pb2+
Pb
6.0
*
4.0
Ca
4.0
Mg
Pb
6.4
CO3
Pb
9.9
(CO3)2

CHAPTER 2

IONOREGULATORY DISRUPTION AS THE ACUTE TOXIC MECHANISM FOR LEAD IN THE RAINBOW TROUT (*Oncorhynchus mykiss*)

Abstract

The mechanism for acute toxicity of lead (Pb) in rainbow trout (*Oncorhynchus mykiss*) was investigated at Pb concentrations close to the 96 h LC50 of 1.0 mg dissolved Pb L^{-1} (0.8 – 1.4, 95% C.I.) determined in dechlorinated Hamilton city tap water (from Lake Ontario, hardness = 140 mg L^{-1} $CaCO_3$). Tissue Pb accumulations associated with death were highest in the gill, followed by kidney and liver. Significant ionoregulatory impacts were observed in adult rainbow trout (200-300g) fitted with indwelling dorsal aortic catheters and exposed to 1.1 ± 0.04 mg dissolved Pb L^{-1}. Decreased plasma [Ca^{2+}], [Na^{+}], and [Cl^{-}] occurred after 48 h of exposure through to 120 h, with increases in plasma [Mg^{2+}], ammonia, and cortisol. No marked changes in PaO_2, $PaCO_2$, pH, glucose, or hematological parameters were evident. Branchial Na^{+}/K^{+} ATPase activity in juvenile trout exposed to concentrations close to the 96 h LC50 was inhibited by approximately 40% after 48 h of Pb exposure. Calcium ion flux measurements using ^{45}Ca as a radiotracer showed 65% inhibition of Ca^{2+} influx after 0 h, 12 h, 24 h, or 48 h exposure to the 96 h LC50 concentration of Pb. There was also significant inhibition (40-50%) of both Na^{+} and Cl uptake, measured with ^{22}Na and ^{36}Cl simultaneously.

We conclude that the mechanism of acute toxicity for Pb in rainbow trout occurs by ionoregulatory disruption rather than respiratory or acid/base distress at Pb concentrations close to the 96 h LC50 in moderately hard water.

Introduction

Lead, a group IVA element in the periodic table, is a naturally occurring metal present in the earth's crust, rock, soil, and water. Lead enters aquatic environments by a number of pathways. The earth's crust, geologic weathering phenomena, and volcanic activity account for natural sources, but most waterborne lead derives from human activities such as mining and smelting, coal burning, cement manufacturing, and use in gasoline, batteries, and paint (World Health Organization, 1995). Contamination of water by such practices is the primary cause of lead poisoning in fish (Sorensen, 1991).

Under normal conditions, waterborne lead falls within the range of 0.0006 to 0.12 mg L^{-1} (Demayo et al., 1982) though concentrations as high as 0.89 mg L^{-1} have been reported (Research Triangle Institute, 1999). In hard water, lead readily complexes to form $Pb(CO_3)$. Such complexes are less available for uptake and therefore, less toxic to fish (Davies et al., 1976; Holcombe et al., 1976). Calcium, a prominent component of hard water, is believed to compete with lead for uptake, contributing to the protective effects of water hardness. Lead species such as Pb^{2+} and $Pb(OH)^{+}$ that commonly occur in soft, low pH waters are more available and toxic (Davies et al., 1976; Hodson et al., 1978).

Although a number of physiological effects of lead have been well characterized in various fish species (Davies et al., 1976; Hodson, 1976; Hodson et al., 1977; Weber et al., 1991; Weber, 1993), relative to other metals, little is known about the acute toxic mechanism of lead in fish. Circumstantial evidence suggests that a lead/calcium antagonism exists (Hodson et al., 1978; Varanasi and Gmur, 1978; Settle and Patterson, 1980) similar to that discovered for cadmium (Verbost et al.,

1987; Verbost et al., 1989) and zinc (Spry and Wood, 1985), by which these metals may directly compete with Ca^{2+} for uptake at calcium binding sites. This would result in hypocalcemia and death of the fish. Such a relationship, however, has not been proven directly. In addition to this possible mechanism, lead may also disrupt the balance of ions such as Na^+ and Cl^- as exhibited by metals like copper (Lauren and McDonald, 1985) and silver (Wood et al., 1996; Morgan et al., 1997). Ahern and Morris (1998) observed disruption of Na^+ balance and a 40% inhibition of Na^+/K^+ ATPase activity in crayfish chronically exposed to 0.5 mg Pb L^{-1}. Another possibility is that respiratory effects may occur similar to those caused by aluminum at moderately acidic pH (Playle et al., 1989) or by nickel at circumneutral pH (Pane et al., 2002). It is unknown whether any of these mechanisms explain the toxic action of lead in fish.

Detailed knowledge of the acute toxic mechanisms for a number of water-borne metals (e.g. copper, silver) has been established, allowing development of models used to predict toxicity in many different water chemistries. The Biotic Ligand Model (BLM) is one example (McGeer et al., 2000; DiToro et al., 2001). Currently, an accepted model for lead does not exist, a situation that is at least partially due to the lack of understanding of its acute toxic mechanism. Therefore, the objective of this study was to determine the mechanism of acute toxicity of lead in the rainbow trout (*Oncorhynchus mykiss*). The experimental approach used was similar to that earlier employed for silver (Wood et al., 1996) and nickel (Pane et al., 2002). It was designed to provide an appropriate 'diagnosis' of the nature of acute physiological disturbances within the fish when they were faced with a lead challenge at the acute level (i.e. close to the 96 h LC50).

Materials and Methods

Experimental animals

Juvenile rainbow trout (1.5 – 3.5 g), for use in the LC50 tests, flux experiments, Na^+/K^+ ATPase measurements, and adult rainbow trout (200 – 300 g), for use in cannulated fish studies, were obtained from Humber Springs Trout Farm in Orangeville, Ontario. Fish were held in dechlorinated City of Hamilton tap water (from Lake Ontario) at a temperature of 7 - 12°C, and fed commercial trout pellets at a ration of 1 % total body weight per day. Water composition was Ca^{2+} = 1.0, Mg^{2+} = 0.2, Na^+ = 0.6, Cl^- = 0.8, K^+ = 0.05 (mM), total Pb = 0.68 μg L^{-1}, dissolved organic carbon (DOC) = 3 mg L^{-1}, hardness (as $CaCO_3$) of approximately 140 mg L^{-1}, and pH = 7.9 – 8.0. Experimental animals were starved 72 hours prior to and throughout all experiments.

96 h LC50 and bioaccumulation

The 96 h LC50 determination for rainbow trout was carried out under flow-through conditions following closely methods outlined in Sprague (1969). Juvenile trout were transferred to one of twelve 20 L exposure tanks (15 fish per tank) fed at a rate of 600 ml min^{-1} from a head reservoir and a series of mixing tanks with aerated dechlorinated Hamilton city tap water. Trout were allowed to acclimate to these conditions for a period of 48 h before exposure to nominal total Pb concentrations of 0.25, 0.5, 1, 3, 5 mg L^{-1} plus one control treatment (2 tanks per treatment). Pb exposure was carried out by dripping a stock solution of $Pb(NO_3)_2$ (Sigma-Aldrich) dissolved in NANOpure water (NANOpure II; Sybron/Barnstead, Boston MA) at a

rate of 1 ml min^{-1} into mixing tanks. At t = 0 h, the tanks were spiked using the stock solution to bring exposure water to the appropriate concentration.

Water samples were obtained daily, filtered (Acrodisc 0.45 μm filter; Pall Corporation, MI) and unfiltered. Filtered water samples were taken to quantify the concentration of dissolved Pb in solution. Water drawn was placed in clean plastic scintillation vials and acidified to 1% HNO_3 for storage until samples were analysed for total and dissolved Pb concentrations. No glassware was employed. Mortality was monitored every six hours. At each 6 h sampling point, dead fish were removed and gill, kidney, and liver samples were excised for tissue lead analysis. Gills were blotted dry and tissues were weighed, digested at 55°C for 48 hours in 1N HNO_3 (Fisher Scientific; trace metal grade), centrifuged, and the supernatant drawn off for total Pb measurement. Both water samples and tissue digests were analyzed for Pb using graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA; Varian, Australia) against a certified multi-element standard (Inorganic Ventures, Inc.). QA/QC procedures include GFAAS and FAAS performance tests twice yearly, multiple determinations on the same samples, multiple blanks, addition-recovery tests, and the sample-addition method for quantification of low-level samples.

Cannulated fish studies

Experimental protocol

The nominal total Pb exposure concentration chosen for this experiment was 1 mg L^{-1}, based on the measured Pb 96 h LC50 in City of Hamilton tap water (see Results). Adult rainbow trout were anaesthetized using MS222 (0.1 g L^{-1} neutralized with NaOH) and surgically fitted with indwelling dorsal aortic catheters (PE50

tubing) (Soivio et al., 1972) filled with Cortland saline (Wolf, 1963). Immediately following surgery, fish were transferred to individual, darkened plexiglass chambers (volume = 8 L) served with individual aeration. Control chambers were fed from a vigorously aerated head tank at a rate of 100 ml min^{-1}. Pb exposure chambers received an equal flow rate but from an aerated mixing tank receiving a 1 ml min^{-1} drip of $Pb(NO_3)_2$ stock solution. At t = 0 h, Pb-exposed chambers were spiked with an appropriate volume of stock solution to achieve desired Pb concentrations.

Control and Pb treatments were run simultaneously using 8 control fish and 7 Pb-exposed fish. In both groups, blood samples (1 ml, with saline and red blood cell replacement) were taken prior to Pb exposure (t = 0 h) and at 24 h, 48 h, 72 h, 96 h, and 120 h. Blood was drawn anaerobically into an ice-cold, gas-tight Hamilton syringe for analysis of arterial pH (pH_a), O_2 tension (P_aO_2), plasma total CO_2 (C_aCO_2), hematocrit (Ht), hemoglobin (Hb), and plasma levels of Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, total Pb, glucose, lactate, cortisol, total protein, and total ammonia. Plasma was separated by centrifugation at 13,000 g and frozen at -80°C for these analyses. Erythrocytes were gently re-suspended in Cortland saline (Wolf, 1963) and re-injected into the fish in a total volume of 1.0 ml. Water samples, filtered (0.45μm) and unfiltered, were drawn simultaneously from in front of the mouth and processed as in section 2.2. Unfiltered samples were also taken for measurement of inspired oxygen tension (P_IO_2) and pH_I.

Analytical Methods

For most blood parameters, the analytical procedures used followed those outlined in Wood et al. (1988). Radiometer electrodes and meters were used in the

analysis of pHa, PaO_2, inspired water pH (pH_I), and O_2 tension (P_IO_2). Hb measurements employed the colorimetric cyanmethemoglobin method (Sigma-Aldrich). Ht was measured directly from micro-hematocrit tubes, spun at 5000g for 4 min and the plasma removed anaerobically for measurement of total CO_2 (C_aCO_2) with a Corning 965 CO_2 analyzer. Lactate was measured enzymatically (L-lactate dehydrogenase/NADH; Sigma-Aldrich) on samples deproteinized with ice cold 8% perchloric acid. Glucose was measured enzymatically from the same acid extracts neutralized with 1M K_2CO_3 using the hexokinase/glucose-6-phosphate dehydrogenase method (Sigma Aldrich). Plasma cortisol was determined using a ^{125}I radioimmunoassay (ICN Biomedicals; Montreal, Quebec). Total plasma ammonia concentration was measured enzymatically (glutamate dehydrogenase/NADP; Sigma Aldrich). Plasma Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations were determined by flame atomic absorption spectrophotometry (FAAS; 220FS SpectrAA; Varian, Australia), and plasma Pb by GFAAS. Plasma was diluted with 0.2% La for plasma Ca^{2+} determination. Plasma Cl^- was measured by coulometric titration (Radiometer CMT10).

Na^+/K^+ ATPase activity

Pb exposure was carried out using methods similar to those used during the 96 h LC50 experiment (20 L tanks, 10 fish per tank, flow-through exposure at 600 ml min^{-1}, one control and five exposure tanks, each tank being used for a different exposure time to a lead level close to the 96 h LC50 (see Results)). Sampling for gill tissue took place at 0, 24, 48, 72, 96, and 120 h. Fish were removed from exposure water, killed immediately with a blow to the head, and dissected on ice. Excised gills

were then placed into a 1.5 ml bullet tube, transferred to liquid nitrogen, and stored at -80°C. Na^+/K^+ ATPase activity was determined using the microplate method outlined by McCormick (1993). For replicate measurements on the same samples, the coefficient of variation was 0.19. To ensure accurate interpretation of the Na^+/K^+ ATPase data, the effect of Pb on the coupling enzymes used in this assay (PK and LDH) was assessed *in vitro* via the addition of $Pb(NO_3)_2$ to the homogenate at the concentrations found in experimental samples; and was found to be insignificant. Protein was measured by the Bradford protein assay using bovine serum albumin protein standards (Sigma-Aldrich).

Ion flux experiments

Ca^{2+} influx measurements

Ca^{2+} influx determinations were carried out on control and experimental fish (juvenile rainbow trout) simultaneously. Experimental fish were pre-exposed to Pb prior to the 2 h flux periods for 0 h, 12 h, 24 h, or 48 h at concentrations close to the resulting 96 h LC50 (see Results). The exposure method and conditions were identical to those used in the 96 h LC50 tests. Fluxes were measured at the time intervals 0-2 h, 12-14 h, 24-26 h, and 48-50 h, using 250-ml Pyrex glass beakers fitted with a steady air supply and filled with 60 ml of control or Pb-containing dechlorinated City of Hamilton tap water (n = 8 for each treatment). The beakers were partially submerged on a light-shielded wet table receiving a constant water flow (for temperature control) for the entire flux period. Juvenile trout were transferred from exposure chambers to the flux beakers and were allowed a settling period of 0.5 h. After 0.5 h elapsed, ^{45}Ca radiotracer (as $CaCl_2$, specific activity = 15.538 mCi mg$^-$

[1], Perkin-Elmer, USA) was added to each beaker (6 μCi per chamber) and allowed to equilibrate for 0.25 h. Following equilibration, water samples were taken for the determination of initial ion concentration (800 μl) and for radioisotope counting (200 μl) (CPM). Equal volumes were taken at the end of the 2 h radiotracer exposure period for final ion and radioactivity measurements. Trout were removed from flux chambers at this time and killed with a blow to the head. Immediately thereafter, the dead fish were rinsed for 1 min in 1 mM EDTA (ethylenediamine-tetraacetic acid; Sigma-Aldrich) followed by a 1 min rinse in a 5 mM cold Ca^{2+} solution ($Ca(NO_3)_2$) (Sigma-Aldrich) to remove all surface-bound ^{45}Ca. Fish were then blotted dry, weighed, wrapped in foil, frozen in liquid nitrogen, and stored at -80°C.

^{45}Ca radioactivity was measured by adding 200 μl water samples to 2 ml of aqueous counting scintillant (ACS™; Amersham) followed by scintillation counting (Rackbeta 1217; LKB Wallac, Turka, Finland). Water samples taken for the determination of total [Ca^{2+}] were diluted with 0.2% La^{3+} and analyzed by FAAS using the Varian 220FS Spectra AA.

Frozen whole fish were homogenized by grinding in liquid nitrogen, using methods similar to those of Hogstrand et al. (1994). Tissue aliquots (approximately 100 mg, exact weight recorded), in duplicate, were transferred to 20 ml glass scintillation vials and solubilized at 50°C for 24 hours in 1 ml of NCS-II™ tissue solubilizer (Amersham). The resulting digest was neutralized using glacial acetic acid (~30μl) and diluted with 10 ml of organic counting scintillant (OCS™; Amersham). Samples were stored in darkness overnight to reduce chemoluminescence. ^{45}Ca radioactivity was measured by scintillation counting and quench-corrected to the same

counting efficiency as water samples by the method of external standard ratios, using a ^{45}Ca quench curve generated from the tissue of interest in the same counting cocktail.

Na^+/Cl^- influx measurements

Methods used for Pb-exposure and flux measurements of Na^+ and Cl^- were similar to those of Ca^{2+} with a few differences. Radiotracers used were ^{22}Na (as NaCl, specific activity = 690.19 mCi mg^{-1}, NEN Life Science Products, Inc, Boston MA) and ^{36}Cl (as NaCl, specific activity = 0.012 mCi mg^{-1}, ICN Biomaterials, Inc, Irvine CA). These isotopes were added together for each round of fluxes at 0.2μCi per flux beaker. Water samples were taken at 0.25 h and at the end of the 2 h flux period for radioisotope counting (1 mL) and for measurement of ion concentration (1 mL). At the end of the flux period, the fish were removed from flux chambers and killed with a blow to the head. Fish were then blotted dry, weighed, placed in plastic scintillation vials, and stored at -20°C for radioisotope analysis. This method assumes that flux measurements reflect exchanges at the gills, consistent with the lack of absorption of monovalent ions such as Na^+ and Cl^- to plastic or fish surfaces (Wood, 1992).

^{22}Na is a dual γ and β emitter while ^{36}Cl is a pure β emitter. Exposure water samples were first measured for ^{22}Na by γ counting (Minaxi γ; Canberra-Packard, Meridan, CT), followed by addition of 5 mL of scintillation fluid (ACS; Amersham) to the same water samples for ^{36}Cl and ^{22}Na scintillation counting (Rackbeta 1217; LKB Wallac, Turka, Finland). Differences in counting efficiencies for ^{22}Na between the two instruments were corrected using common standards, and ^{36}Cl β radioactivity

was measured by subtracting the corrected ^{22}Na activity from the total activity measured by scintillation counting. [Na^+] was measured by FAAS, while [Cl^-] was analyzed by the mercuric thiocyanate spectrophotometric method (Zall et al., 1956). The concentrations of the two radioisotopes were also measured in the fish. Whole-body ^{22}Na was measured by γ counting. For ^{36}Cl determination, whole fish were digested in 1N HNO_3 (Fisher Scientific; trace metal grade) at 60° C for 48 hours, homogenized by vortexing, an aliquot removed (1.5 mL), centrifuged at 13,000g for 10 minutes, and the supernatant (1 mL) added to 5 mL of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT). ^{22}Na plus ^{36}Cl radioactivity was measured by scintillation counting and corrected as above to quantify only ^{36}Cl β emissions. Quenching observed for ^{36}Cl was negligible.

Calculations and Statistical Analysis

The 96 h LC50 for Pb with upper and lower 95% confidence intervals was calculated using probit analysis on SPSS statistical software.

Calculations of P_aCO_2 and plasma HCO_3^- in cannulated adult rainbow trout were made using equations outlined in Playle et al. (1989) using the Henderson-Hasselbach equation and values for CO_2 solubility and apparent pK (pK') in trout plasma at the corresponding temperature from Boutilier et al. (1984). Mean cell hemoglobin concentration (g Hb ml^{-1} of red blood cells) was calculated as the ratio of simultaneous Hb to Ht measurements.

Unidirectional calcium, sodium, and chloride influx rates in juvenile rainbow trout were calculated using the following formula:

$$J_{in}^{Ion} = \frac{cpm\, g\, fish^{-1}}{1/2\left[\frac{cpm_i}{[Ion]_i} + \frac{cpm_f}{[Ion]_f}\right]t}$$

where cpm_i, cpm_f, and t are as described in flux procedures, and $[Ion]_i$ and $[Ion]_f$ are initial and final ion concentrations in the flux water (Wood, 1992).

Calculated data are expressed as mean ± 1 SEM. Experimental means were compared to corresponding control mean values by an unpaired two-tailed Student's t-test. Time-dependent responses in both control and experimental groups were tested against initial 0 h measurements using a one-way ANOVA with a two-sided Dunnett's post hoc multiple comparison. In addition, two-way ANOVA tests were run on data obtained from adult cannulated trout to investigate the possible significance of time on measured parameters. Where time was a factor, a Tukey honest significant difference (HSD) post-hoc comparison was run. All statistical significance is calculated at $p < 0.05$.

Results

96 h LC50 and tissue lead distribution

The acute lead 96 h LC50 for juvenile rainbow trout in dechlorinated Hamilton city tap water was calculated to be 1.0 mg L^{-1} as dissolved Pb (total Pb = 1.04 mg L^{-1}), with lower and upper 95% confidence intervals of 0.8 and 1.4 mg Pb L^{-1}, respectively.

The tissue lead distribution associated with acute mortality at waterborne Pb concentrations close to the 96 h LC50 is given in Fig. 2-1. Of the tissues sampled, Pb burden was greatest in the gill, reaching concentrations close to 200 μg g^{-1} wet weight

in Pb-exposed fish. This was 343 times greater than Pb measured in the gills of control fish. The kidney also showed significant accumulation with a burden of approximately 35 $\mu g\ g^{-1}$ wet weight that was about 5 times greater than control burdens. Liver Pb was approximately 16 $\mu g\ g^{-1}$ wet weight versus control concentrations of about 3 $\mu g\ g^{-1}$ wet weight.

Cannulated fish studies

Physiological responses to lead exposure

The mean measured dissolved Pb concentration during the exposure period for these adult fish was 1.1 ± 0.04 mg L^{-1} (total Pb = 1.4 ± 0.15 mg L^{-1}), which was close to the 96 h LC50 of 1.0 mg L^{-1} for juvenile rainbow trout. The concentration of total Pb in control water was 0.7 ± 0.15 $\mu g\ L^{-1}$.

Rainbow trout exposed to acute levels of $Pb(NO_3)_2$ did not appear to be under any degree of respiratory distress. Measured PaO_2 (Fig. 2-2a) in Pb-exposed trout did not show any significant changes when compared to control fish, and did not deviate from levels observed at t = 0 h. Similarly, $PaCO_2$ did not change significantly in Pb-exposed or control individuals, though there was a small, non-significant increase at t = 120 h in control individuals (Fig. 2-2b). Ventilation rate remained stable (Fig. 2-2c) and plasma lactate unchanged (Fig. 2-2d) throughout the exposure period.

Acid-base measurements showed that pH_a also remained very stable (Fig. 2-3a). While no apparent respiratory or metabolic acidosis was evident, a progressive non-significant increase in plasma HCO_3^- occurred in Pb-exposed fish after 48 h of exposure (Fig. 2-3b).

Glucose, a classic indicator of stress, did not change significantly in control or Pb-exposed fish (Fig. 2-4a). However, significant increases in plasma cortisol levels were observed in Pb-exposed fish at 96 and 120 h while control values remained relatively stable (Fig. 2-4b). In addition to an observed cortisol elevation, Pb-exposed fish appeared to be retaining ammonia as plasma concentrations showed a progressive increase from 48 to 120 h of exposure that resulted in levels significantly greater than in control fish (Fig. 2-4c).

Ionoregulatory impacts

Plasma Pb increased dramatically from background concentrations of 24 μg L^{-1} to approximately 170 μg L^{-1} by 24 h in Pb-exposed fish (Fig. 2-5a). However, Pb concentrations did not continue to increase but stabilized from 24 to 120 h. Control plasma Pb levels remained low and unchanged (approximately 20 μg L^{-1}).

Significant hypocalcemia was observed in the Pb treatment (Fig. 2-5b). Plasma Ca^{2+} concentrations were impacted after 48 h of exposure, showing a 31% reduction by 72 h and significantly lower concentrations than in the control treatment at 72 and 96 h. Control values remained stable. While Pb appeared to affect Ca^{2+} concentrations in exposed fish, it also caused a small increase in plasma Mg^{2+} (approximately 36%) that was significantly greater than control values at 96 and 120 h (Fig. 2-5c).

Plasma K^{+} did not show significant changes due to treatment, however, there was an overall significant time effect on concentrations after repeated measuring (Fig 2-6b). Pb-exposed fish exhibited significant decreases in plasma Na^{+} and Cl^{-} concentrations. Plasma Na^{+} (Fig. 2-6b) fell rapidly after 48 h in Pb-exposed fish from

about 150 mM to 124 mM (18% decrease). Conversely, control values remained normal and relatively stable. Plasma Cl^- showed a similar trend with a 15 % decrease, falling to 105 mM by 120 h (Fig. 2-6c). Again, control fish maintained stable plasma Cl^- levels (approximately 130 mM).

Hematology

Continuous daily blood sampling caused hemoglobin (Hb) and hematocrit (Ht) to decrease over time in both control and Pb treatments (Fig. 2-7a and 2-7b), despite the fact that the majority of the red blood cells were replaced after each sampling. This trend was significant only in control fish which exhibited a significant decrease in Ht at 96 h relative to t = 0 h and significantly lower [Hb] and Ht at 72 and 96 h compared to experimental fish. Mean cell hemoglobin concentration did not change, however, (Fig. 2-7c) showing that erythrocyte swelling did not occur. Plasma protein remained unchanged in both control and Pb-exposed fish (Fig.2-7d).

Na^+/K^+ ATPase activity

Rainbow trout were exposed to a dissolved Pb concentration of 1.5 ± 0.04 mg L^{-1} (total Pb = 1.6 ± 0.11 mg L^{-1}). When compared to values for unexposed fish at t = 0 h (control sampling), a significant 40% reduction in activity occurred by 48 h, and the inhibition remained stable and significant through to 120 h of exposure (Fig. 2-8).

Ion flux measurements

Ca^{2+} influx measurements

Calcium influx rates were significantly inhibited in juvenile trout through to 48 h compared to control values during exposure to 1.2 ± 0.04 mg L^{-1} of dissolved Pb (total Pb = 1.7 ± 0.25 mg L^{-1}), a concentration close to the 96 h LC50 for rainbow trout in Hamilton city dechlorinated tap water. The amount of total Pb in control exposure water was 0.85 ± 0.05 μg L^{-1}. Inhibition was immediate with influx rates being reduced in the first 2 h (initial flux period), and remaining stable at about 65% inhibition through to 48 h. No apparent time-dependent changes in uptake rates that would indicate recovery or up-regulation of calcium influx occurred.

Na^+ and Cl^- flux measurements

Figure 2-9b and 2-9c illustrate influx measurements for sodium and chloride, respectively, in control fish or fish exposed to 0.89 ± 0.05 mgPb L^{-1} dissolved Pb (total Pb = 0.98 ± 0.07 mg L^{-1}). The amount of total Pb in control exposure water was 1.1 ± 0.17 μg L^{-1}. Both sodium and chloride uptake were significantly inhibited in Pb-exposed fish compared to rates measured in control fish. Inhibition took place immediately during the first 2 h flux period and continued at a stable value of about 50 % at 12-14 h, 24-26 h, and 48-50 h. Chloride uptake showed similar reductions of about 40% at 0-2 h, 12-14 h, 24-26 h, and 48-50 h.

Discussion

96 h LC50 and tissue accumulation

Considering the water hardness (140 mg L^{-1} as $CaCO_3$) and exposure method (flow-through) used, the 96 h LC50 of 1.0 mg dissolved Pb L^{-1} (1.04 mg L^{-1} total Pb) determined in this study is surprisingly close to previously reported LC50 values

(expressed as dissolved Pb) which used very different exposure conditions. For example, Davies et al. (1976) reported an LC50 obtained from a static-renewal method in water with hardness = 353 mg L^{-1} (as $CaCO_3$) of 542 mg L^{-1} total Pb, of which only 1.47 mg Pb L^{-1} was dissolved. Davies et al. (1976) also determined an LC50 in relatively soft water (28 mg L^{-1} as $CaCO_3$) of 1.32 mg L^{-1} dissolved Pb. Hodson et al. (1978) reported a value of 2.4 mg Pb L^{-1} in a 21-day LC50 done in Lake Ontario water (hardness = 135 mg L^{-1} as $CaCO_3$). Holcombe et al. (1976), using a flow-through method, reported an LC50 of 3.36 mg L^{-1} dissolved Pb (total Pb = 4.10 mg L^{-1}) using adult brook trout in relatively soft water (44 mg L^{-1} as $CaCO_3$). This slightly higher value may reflect a fish species or age difference. With the apparent uniformity of dissolved Pb LC50 values, despite a wide range of water qualities and total Pb LC50's, it appears that future research into the effects of water quality on Pb toxicity should concentrate on the dissolved component only. In the present study, LC50 measurements were useful in establishing an acute toxic level of Pb under flow-through conditions in water of our hardness that could be used in further acute studies.

Tissue accumulation data (Fig. 2-1) point to the gill as the probable primary site for the acute toxic action of Pb based on the high Pb-burden measured here compared to other soft tissues sampled. This is consistent with the fact that the gill is the primary site of Pb uptake in fish (Varanasi and Gmur, 1978) and that a strong relationship may exist between gill metal burden and toxicity as has previously been demonstrated for other metals (Playle et al., 1993; Playle, 1998; Di Toro et al., 2001). Chronically exposed rainbow trout also show elevated gill-Pb concentrations relative to other soft tissues such as the kidney and liver, accumulating approximately 15 μg g^{-1} wet weight after prolonged exposure to 0.1 mg Pb L^{-1} (Hodson et al., 1978). This

accumulation is less than that reported in the present study, which could reflect a difference in rates of uptake in acute versus chronic Pb exposures. It is clear that Pb also crossed the gill and entered the fish as significant accumulation occurred in the kidney and liver, and Pb was substantially elevated in the blood plasma (Fig 2-5a). In addition to the gill, the intestine may have been a route of uptake as demonstrated in Pb-exposed goldfish (Tao et al., 1999). Kidney Pb could contribute to acute toxicity, though studies investigating the nephrotoxic effects of Pb have not yet been performed in fish. Studies on the nephrotoxicity of Cd, initiated because selective accumulation was observed in the kidney tissue during chronic exposure (Sangalang and Freeman, 1979), found that disturbances in electrolyte balance do not result from impairment of renal function (Giles, 1984). Whether this is the case for Pb is still unclear. Similarly, the possible contribution of the liver Pb accumulation to acute toxicity is unknown.

Acute ionoregulatory disturbance

Cannulated fish exposed to the Pb 96 h LC50 showed significant ionoregulatory impairment after 48 h of exposure while experiencing no apparent respiratory or acid/base disturbance. The observed stability of PaO_2, $PaCO_2$, pHa, plasma lactate, and ventilation rate in control and experimental trout over the course of the 120 h experiment contrasts with the clear respiratory toxicity induced by Al at moderately acidic pH (Playle et al., 1989) or by Ni at circumneutral pH (Pane et al., 2002). Exposure to Al and Ni results in substantial decreases in $PaO_2$and pHa, and increases in $PaCO_2$, blood lactate, and ventilation rate.

The time course of accumulation of Pb in the plasma of experimental fish saturated quickly, following a rapid 7-fold increase over the first 24 h. This is similar to metals such as Ag, Cu, or Cd, which show saturation over time (Wood et al., 1996; Richards and Playle, 1999; Wood et al., 1999). Part of the explanation may be the ability of erythrocytes to sequester Pb from the plasma (Holcombe et al., 1976; Hodson et al., 1977; Johansson-Sjobeck and Larsson, 1979). In addition, the saturation phenomenon may reflect clearance of Pb to the tissues and reduced uptake at the gill over time, possibilities which could be tested in future studies. The effect of Pb on hematological parameters was minimal in the present study (Fig.2-7).

While the plasma Pb elevation saturated by 24 h, the fall in plasma Ca^{2+} in experimental fish was not significant until 48 h (Fig. 2-5d). This agrees with existing circumstantial evidence in favour of a Pb/Ca^{2+} antagonism (Hodson et al., 1978; Varanasi and Gmur, 1978; Settle and Patterson, 1980), and with similar antagonism and hypocalcemic responses induced by exposure to Cd (Giles, 1984; Verbost et al., 1987; Reid and McDonald, 1988; Wicklund-Glynn et al., 1994) or Zn (Spry and Wood, 1985; Hogstrand et al., 1994). The observed hypocalcemia is also consistent with the observed inhibition of Ca^{2+} influx by Pb in juvenile rainbow trout (Fig. 2-9a), but contrast to Sayer et al. (1989) who did not observe an effect on Ca^{2+} uptake in brown trout fry exposed to Pb in soft, acidic water. It is possible that direct competition for uptake between these two divalent ions at the same apical sites on the gill ionocytes is the basis of inhibition. This competition would eventually lead to hypocalcemia similar to that observed in cannulated fish in this study. This phenomenon has been seen for Cd, where evidence suggests that both Ca^{2+} and Cd^{2+} are transported through the gill epithelium by chloride cells (Perry and Wood, 1985;

Verbost et al., 1987; Perry and Flik, 1988; Verbost et al., 1989; Perry et al., 1992; Flik et al., 1993) passing through the apical membrane passively via voltage-independent Ca^{2+} channels driven by electrochemical gradients (Perry and Flik, 1988; Verbost et al., 1989). Pb may also inhibit the basolateral transport mechanism, which is thought to involve a high affinity Ca^{2+} ATPase and/or a Na^{+}/Ca^{2+} exchanger (Flik et al., 1993). Competitive interaction between Pb^{2+} and Ca^{2+}could also explain the protective effects of increased water hardness as demonstrated in fish exposed to Pb (Sorensen, 1991). Recently, we have also found Pb inhibition of Ca^{2+}uptake at much lower Pb concentrations (J. Rogers and C.M. Wood, unpublished data), the severity of the inhibition increasing as Pb concentrations approached the LC50. It is also possible Ca^{2+} efflux is stimulated in the presence of Pb. This was observed in soft water flux measurements by Sayer et al. (1991) in brown trout and may be a component of the hypocalcemia and disturbed Ca^{2+} homeostasis observed in this study.

Magnesium is another tightly regulated ion in the rainbow trout, normally maintained at concentrations below 1 mM to maintain stable cellular and enzymatic functions (Bijvelds et al., 1998). The increase in Mg concentration observed in our experimental fish did not surmount this threshold, however, relative to control values, a significant increase was evident. Increased plasma concentrations of K were not observed suggesting that the Mg elevation did not result from red cell hemolysis. Possibly, it was in some way associated with the hypocalcemic response. Although it is widely accepted that these cations enter fish via separate routes (Hardwick et al., 1990; Kayne and Lee, 1993; Marshall, 2002), circumstantial evidence exists for a Ca^{2+}/Mg^{2+} relationship. For example, low Mg^{2+} intake induces high body calcium

concentrations in rainbow trout (Cowey et al., 1977). Mg^{2+} is also thought to move passively over apical membranes (gill and intestinal) down an electrochemical gradient (Flik et al., 1993). Ca^{2+} likely plays a role in this movement, so theobserved hypocalcemia in Pb-exposed fish may cause disruption of the electrochemical gradient established at the apical surface.

Although no significant changes were observed with respect to K^+ regulation, (Fig. 2-6a), Na^+ and Cl^- balance was severely disrupted in experimental fish. Typically, divalent metals such as Pb, Cd, or Zn are viewed as calcium antagonists; however, the ionoregulatory impacts of Pb appear to include these monovalent ions. Similar to the response to acute Ag exposure (Wood et al, 1996), net loss of Na^+ and Cl^- at the gill through the observed inhibition of influx (Fig. 2-9b,c), would result in the observed gradual decreases in plasma Na^+ and Cl^- (Fig. 2-6b,c). Inhibition of influx could occur at a number of levels; competition for uptake at the apical surface, inhibition of uptake at Na^+ channels or Na^+ and Cl^-exchangers, inhibition of the apical H^+ ATPase which is thought to polarize the Na^+ channel, inhibition of carbonic anhydrase that would limit the supply of H^+, NH_4^+, and HCO_3^- for these exchangers, or inhibition of the Na^+/K^+ ATPase at the baso-lateral membrane, which indirectly energizes the uptake mechanisms. Clearly the latter phenomenon occurs (Fig. 2-8), which is consistent with enzyme inhibition previously observed in Pb-exposed tilapia (Ay et al., 1999), but contrast to Sola et al. (1994) who did not observe an adverse effect of Pb on Na^+/K^+ ATPase activity in rainbow trout exposed to 1 mg Pb L^{-1}. From a time-course perspective, significant inhibition of Na^+/K^+ ATPase was observed during the time period where plasma Na^+ and Cl^- were found to decrease in cannulated trout. However, it is unclear whether the decrease in plasma Cl^- is

electrochemically linked to inhibition of Na^+ transport or whether Pb acts to directly inhibit Cl^- transport by HCO_3^-/Cl^- exchange (Wood and Goss, 1990; Sullivan et al., 1995; Wilson et al., 2000) or active transport by a HCO_3^- - dependent anion ATPase (Kerstetter and Kirschner, 1994). Sola et al. (1994) did not observe significant impairment of HCO_3^--ATPase activity in rainbow trout exposed to 1 mg Pb L^{-1}. While ion disruption resulting from inhibition of uptake was observed in this study, future research investigating the possible role of the efflux component in Na^+, Cl^-, and Ca^{2+} losses is necessary.

Na^+/K^+ ATPase inhibition upon Pb-exposure was not as severe as that observed in rainbow trout exposed to a 96 h LC50 level of Ag (10 μg L^{-1}) for 48 h in the same water quality (85% inhibition) (Morgan et al., 1997). The mechanisms of inhibition may be different for Ag and Pb. While Ag has a high-affinity for Na^+/K^+ ATPase based on strong binding to sulfhydryl groups (Morgan et al., 1997) resulting in potent inhibition of the enzyme, the less potent inhibition caused by Pb may result from Pb binding to carboxyl groups at the active site of Na^+/K^+ ATPase, causing a reduction in phosphorylation activity, a phenomenon demonstrated in vitro in human erythrocytes by Ong and Lee (1980a). This reduction in activity would result in a reduced rate of ion uptake, which is consistent with the ion disruption observed in this study. Generally, about a 40 mM reduction in plasma Na^+ results in death of the fish, as shown for Ag after 6 days of exposure (Wood et al., 1996). Prolonged exposure to Pb would probably result in similar mortality as plasma Na^+ dropped by 26 mM after 5 days of exposure in cannulated adult rainbow trout.

Low concentrations of plasma Na^+ and Cl^- observed after 48 h of Pb exposure (Fig. 2-6b,c) correspond with significant increases in plasma total ammonia at this

time (Fig. 2-4c). Ammonia excretion, the mechanism of which is still controversial, occurs at the fish gill by passive diffusion, by indirect coupling to H^+ excretion/Na^+ uptake, or by Na^+/NH_4^+ exchange (Wilkie, 1997). Experiments using Na^+ transport blockers show that ammonia excretion is simultaneously inhibited (Kerstetter and Keeler, 1976; Payan, 1978; Wright and Wood, 1985; Wilson et al., 1994), supporting the existence of a direct or indirect exchange mechanism. Thus, there may be an ionoregulatory basis for the observed increases in plasma total ammonia that accompany decreases in plasma Na^+ after 48 h of Pb exposure. Pb inhibition of Na^+ transport, therefore, would be a fitting explanation for observed rises in plasma total ammonia and would support an ionoregulatory rather than a respiratory or stress-related disturbance that could cause similar plasma ammonia elevations. This phenomenon has also been observed in fish exposed to Ag (Webb and Wood, 1998), and Cu (Beaumont et al., 1995).

Stress indices measured showed no change in plasma glucose concentrations, however, under ionoregulatory distress, slow activation of the pituitary interrenal axis likely resulted in the cortisol mobilization observed in cannulated adult trout at 96 h and 120 h (Fig. 2-4b). Perry and Wood (1985) showed that cortisol treatment in rainbow trout increased influx rates for Ca^{2+} (J_{in}^{Ca2+}) compared to untreated controls by cortisol-stimulated proliferation of lamellar chloride cells. Therefore, an ionoregulatory disruption such as hypocalcemia may have triggered the compensatory release of cortisol in Pb-exposed trout.

Conclusions

This study presents strong evidence that the mechanism of waterborne Pb toxicity in fish during acute exposure is ionoregulatory distress. Using the rainbow trout in moderately hard water as a model system, the toxic action of Pb appears to be midway between other known acute ionoregulatory toxicants like Ag and Cu which affect Na^+ and Cl^- balance, and Zn and Cd which disrupt Ca^{2+} homeostasis (see Introduction). This is in direct contrast with the toxic mechanisms of Al and Ni, which are primarily respiratory in nature. Data presented also open the possibility of predictive modelling approaches such as the Biotic Ligand Model (BLM), which are based on a toxic metal binding to the gills in competition with protective and nutrient ions in the water column (Playle et al., 1993; Playle, 1998; McGeer et al., 2000; Di Toro et al., 2001). Binding models for Pb are presently being developed (MacDonald et al., 2002), and based on their predictions and the physiological evidence presented in this study, it appears that Pb is capable of out-competing Na^+ and Ca^{2+} for ion transport sites at the freshwater fish gill (see Fig. 1-2). In seawater, the physiology of Pb toxicity and the acute toxic mechanism of action may change. However, ionoregulation in marine fish follows different principles, with active transport processes dedicated to net ion excretion at the gills, rather than net ion uptake. Therefore, the effects of Pb in seawater cannot be extrapolated from freshwater data. Future research on the physiological effects of Pb in marine fish is needed.

This study has opened up promising avenues for further research. Kinetic analysis of Pb^{2+}/Ca^{2+} interaction similar to that done for Zn (Hogstrand et al., 1995), investigation into the effect of Pb^{2+} on the function of Ca^{2+}-ATPase in the gills, and research into mechanisms of Na^+/Cl^- disruption are key aspects of Pb-toxicity that require further investigation. In addition, the possibility of Pb-induced nephrotoxicity

as a component of the observed ionoregulatory distress is an important topic for future study.

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Fig. 2-1

Pb accumulation in tissues of juvenile rainbow trout exposed to control conditions (black bars) or elevated waterborne Pb (grey bars). The data represent the concentrations occurring at death in rainbow trout exposed to the 96 h LC50 concentration during the 96 h LC50 test. Pb concentrations are expressed as $\mu g\ g^{-1}$ $tissue^{-1}$wet weight. Data are expressed as mean $\pm$ 1 SEM. Asterisk "*" indicate significant difference from control values in analysis performed on log-transformed data ($p<0.05$; two-tailed Student's t-test).

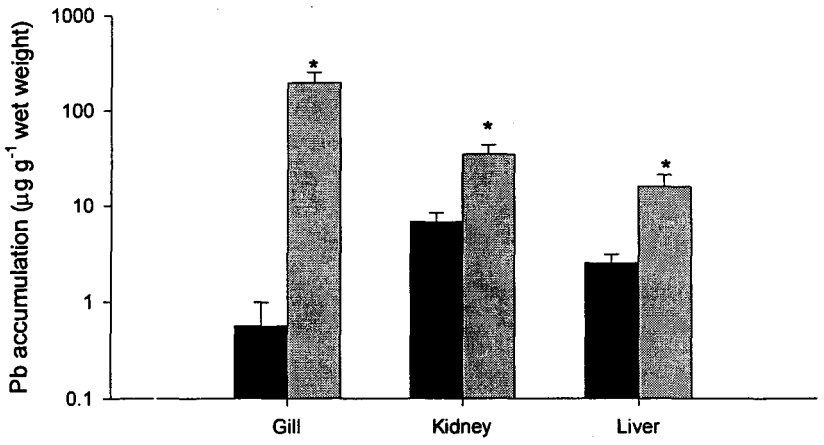

Pb accumulation (µg g-1 wet weight)
1000
100
10
1
0.1
*
*
*
Gill
Kidney
Liver

Fig. 2-2

Respiratory parameters obtained from cannulated adult rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb L^{-1}. (a) Arterial oxygen tension (PaO_2). (b) Arterial carbon dioxide tension ($PaCO_2$). (c) Ventilation rate. (d) Plasma lactate. Data are expressed as mean ± 1 SEM. There were no significant differences ($p>0.05$).

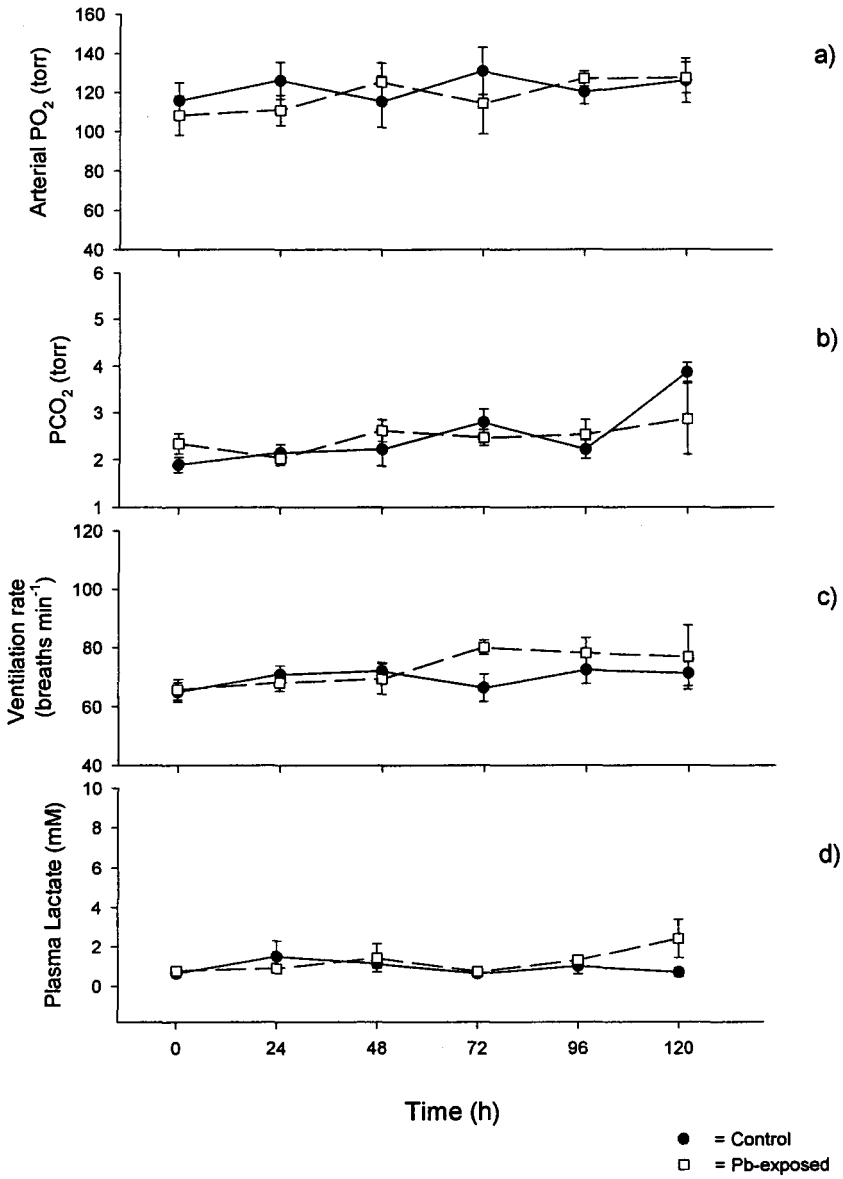

Arterial PO_2 (torr)
160
140
120
100
80
60
40
a)
PCO_2 (torr)
6
5
4
3
2
1
b)
Ventilation rate (breaths min^{-1})
120
100
80
60
40
c)
Plasma Lactate (mM)
10
8
6
4
2
0
d)
0
24
48
72
96
120
Time (h)
● = Control
□ = Pb-exposed

Fig. 2-3

Acid/base parameters obtained from cannulated rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb L^{-1}. (a) Arterial pH. (b) Arterial plasma [HCO_3^-]. Data are expressed as mean ± 1 SEM. There were no significant differences ($p>0.05$).

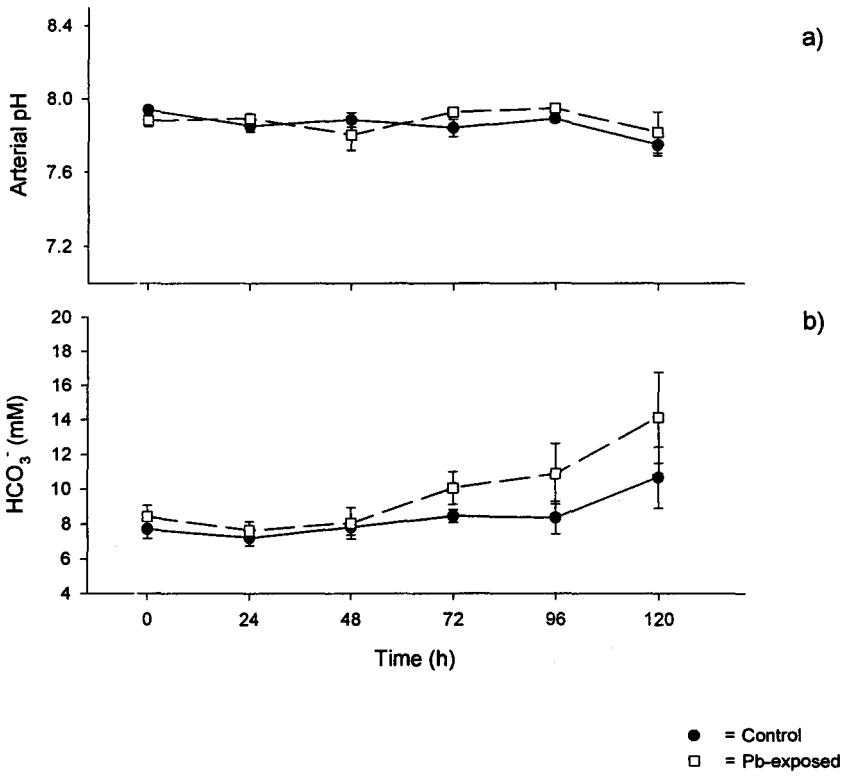

a)
b)
Arterial pH
8.4
8.0
7.6
7.2
HCO_3^- (mM)
20
18
16
14
12
10
8
6
4
0
24
48
72
96
120
Time (h)
● = Control
□ = Pb-exposed

Fig. 2-4

Stress indices in arterial blood plasma obtained from cannulated adult rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb L^{-1}. (a) Arterial plasma [glucose]. (b) Arterial plasma [cortisol]. (c) Arterial plasma total ammonia. Data are expressed as mean ± 1 SEM. Asterisk "*" indicates significant difference ($p<0.05$; two-tailed Student's t-test) from simultaneous control mean. "+" indicates significant difference ($p<0.05$; Dunnett's one-way ANOVA) from 0 h control mean.

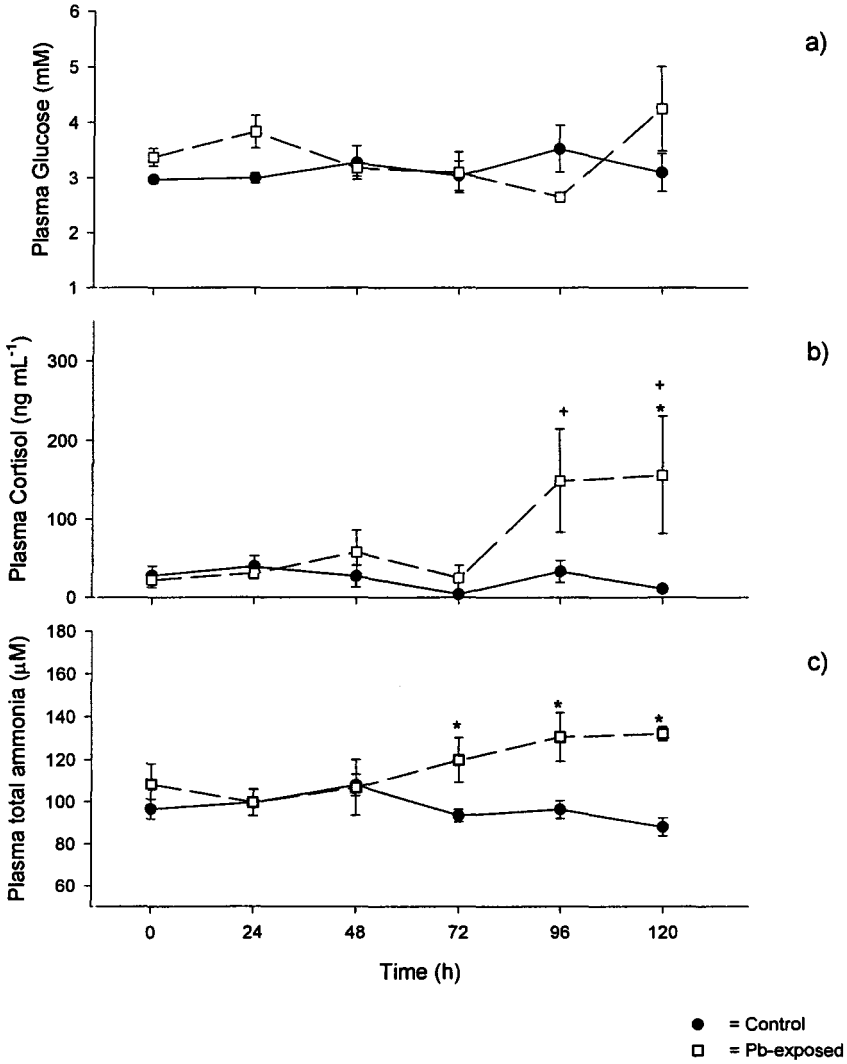

a)
b)
c)
Plasma Glucose (mM)
Plasma Cortisol (ng mL-1)
Plasma total ammonia (μM)
Time (h)
0
24
48
72
96
120
= Control
= Pb-exposed

Fig. 2-5

Plasma ion concentrations obtained from cannulated adult rainbow trout exposed to control conditions or 1.1 ±0.04 mg dissolved Pb L^{-1}. (a) Arterial plasma [Pb^{2+}]. (b) Arterial plasma [Ca^{2+}]. (c) Arterial plasma [Mg^{2+}]. Data are expressed as mean ± 1 SEM. Other details as in legend of Fig. 2-4.

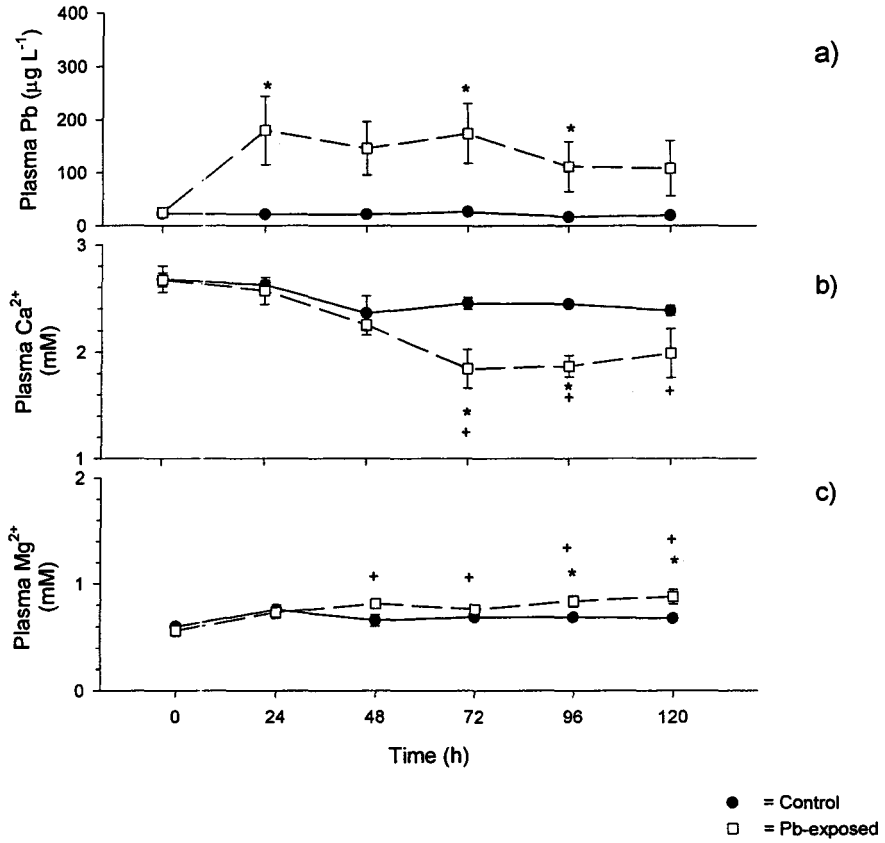

a)
b)
c)
Plasma Pb (µg L-1)
Plasma Ca2+ (mM)
Plasma Mg2+ (mM)
400
300
200
100
0
3
2
1
2
1
0
0
24
48
72
96
120
Time (h)
= Control
= Pb-exposed

Fig. 2-6

Plasma ion concentrations obtained from cannulated rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb L^{-1}. (a) Arterial plasma [K^+]. (b) Arterial plasma [Na^+]. (c) Arterial plasma [Cl^-]. Data are expressed as mean ± 1 SEM. Other details as in legend of Fig. 2-4. "**" indicates significant difference from 24 h control mean due to time-effect ($p<0.05$; two-way ANOVA with Tukey honest significant difference (HSD) test.

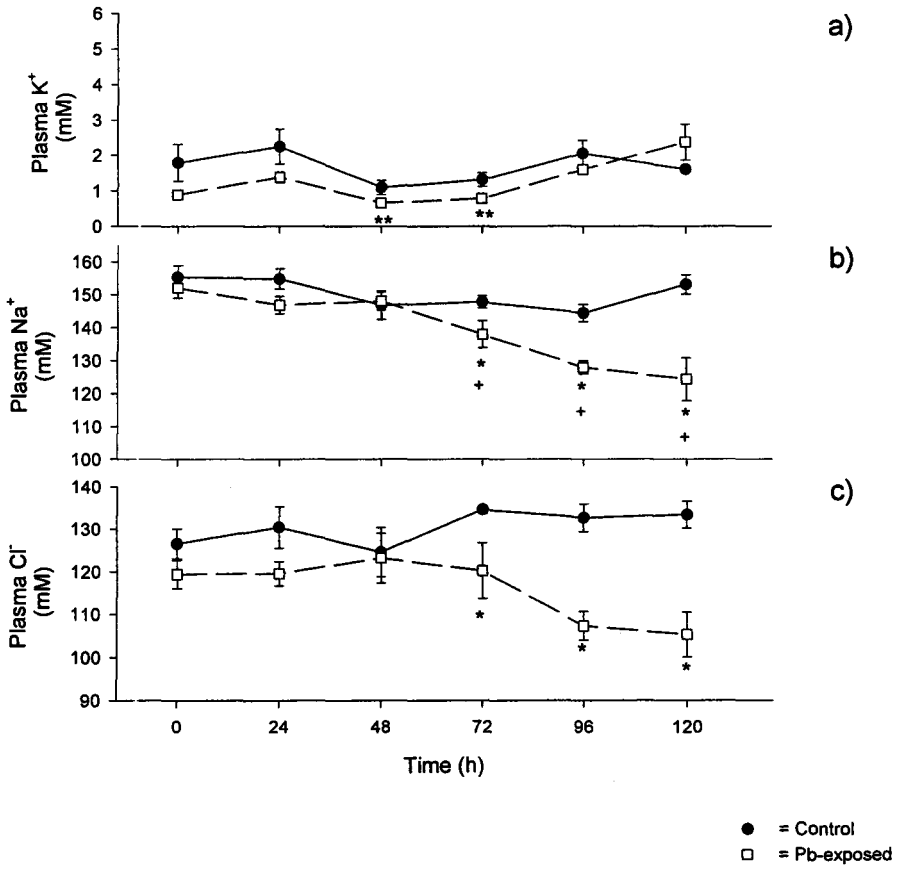

a)
b)
c)
Plasma K+ (mM)
Plasma Na+ (mM)
Plasma Cl- (mM)
Time (h)
0
24
48
72
96
120
= Control
= Pb-exposed

Fig. 2-7

Hematological parameters obtained from cannulated rainbow trout exposed to control conditions or 1.1 ± 0.04 mg dissolved Pb L^{-1}. (a) Blood [Hb]. (b) Hematocrit (Ht). (c) Mean cellular hemoglobin concentration (MCHC). (d) Arterial plasma [protein]. Data are expressed as mean ± 1 SEM. Other details as in legend of Fig. 2-4.

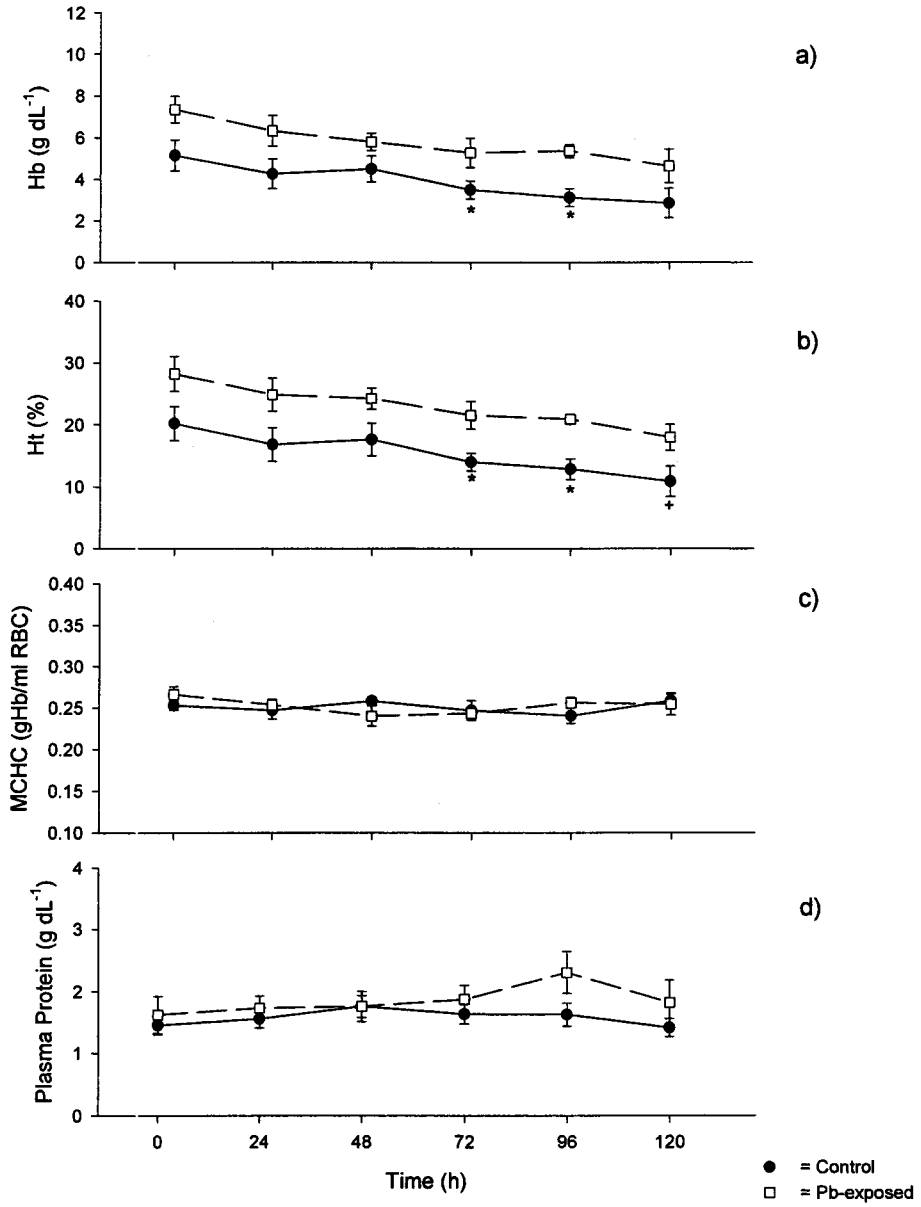

a)
Hb (g dL-1)
12
10
8
6
4
2
0
b)
Ht (%)
40
30
20
10
0
c)
MCHC (gHb/ml RBC)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
d)
Plasma Protein (g dL-1)
4
3
2
1
0
0
24
48
72
96
120
Time (h)
● = Control
□ = Pb-exposed

Fig. 2-8

Time course analysis of branchial Na^+/K^+ ATPase activity in juvenile rainbow trout at control sampling (c) (t = 0 h) and after exposure to 1.5 ± 0.04 mg dissolved Pb L^{-1} for up to 120 h. Data are expressed as mean ± 1 SEM (N = 7-8). "+" indicates significant difference ($p<0.05$; Dunnett's one-way ANOVA) from control sampling mean.

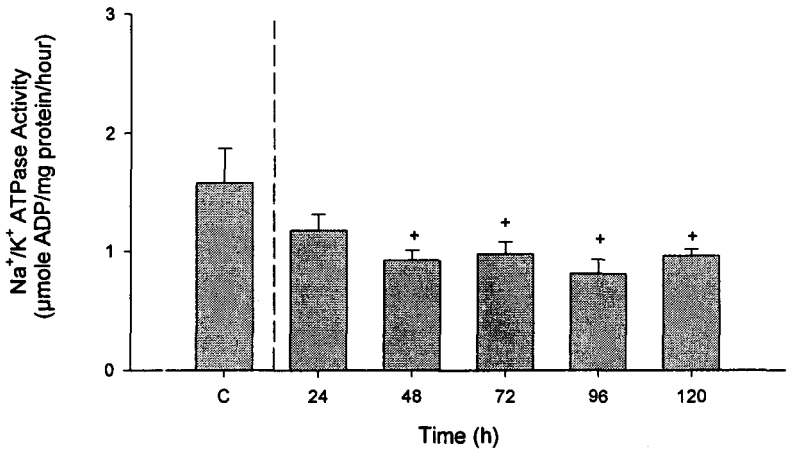
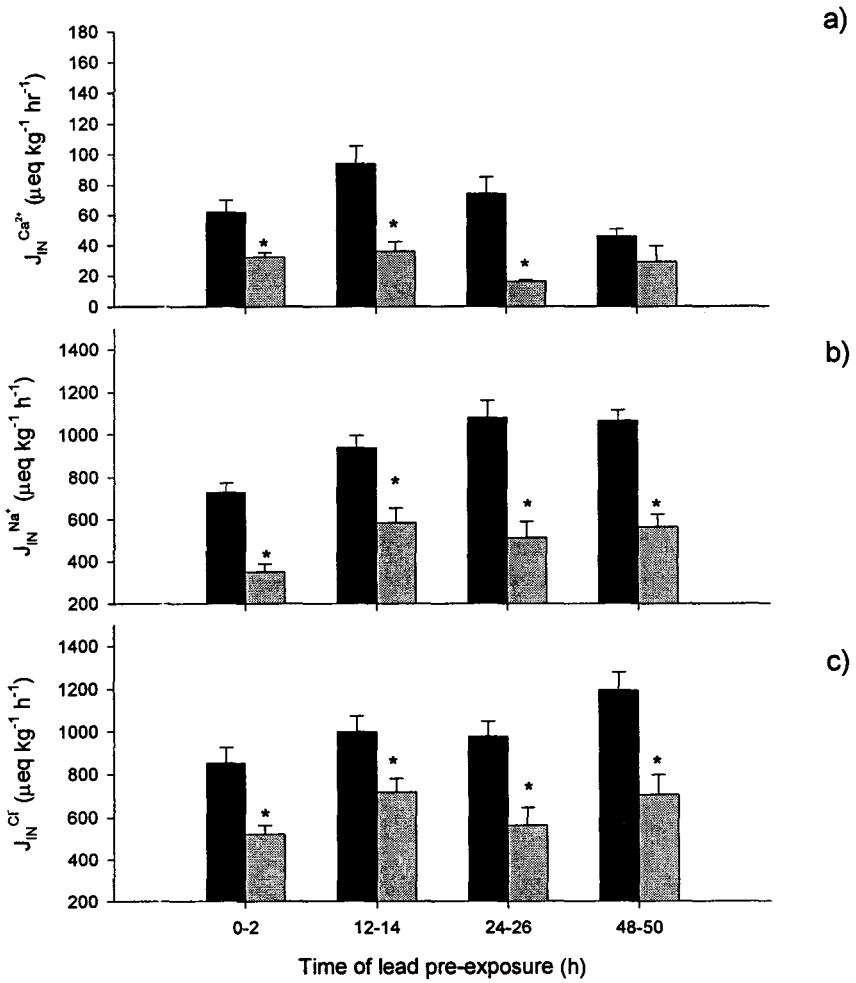

Na+/K+ ATPase Activity
(µmole ADP/mg protein/hour)
3
2
1
0
C
24
48
72
96
120
Time (h)

Fig. 2-9

Ion flux measurements in juvenile rainbow trout for a) calcium influx in control water (black bars) or water with 1.2 ± 0.04 mg dissolved Pb L^{-1} (grey bars) after exposure to control conditions or 1.2 ± 0.04 mg dissolved Pb L^{-1} for 0, 12, 24, or 48 h b) sodium uptake in control water (black bars) or water with 0.89 ±0.05 mg dissolved Pb L^{-1} after exposure for 0, 12, 24, or 48 h, and c) chloride uptake in control water (black bars) or water with 0.89 ± 0.05 mg dissolved Pb L^{-1} after exposure for 0, 12, 24, or 48 h. Asterisk "*" indicates significant difference ($p<0.05$; two-tailed Student's t-test) from corresponding control means (N = 7-8).


a)
b)
c)
$J_{IN}^{Ca^{2+}}$ (µeq kg^{-1} hr^{-1})
$J_{IN}^{Na^{+}}$ (µeq kg^{-1} h^{-1})
$J_{IN}^{Cl^{-}}$ (µeq kg^{-1} h^{-1})
0-2
12-14
24-26
48-50
Time of lead pre-exposure (h)

CHAPTER 3

CHARACTERIZATION OF BRANCHIAL LEAD-CALCIUM INTERACTION IN THE FRESHWATER RAINBOW TROUT (*Oncorhynchus mykiss*)

Abstract

The mechanism of branchial Pb uptake and interplay with Ca^{2+} transport was investigated in the freshwater rainbow trout (*Oncorhynchus mykiss*). Pb significantly reduced Ca^{2+} influx by approximately 40 % and 30% after exposure to 2.3 ±0.1 and 1.4 ± 0.2 μM dissolved Pb, respectively, for 0– 48 h. Acute inhibition of Ca^{2+} influx by Pb exhibited typical Michaelis-Menten kinetics with an approximate 16-fold increase in K_m while J_{max} values did not significantly change, yielding an inhibitor constant ($K_{i, Pb}$) of 0.48 μM. Branchial Pb accumulation was reduced with increasing waterborne Ca^{2+} concentrations, suggesting a protective effect of Ca^{2+} against Pb uptake at the gill. The apical entries of Ca^{2+} and Pb were both inhibited (55 and 77 % respectively) by the addition of La^{3+} (1 μM) to the exposure water. The use of Cd (1 μM) and Zn (100 μM) as voltage-independent calcium channel blockers also reduced branchial Pb uptake by approximately 56 % and 47% respectively. Nifedipine and verapamil (up to 100 μM), both voltage-dependent calcium channel blockers, had no effect on gill Pb accumulation. $CaCl_2$ injection reduced both Ca^{2+} and Pb uptake by the gills. This suggests transport of Pb through apical voltage-independent calcium channels, similar to the entry of Ca^{2+}. High-affinity Ca^{2+}-ATPase activity was not acutely affected by Pb, but by 96 h a significant 80 % reduction in activity occurred during exposure to 5.5 ± 0.4 μM dissolved Pb, indicating a possible non-competitive

component to Pb-induced Ca^{2+} disruption. The effect of Pb on Ca^{2+} efflux was investigated and found to be insignificant. We conclude that uptake of Pb occurs, at least in part, by the same mechanism as Ca^{2+}, which results in disruption of Ca^{2+} influx and ultimately Ca^{2+} homeostasis.

Introduction

Lead is a common contaminant in the natural environment that can enter the water column through geologic weathering and volcanic action, or by various anthropogenic practices including smelting, coal burning, and use in gasoline, batteries, and paint (World Health Organization, 1995). Though waterborne Pb concentrations do not normally exceed 0.6 μM (Demayo et al., 1982), levels as high as 4.3 μM have previously been reported (USEPA, 1986). Contamination of water through anthropogenic practices is the primary cause of lead poisoning in fish (Sorensen, 1991).

Recent evidence has shown that the acute toxic mechanism for waterborne lead in the rainbow trout (*Oncorhynchus mykiss*) is ionoregulatory disruption, with observed effects on Na^+ and Cl^- balance and Ca^{2+} homeostasis (Rogers et al., 2003). These effects are manifested through an inhibition of ion influx and corresponding decreases in the plasma levels of these ions. This places Pb midway between other known acute ionoregulatory toxicants like Cu and Ag, which affect Na^+ and Cl^- balance (Lauren and McDonald, 1985; Wood et al., 1996; Morgan et al., 1997), and Zn and Cd, which disrupt Ca^{2+} homeostasis (Spry and Wood, 1985; Verbost et al., 1987).

Branchial uptake of Ca^{2+} is thought to be primarily by passive movement through apical voltage-insensitive channels in the "chloride" cells of the fish gill (Flik et al., 1993). Once entering the chloride cell, Ca^{2+} is transported via Ca^{2+}-binding proteins to the basolateral membrane where it is actively extruded into the circulation by way of a high-affinity Ca^{2+}-ATPase enzyme (Flik et al., 1985; Verbost et al., 1994; Marshall 2002) and/or a Na^+/Ca^{2+} exchange mechanism (Flik et al., 1994, 1997;

Verbost et al., 1997). Pb could potentially have an impact at any one of these steps of calcium entry. Recently, MacDonald et al. (2002) speculated that Pb disrupts Ca^{2+}-homeostasis by competitive inhibition at apical Ca^{2+}channels in the fish gill, therefore, entering the fish by the same mechanism as Ca^{2+}. There is an abundance of circumstantial evidence in support of this relationship. For example, the toxicity of waterborne Pb is greatly reduced with increasing water hardness, the Ca^{2+}component likely exerting protective effects by inhibiting entry of Pb (Sorensen, 1991). Elevated dietary Ca^{2+} levels have also been shown to reduce Pb uptake in fish (Varanasi and Gmur, 1978). Once crossing the gill and entering the systemic circulation, Pb accumulates in bone, suggesting similarities between the handling of Pb and Ca^{2+} within the organism (Davies et al., 1976; Holcombe et al., 1976; Settle and Patterson, 1980). These indirect relationships suggest that Pb may share one or multiple points of entry with calcium; however, despite such circumstantial evidence, this relationship has not been proven directly.

Using both physiological and pharmacological techniques, metals like Cd and Zn have been shown to be Ca^{2+} antagonists. Both metals have been found to reduce rates of Ca^{2+} influx (Verbost et al., 1987; Spry and Wood, 1985) resulting in hypocalcemia. Kinetic analyses of Ca^{2+} interaction with both Zn (Spry and Wood, 1989; Hogstrand et al., 1994, 1998) and Cd (Niyogi and Wood, submitted) have demonstrated typical Michaelis-Menten relationships; increases in K_m values (decreases in the affinity of the apical calcium channel) with little or no change in the maximal rate of uptake (J_{max}). This suggests direct competition. Additionally, apical calcium channel blockers such as La^{3+} have been shown to reduce the rate of Ca^{2+}, Cd, and Zn uptake, suggesting that these ions share the same lanthanum-inhibitable

route of uptake (Verbost et al., 1989; Hogstrand et al., 1995, 1996). Using the rainbow trout as a model species, the objective of the present study was to characterize the branchial interaction of Pb and Ca^{2+} by incorporating kinetic analysis and the use of apical channel blockers (both voltage sensitive and voltage insensitive). The potential stimulation of Ca^{2+}-efflux, as reported in Pb-exposed brown trout (Sayer et al., 1991), and possible inhibition of high-affinity Ca^{2+}-ATPase were also investigated as potential factors in Pb-induced disruption of Ca^{2+}-homeostasis. Characterization of Pb binding to the rainbow trout gill may aid in the development of water-chemistry based predictive models for Pb, such as the Biotic Ligand Model (BLM) (McGeer et al., 2000; Paquin et al., 2000; DiToro et al., 2001; MacDonald et al., 2002). This process requires further understanding and characterization of key binding sites involved in Pb toxicity.

Materials and Methods

Experimental Animals

Juvenile rainbow trout (3 - 10 g), for use in influx experiments, and experiments using calcium channel blockers and $CaCl_2$ injection, larger juvenile trout (40 – 60 g) for use in Ca^{2+}-efflux measurements, and adult rainbow trout (200 –300 g) for use in the high-affinity Ca^{2+}-ATPase assay, were obtained from Humber Springs Trout Farm in Orangeville, Ontario. Fish were held in dechlorinated City of Hamilton tap water (from Lake Ontario) at a temperature of 7 - 12°C, and fed commercial trout pellets at a ration of 1 % total body weight per day. Water composition was Ca^{2+} = 1.0, Mg^{2+} = 0.2, Na^{+} = 0.6, Cl^{-} = 0.8, K^{+} = 0.05 (mM), total Pb = 0.003 μM (0.68 μg L^{-1}), dissolved organic carbon (DOC) = 3 mg L^{-1}, hardness (as $CaCO_3$) of approximately 140 mg L^{-1}, and pH = 7.9 – 8.0. Experiments were conducted at a temperature of 9 - 12°C and experimental animals starved 72 hours prior to and throughout all experiments.

Ca^{2+} influx measurements

Ca^{2+} influx determinations were carried out using methods almost identical to those outlined in Rogers et al. (2003), differing only in Pb-exposure concentrations used and duration of the pre-exposure period. In brief, Ca^{2+} influx measurements were made by relating the specific activity of ^{45}Ca in the exposure water to the accumulation of isotope in the fish following the flux period. In the present study, influx measurements were made under control conditions and at Pb concentrations that were approximately 50% and 25% of the 96 h LC50 of 4.8 μM dissolved Pb determined in City of Hamilton dechlorinated tap water by Rogers et al. (2003). The

nominal exposure concentrations implemented were 2.4 μM Pb and 1.2 μM Pb. Juvenile rainbow trout were subject to pre-flux exposure periods of 0 h, 12 h, and 24 h before undergoing a flux period of 2 h in control water or Pb-containing water.

Kinetic analysis of the interaction between Pb and Ca^{2+}

The differential effects of Pb on unidirectional calcium influx were assessed *in vivo* using methods similar to those outlined in Zohouri et al. (2001). Sixteen polyethylene bags representing a control and three Pb concentrations, and a series of four different calcium concentrations, were filled with 3 L of calcium-free synthetic water (0.7 mM Na^+ and Cl^- added as NaCl, $KHCO_3$: 1.9 mM, pH 8.0). Each bag was fitted with an air line and placed on a water bath for temperature control. Three bags of each set were spiked with a $Pb(NO_3)_2$ stock solution (Sigma; Aldrich) to obtain nominal Pb concentrations of 0.48, 2.4, and 4.8 μM Pb (control = 0 μM Pb). Each bag was then spiked with $Ca(NO_3)_2$ (Fisher Scientific, Canada) to achieve nominal calcium concentrations of 150, 300, 600, and 1200 μM. Finally, flux bags were injected with 7 μCi L^{-1} ^{45}Ca (as $CaCl_2$, specific activity = 0.14 μCi mol^{-1}, Perkin-Elmer, USA).

Juvenile rainbow trout were transferred to each of the sixteen flux bags (7 fish per bag) and an initial 15-minute 'settling' period was allowed for acclimation and isotopic equilibration. The exposure period was 3 h in length with initial and final water samples (5 ml) drawn in duplicate for determination of ^{45}Ca activity and total calcium concentration. Water samples were also drawn for determination of total Pb (unfiltered) and dissolved Pb (filtered; 0.45 μm filter) concentrations. These samples were stored in plastic scintillation vials at 1% HNO_3 for analysis. Following the 3 h

flux period, fish were removed and killed with a single blow to the head. The fish were then rinsed for 1 min in 1 mM EDTA (ethylenediamine-tetraacetic acid; Sigma-Aldrich) followed by a 1 min rinse in a 5 mM cold Ca^{2+} solution ($Ca(NO_3)_2$) (Sigma-Aldrich) to remove all surface-bound ^{45}Ca. Whole bodies were blotted dry, placed in scintillation vials, and digested in 1N HNO_3 (Fisher Scientific; trace metal grade) at 55°C for 48 h. Samples were then homogenized by vortexing, an aliquot removed (1.5 mL), centrifuged at 13,000g for 10 minutes, and the supernatant (1 mL) added to 5 mL of an acid-compatible scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT). ^{45}Ca radioactivity was measured by scintillation counting (Rackbeta 1217; LKB Wallac, Turka, Finland). Water samples taken for determination of ^{45}Ca radioactivity were added to 10 ml of aqueous counting scintillant (ACS™: Amersham) and scintillation counted as above. ^{45}Ca radioactivity was quench-corrected to the same counting efficiency as water samples by the method of external standard ratios, using a ^{45}Ca quench curve generated from the tissue of interest in the same counting cocktail.

Water samples taken for the determination of total $[Ca^{2+}]$ were diluted with 0.2% La^{3+} and analyzed by flame atomic absorption spectrophotometry (FAAS) using the Varian 220FS Spectra AA. Determination of total and dissolved waterborne Pb concentrations was done using graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA; Varian, Australia) against a certified multi-element standard (Inorganic Ventures, Inc.).

The effect of calcium on branchial lead accumulation

The effect of waterborne calcium concentration on the branchial accumulation of Pb in juvenile rainbow trout was assessed using methods similar to those implemented in the kinetic analysis of Pb and Ca^{2+}, though precision was lower due to the lack of a suitable Pb radioisotope. Following a 3 h exposure period, experimental animals were removed from the flux bags, killed with a blow to the head, and the gills dissected. The gills were rinsed for 1 min in de-ionized water, blotted dry, weighed, and digested in 1N HNO_3 at 55°C for 48 h. Samples were then homogenized by vortexing, an aliquot removed (approximately 1.5 ml), centrifuged at 13,000 g for 10 minutes, and the supernatant analyzed for total Pb concentration using GFAAS.

Calcium-channel blocker experiments

The role of apical calcium channels in mediating branchial Pb uptake was investigated by the use of the voltage-independent blockers, La^{3+}, Cd, and Zn, and the voltage-dependent channel blockers nifedipine and verapamil. In assessing the effects of La^{3+} on branchial Pb accumulation, a series of six polyethylene bags representing one control (0 μM La^{3+}) and five La^{3+} concentrations were filled with 3 L of synthetically modified water obtained by reverse osmosis (Na^+ and Cl^- added as NaCl = 0.7 mM), made carbonate-free to reduce complexation of waterborne La^{3+} (Verbost et al., 1987; Hogstrand et al., 1996) and calcium-free to maximize branchial Pb accumulation. Flux bags were then spiked with a $LaCl_3$ (Sigma-Aldrich) stock solution to achieve nominal waterborne La^{3+} concentrations of 0.0001 to 1 μM. Juvenile rainbow trout were transferred to each of the six flux bags (7 fish per bag)

and allowed a 10 min acclimation period for equilibration with waterborne La^{3+}. The bags were subsequently spiked with a $Pb(NO_3)_2$ stock solution to achieve a nominal Pb concentration of 4.8 μM, followed by an additional acclimation period to allow for Pb equilibration. Water samples were taken, filtered and unfiltered, for determination of total and dissolved Pb concentrations. The fish were then exposed for 3 h. Following exposure, fish were removed from flux bags and the gills were dissected. Processing of the dissected gills for total Pb concentrations used procedures identical to those outlined above. Using ^{45}Ca as a radiotracer, simultaneous measurements of Ca^{2+} influx were made at control and 1 μM La^{3+} to assess the effect of La^{3+} on Ca^{2+} uptake. Influx measurements were performed using a waterborne Ca^{2+} concentration of 600 μM. Further procedures used were identical to those outlined above under calcium influx measurements. The effects of Cd and Zn on branchial Pb accumulation and Ca^{2+} influx were assessed using methods almost identical to those used for La^{3+}. For Cd, flux bags were spiked with a stock solution of $Cd(NO_3)_2$to achieve concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 μM Cd. For Zn, bags were spiked with $Zn(SO_4)_2$ to achieve concentrations of 0.01, 0.1, 1, 10, and 100 μM Zn. These experiments also included measurements of gill Cd burden from the same gill digests analyzed for total Pb, using GFAAS.

The use of nifedipine and verapamil as L-type calcium channel blockers was evaluated using methods almost identical to those used for La^{3+}, Cd, and Zn with the exception of waterborne concentrations employed. For nifedipine, bags were spiked with a stock solution made using 75 % ethanol (to solubilize the blocking agent; final ethanol concentration in the flux medium was 0.1%) to achieve concentrations of 0.1, 1, 10, and 100 μM nifedipine. A control was run by spiking nifedipine-free water

with ethanol to control for the effects of ethanol on Pb accumulation and to maintain consistency between blocker experiments. For verapamil, flux bags were spiked with a stock solution made using NANOpure water (NANOpure II; Sybron/Barnstead, Boston MA) to achieve final concentrations of 0.1, 1, 10, and 100 μM verapamil.

Effect of $CaCl_2$ injection on Ca^{2+} influx and branchial Pb accumulation

Methods used in testing the effects of calcium chloride injection on Ca^{2+} influx and branchial Pb accumulation followed closely those outlined in Hogstrand et al. (1996). Ionic calcium was injected into juvenile rainbow trout in an effort to reduce uptake of Ca^{2+} and Pb through the stanniocalcin-regulated pathway. In the present study, fish were injected intraperitoneally with 0.22 μM Ca^{2+} g body weight^{-1} using an injection solution made from $CaCl_2 \bullet H_2O$ dissolved in 0.9 % NaCl. Control fish were sham injected with 0.9 % NaCl (vehicle only). Approximately 30 minutes following injection, measurements of Ca^{2+} influx were performed on experimental and control fish (n = 8 per treatment) using methods described in the previous section. Branchial Pb accumulation was measured in a separate group of control and experimental fish (n = 8 per treatment), again using methods outlined above.

Determination of Ca^{2+}-efflux

The possibility of Pb-induced stimulation of Ca^{2+}-efflux was investigated by following closely the methods of Perry and Flik (1988). Fish were subject to either no pre-flux exposure (control), or a pre-exposure period of 24 or 48 h to waterborne Pb concentrations close to the 96 h LC50 of 4.8 μM. Pb exposure was carried out by dripping a stock solution of $Pb(NO_3)_2$ (Sigma-Aldrich) dissolved in NANOpure water

(NANOpure II; Sybron/Barnstead, Boston MA) at a rate of 1 ml min^{-1} into a mixing tank fed with 500 ml min^{-1} of City of Hamilton dechlorinated tap-water. The mixing tank then fed an exposure tank (~200 L) holding 20 rainbow trout. At t = 0 h, the exposure tank was spiked with the Pb stock solution to achieve the appropriate Pb concentration.

After the appropriate pre-exposure period, rainbow trout were anaesthetized using MS-222 and given an intra-peritoneal injection of a ^{45}Ca solution (30 μCi ^{45}Ca in 1 ml saline) in preparation for efflux measurements. A period of 8– 12 h was allowed for recovery of the fish, and for isotopic equilibration. Fish were then transferred to darkened, plexiglass boxes (volume = 450 ml), each fitted with an air supply. The boxes were filled with either Pb-free water or water spiked with $Pb(NO_3)_2$ to achieve a nominal Pb concentration of 4.8 μM, and placed in a water bath for temperature control. Following a 0.5 h settling period, a 3 h Ca^{2+} flux measurement was started. Initial water samples were taken in duplicate for ^{45}Ca activity and total calcium concentration (5 ml), and for determination of dissolved Pb concentrations (10 ml). At the end of the flux period, comparable final water samples were drawn, fish were removed from their respective exposure boxes and anaesthetized with MS-222. A terminal blood sample was taken from each fish by caudal puncture, centrifuged at 13, 000 g, and the plasma frozen for measurement of ^{45}Ca activity and total calcium concentration. Ca^{2+}-efflux was calculated using the following formula:

$$J_{out}^{Ca^{2+}} = \frac{(R_i - R_f) * V}{SA * W * t}$$

where R_i and R_f are initial and final water radioactivities in counts per minute per millilitre, V is the volume of the external flux medium in millilitres, SA is the t = 3 h specific activity of the plasma in counts per minute per micromole, *t* is the duration of the flux period in hours, and W the fish weight in kg. Water samples were analysed using methods identical to those outlined in the Pb/Ca^{2+} kinetic experiments.

The effect of Pb on high-affinity Ca^{2+}-ATPase activity

High-affinity Ca^{2+}-ATPase activity was assayed in adult rainbow trout exposed to control conditions or to a nominal Pb concentration of 4.8 μM for 3h, 24 h or 96 h in City of Hamilton dechlorinated tap water. Pb exposure was carried out using methods identical to those used in measurements of Ca^{2+}-efflux. Isolation of the basolateral membrane from gill epithelium was carried out to assay enzyme activity. Procedures followed closely those outlined in Perry and Flik (1988). Trout were anaesthetized with MS-222 and injected intravenously with 2500 U of sodium heparin dissolved in 1 ml of saline. After 20 minutes, the fish were decapitated just posterior to the pectoral fins. The head was placed onto an operating table where the gills, irrigated with freshwater, were perfused with ice-cold isotonic saline (0.6% NaCl) containing 20 U/ml of sodium heparin. Perfusion was carried out at a pressure of 60 cmH_2O via a catheter (PE 60 tubing) inserted into the bulbus arteriosus until the gills appeared to be free of trapped red blood cells. Following perfusion, the gill basket was quickly excised. Further preparations were carried out on ice (0-4℃) from this point.

The gill epithelium was scraped from the gill arches onto a glass plate using a glass microscope slide. The scrapings were then placed into a douncer with a loose

pestle and homogenized in 15 ml of a hypotonic buffer consisting of 25 mM NaCl and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-TRIS (hydroxymethyl) aminomethane (TRIS) (pH = 7.4). The volume was brought to 50 ml and the homogenate centrifuged for 15 minutes at 550 g (Beckman Ti 70 Rotor) to remove nuclei and cellular debris. Membranes were then collected by ultracentrifugation of the remaining supernatant at 30 000 g for 30 minutes. The resulting pellet fixed to the centrifuge tube, was resuspended with 100 strokes of a douncer in 15 ml of an isotonic buffer containing 250 mM sucrose, 12.5 mM NaCl, 0.5 mM dihydro-ethylenediaminetriacetic acid (H_2EDTA), and 5 mM HEPES-TRIS (pH = 7.5). The volume of the suspension was then brought to 30 ml with the same buffer. The resulting suspension was then centrifuged differentially in the following manner: 1 000 g for 10 minutes, 10 000 g for 10 minutes, 30 000 g for 30 minutes. The resulting pellet was obtaining by adding 300 µl of a buffer containing 20 mM HEPES-TRIS (pH = 7.4) and 200 mM sucrose, and resuspended by passing the mixture through a 23-gauge syringe needle. The membrane preparations contained approximately 3 mg mL^{-1} of bovine serum albumin protein equivalents and were used on the same day of isolation.

High-affinity Ca^{2+}-ATPase activity was assayed in the presence of oligomycin B (5 µg mL^{-1}) and 5 mM sodium azide, at 1.0 µM and 0 µM free Ca^{2+} in an assay medium containing 100 mM NaCl, 0.1 mM ouabain, 0.5 mM ethylene glycol-bis(aminoethyl ether) – *N,N,N',N'*, tetraacetic acid (EGTA), 0.5 mM *N*-hyroxyethylenediaminetriacetic acid (HEEDTA), 5 mM $MgCl_2$, 3 mM Na_2ATP, and 20 mM HEPES-TRIS (pH = 7.4). Membrane vesicles were permeabilized with 20 µg saponin per mg membrane protein. High-affinity Ca^{2+}-ATPase activity was measured

as the difference in inorganic phosphate release (P_i) in the presence and absence of Ca^{2+} in the assay medium.

The *in vitro* effect of Pb on Ca^{2+}-ATPase activity was tested by adding Pb (from a $Pb(NO_3)_2$ stock solution) to the final membrane suspension, just prior to addition to the assay reaction medium, at concentrations similar to those measured in the final membrane suspension from the *in vivo* exposure. Finally, branchial Pb accumulation was measured in a separate group of fish, similar in size and exposed to control conditions, or to 4.8 μM Pb for 3h, 24h, or 96h. Procedures used for measurement of gill Pb burden were identical to those outlined in *Calcium-channel blocker experiments.*

Statistical Analysis

In kinetic experiments, Michaelis-Menten analysis of the relationship between the rate of Ca^{2+} influx (J_{in}^{Ca2+}) and waterborne calcium (substrate) were performed using Lineweaver-Burk linear regression (double reciprocal plots), where J_{max} (maximum rate of influx) = inverse of the y-intercept of the regression line, and $-K_m$ (Michaelis constant) = inverse of the x-intercept of the regression line. For comparison, data were also analysed by Eadie-Hofstee plots and by non-linear regression.

Calculated data are expressed as mean ± 1 SEM (N). Experimental means were compared to corresponding control mean values by an unpaired two-tailed Student's t-test. Time-dependent responses in both control and experimental groups were tested against initial 0 h measurements using a one-way ANOVA with a two-

sided Dunnett's post hoc multiple comparison. All statistical significance was calculated at $p < 0.05$.

Results

Effect of Pb on Ca^{2+} influx

Juvenile rainbow trout exposed to 2.3 ±0.1 μM dissolved Pb showed significant reductions in Ca^{2+}-influx rate compared to control fish (Fig. 3-1a). Effects on influx were immediate, with an approximate 40 % inhibition occurring at 0-2 h of Pb exposure, this effect carrying through to 24-26 h, though escaping significance at 12-14 h. Exposure to 1.4 ± 0.2 μM dissolved Pb resulted in similar inhibition of Ca^{2+} influx (Fig. 3-1b), though significant only at 12-14 h where the reduction was 32 %. The concentration of total Pb in control water was 0.002 ±0.0001 μM.

Kinetic analysis of the interaction between Pb and Ca^{2+}

The saturable nature of calcium influx with increasing waterborne calcium concentrations obeyed typical Michaelis-Menten kinetic analysis, in the presence and absence of Pb (Fig. 3-2). Consistent with the immediate inhibition of Ca^{2+}-influx documented in the previous experiment, waterborne Pb exposure resulted in inhibition of calcium influx in juvenile rainbow trout over a 3 h exposure period, the inhibition increasing with increased Pb concentrations. From analysis of a double reciprocal Lineweaver-Burk plot (Fig. 3-3a), it is apparent that K_m values increased significantly with increasing waterborne Pb concentration (Table 3-1), for example, an approximate 16-fold increase in K_m value between control levels and 4.8 μM Pb exposure. While measurements of K_m showed significant increases, J_{max} was not

significantly altered as a function of waterborne Pb concentration (Table 3-1). Other analyses (Eadie-Hofstee, non-linear regression) yielded similar results, though with larger error terms.

Because of the competitive nature of Ca^{2+}-influx inhibition by Pb, an inhibitor constant was determined from a regression plot of apparent Km (measured Km values from Table 1)/ Jmax vs. waterborne Pb concentration (Fig. 3-3b; Segel, 1976). The $K_{i,\,[Pb]}$ (x-intercept of the regression line = $-K_i$) was calculated to be 0.47 μM Pb.

The effect of calcium on branchial Pb accumulation

The effect of waterborne calcium on branchial Pb uptake is shown in Fig. 3-4. Overall, a protective effect of calcium was observed with the amount of branchial Pb accumulation decreasing with increasing waterborne Ca^{2+} concentration. At a waterborne Pb concentration of 0.48 μM, accumulation was consistent over the range of Ca^{2+} concentrations (150 – 1300 μM). With increases in waterborne Pb concentrations to 2.4 and 4.8 μM, the concentration of waterborne Ca^{2+} required to yield the same protective effects increased. For example, upon exposure to 4.8 μM Pb, Pb accumulation at 150 μM Ca^{2+} was approximately 35 times higher than control (0 μM Pb) accumulation while at 1300 μM Ca^{2+}, accumulation was only 6 times greater than control gill Pb burden. At a waterborne Ca^{2+} concentration of approximately 1300 μM, branchial Pb burden measured after exposure to 0.48, 2.4, and 4.8 μM Pb did not differ significantly.

The effect of calcium channel blockers on branchial Pb accumulation

The effect of La^{3+}, a voltage-independent Ca^{2+}-channel inhibitor, on Ca^{2+} uptake and branchial Pb accumulation is shown in Figure 3-5a and 3-5b. From Ca^{2+} influx measurements determined for control conditions at 600 μM Ca^{2+} and 1 μM La^{3+} (added as $LaCl_3$), an approximate 55% reduction in Ca^{2+} uptake occurred in the presence of 1 μM La^{3+}. Upon Pb-exposure, waterborne La^{3+} significantly reduced gill Pb burden in a dose-dependent fashion. In the presence of 0.0001 μM La^{3+}, an approximate 39 % reduction in Pb accumulation occurred compared to control fish exposed to the same Pb concentration in the absence of La^{3+}. This inhibition of Pb uptake was dramatic from 0.001 to 1 μM La^{3+}, with an approximate 77 % reduction (compared to control) in Pb accumulation in the presence of these waterborne La^{3+} concentrations.

Waterborne Cd had similar effects on Ca^{2+} uptake and branchial Pb accumulation. A waterborne concentration of 1 μM Cd reduced the rate of Ca^{2+} influx by approximately 74 % compared to controls (Fig. 3-6a). Branchial Cd accumulation occurred in a dose-dependent fashion when waterborne Cd concentration was increased from control levels to 1 μM, with the largest increase occurring at the highest concentration (data not shown). This dose-dependent uptake corresponded with reduced levels of branchial Pb accumulation (Fig. 3-6b). While gill Cd burden increased by approximately 20 fold, Pb uptake was reduced by 56 %. Results using Zn as a calcium channel blocker were similar. Ca^{2+} uptake was reduced by approximately 43 % in the presence of 100 μM Zn (Fig. 3-7a). This inhibition corresponded with an approximate 47 % reduction in branchial Pb accumulation in fish exposed to Pb in the presence of Zn (Fig. 3-7b).

Voltage-dependent, L-type calcium channel blockers did not appear to impact Ca^{2+} influx or branchial Pb accumulation. Exposure to 100 μM nifedipine or 100 μM verapamil did not significantly reduce the rate of Ca^{2+} influx at 600 μM Ca^{2+} (Fig. 3-8a). The effects of nifedipine on Pb accumulation were found to be not significant compared to controls (Fig. 3-8b). Exposure to verapamil yielded similar results when comparing Pb accumulation in the presence and absence of the blocking agent (Fig. 3-8c).

Effect of $CaCl_2$ injection on Ca^{2+} influx and branchial Pb accumulation

Injection of $CaCl_2$ significantly reduced Ca^{2+} influx by approximately 52 % compared to fish sham injected with vehicle only (Fig. 3-9a). Inhibition of Ca^{2+} influx corresponded with a significant decrease in branchial Pb accumulation in $CaCl_2$ injected fish (Fig. 3-9b). This reduction was approximately 37% compared to controls (NaCl injected).

The role of Ca^{2+} efflux in Pb-induced hypocalcemia

The role of Ca^{2+} efflux in Pb-induced disruption of Ca^{2+}-homeostasis was assessed during exposure to control conditions or 6.3 ±0.1 μM dissolved Pb. Results are summarized in Table 3-2. Rainbow trout exposed to control conditions had an average Ca^{2+}-efflux (J_{out}^{Ca2+}) of approximately 32.8 ± 4.6 nmol g^{-1} h^{-1}. Pb-exposed fish did not show significant deviations from this value after exposures of 3, 27, and 51 h to experimental conditions.

High-affinity Ca^{2+}-ATPase activity

Adult rainbow trout were exposed to a dissolved Pb concentration of 5.5 ± 0.4 μM. When compared to values for unexposed fish at t = 0 h (control sampling), Ca^{2+} ATPase activity after 3 h of Pb exposure was not reduced. However, prolonged exposure resulted in a reduction in activity after 24 h, and a significant 80% inhibition by 96 h of Pb exposure (Fig. 3-10a). Branchial Pb burden increased significantly after 24 h and 96 h in fish exposed to an identical waterborne Pb concentration (Fig. 3-10b). This accumulation appeared to correlate well with the inhibition of high-affinity Ca^{2+}-ATPase activity (Fig. 3-10c). The *in vitro* effects of $Pb(NO_3)_2$ on the Ca^{2+}-ATPase assay were insignificant.

Discussion

Previous research has presented circumstantial evidence that Pb may compete with Ca^{2+} for the same binding sites on the gills of freshwater fish (Davies et al., 1976; Varanasi and Gmur, 1978; Sorensen, 1991; Macdonald et al., 2002; Rogers et al., 2003). In the present study, direct evidence is provided suggesting that Pb does in fact compete with calcium for uptake and that interference with branchial Ca^{2+} transport includes sharing of at least two phases of this transport: an immediate interaction with apical entry and a more slowly developing interaction with high-affinity Ca^{2+}-ATPase.

The observed rapid inhibition of calcium influx (Fig. 3-1) after exposure to 1.4 and 2.3 μM Pb is consistent with previous studies demonstrating reduced Ca^{2+} influx after exposure to Pb concentrations close to 4.8 μM (Rogers et al., 2003). This is contrary to findings presented by Sayer et al. (1989; 1991) that Pb exposure does not result in disruption of Ca^{2+} influx. From the present data (Fig. 3-1,3-2), it is apparent that at exposure concentrations (0.48 μM upwards) that approach environmentally relevant or normal levels (0.003 – 0.58 μM Pb; Demayo et al. (1982)), hypocalcemic effects of Pb are still prominent. Additionally, influx inhibition occurs immediately, suggesting that the interaction may be competitive in nature, similar to metals like Cd (Verbost et al., 1987, 1989) and Zn (Spry and Wood, 1985; Hogstrand et al., 1994, 1996). Therefore, measurements of Ca^{2+} influx were useful in validating a kinetic approach to analyze the relationship between Pb and Ca^{2+}.

The construction of uptake curves at a number of waterborne substrate (Ca^{2+}) concentrations using various waterborne Pb levels allowed characterization of the effect of Pb on branchial Ca^{2+} influx kinetics. From these curves, K_m and J_{max} values

were calculated for each Pb concentration used (Table 3-1). These data were indicative of a typical Michaelis-Menten competitive relationship with a significant increase in K_m value corresponding to increasing waterborne Pb concentrations. Increases in K_m suggest that as waterborne Pb concentrations are elevated, the affinity of the calcium-binding site for the calcium ion decreases due to the presence of a competitor. Consequently, the amount of Ca^{2+} required to achieve half maximum transport is elevated. The stability of J_{max} values obtained from this kinetic study confirmed the competitive nature of the Pb/Ca^{2+} relationship as the maximum rate of Ca^{2+} transport, or the integrity of Ca^{2+} binding sites, remained unchanged. These findings are consistent with those made for Zn (Hogstrand et al., 1994) and for Cd (Niyogi and Wood, submitted), metals that are known calcium antagonists. Kinetic analysis of Pb and Ca^{2+} interaction allowed calculation of an inhibitor constant ($K_{i, Pb}$) for calcium influx of 0.48 μM Pb. Based on this value, it is evident that the affinity of Pb for Ca^{2+} binding sites on the fish gill is less than that for Cd, but greater than that for Zn. Niyogi and Wood (submitted) reported an inhibitor constant of 0.12 μM for Cd in rainbow trout, a value 4 times less than that reported in the present study for Pb. From the data of Hogstrand et al. (1994, 1998), the inhibition constant of Zn appears to be above 2 μM. This variation in affinity for Ca^{2+} binding sites could explain the difference in potency between Pb, Cd, and Zn in terms of acute toxicity; Cd being the most toxic with a 96 h LC50 of 0.2 μM (Hollis et al., 1999) compared to an LC50 of 4.8 μM for Pb (Rogers et al., 2003) and 13.3 μM for Zn (Alsop et al., 1999). All values were determined in the same water quality.

Branchial Pb accumulation was largely dependent upon waterborne Ca^{2+} concentrations as shown in Fig. 3-4. At the lowest waterborne Ca^{2+} concentration of

150 μM, Pb accumulation significantly increased as waterborne Pb concentrations increased from control to 4.8 μM. This relationship changed as Ca^{2+} concentration increased. Accumulation at 0.48, 2.4, and 4.8 μM Pb was not significantly different at higher levels of waterborne Ca^{2+} demonstrating strong protective effects of waterborne Ca^{2+} against branchial Pb uptake. This confirms other existing evidence highlighting the protective effects of water hardness in Pb toxicity (Sorensen, 1991) and suggests that it is the Ca^{2+} component of hard water that dictates the toxicity of dissolved Pb to fish. Richards and Playle (1999) reported similar protective effects of Ca^{2+} against Cd accumulation on the gills in synthetically modified soft water. Alsop and Wood (1999) demonstrated reduced Zn uptake and toxicity with increasing waterborne Ca^{2+} concentration. Considering differential branchial Pb accumulation and kinetic Pb/Ca^{2+} analysis from the present study, it is evident that the gill is likely the major site of a competitive interaction between Pb and Ca^{2+} that contributes to Pb toxicity in the rainbow trout.

Further evidence supporting the existence of a competitive relationship between Pb and Ca^{2+} was acquired through the use of apical calcium channel blockers. While inhibiting Ca^{2+} uptake (Fig. 3-5a), waterborne La^{3+}, a classic Ca^{2+} channel blocker (Weiss, 1974), significantly reduced the amount of Pb accumulation on the gill (Fig. 3-5b). This suggests that apical entry of Pb into the chloride cells probably occurs through a lanthanum-sensitive, voltage-independent apical calcium channel. These observations are similar to those reported for Zn by Hogstrand et al. (1996), with an approximate 53 % decrease in Zn uptake occurring in the presence of 1μM La^{3+} compared to controls. Comhaire et al. (1998) reported a 48 % inhibition of Co^{2+} uptake at low-level La^{3+} but 1 μM La^{3+} resulted in a stimulation of Co^{2+} influx.

A similar effect was observed in the present study at waterborne La^{3+} concentrations exceeding 1 μM (data not shown). Stimulated Pb accumulation may have been due to disruption of apical membrane integrity by high La^{3+} concentrations, resulting in an increase in the diffusive component of Pb uptake. Finally, the results of the present study are consistent with those of Verbost et al. (1987), demonstrating an inhibition of Cd influx by La^{3+}, leading to the conclusion that Cd and Ca^{2+} share the same apical entry sites. This has led to the use of Cd for the purpose of calcium-channel blocker experiments (Comhaire et al., 1998), as in the present study.

The effects of Cd on Ca^{2+} influx were significant (Fig. 3-6a). As gill Cd accumulation increased with increased waterborne Cd concentrations, gill Pb burden was reduced in a dose-dependent fashion compared to uptake observed in the absence of the blocker (Fig. 3-6b). This is consistent with Comhaire et al. (1998), who reported significant inhibition of Co^{2+} uptake upon exposure to similar waterborne Cd concentrations. The inhibition of Pb accumulation by Cd, a metal that traverses the chloride cell apical membrane through voltage-independent, lanthanum-inhibitable channels (Verbost et al., 1989) is further evidence supporting a similar route of entry for Pb. In further support of this theory is the effect of Zn on Pb accumulation (Fig. 3-7b). The ability of Zn, a metal believed to enter fish via Ca^{2+} uptake pathways (Spry and Wood 1985; Hogstrand et al., 1994, 1995, 1996, 1998), to reduce branchial Pb accumulation suggests a similar route of entry for these metals.

The possibility of Pb and Ca^{2+} transport through voltage-dependent, L-type calcium-channels at the apical surface of the chloride cell was discounted by the lack of effect of verapamil and nifedipine (Fig. 3-8 a-c), both voltage-dependent Ca^{2+} channel blockers (Perry and Flik, 1988). Perry and Flik (1988) reported similar

results when assessing the effect of nifedipine on Ca^{2+} uptake leading to the suggestion that Ca^{2+} flux across the apical membrane occurs through voltage-independent Ca^{2+} channels.

Despite obvious effects of voltage-independent calcium channel blockers on branchial Pb accumulation in the rainbow trout, the possibility that Pb could also be binding non-specifically may still exist. Studies using mammalian systems, for example, have demonstrated that calcium antagonists such as Zn and Pb effectively reduce voltage-activated calcium channel currents in rat neuronal cell lines (Busselberg et al., 1992; Platt and Busselberg, 1994; Busselberg et al., 1994; Busselberg, 1995). Lanthanum has also been reported to inhibit voltage-dependent channels, specifically T-type calcium currents in rat hippocampal neurons (Takahashi and Akaike, 1991). These results are in contrast with those of the present study in rainbow trout that suggest an insignificant role for voltage-dependent channels in the transport of Pb based on the presumption that transport is blocked solely by voltage-independent calcium channel blockers. Though it is possible that this lack of specificity reported in mammalian literature may reflect a purely *in vitro* effect compared with the experiments conducted *in vivo* in the present study, caution should be taken in interpreting data derived from the use of calcium channel blockers, especially when utilizing lethal doses that could enhance non-specific binding at the gill surface.

Injection of ionic Ca^{2+} was also employed to manipulate apical Ca^{2+} channels so as to inhibit Ca^{2+} influx via the stanniocalcin-controlled pathway. Previous studies have shown that activation of this pathway results in a decreased permeability of chloride cells to Ca^{2+} entry, thereby reducing influx (Perry et al., 1989; Verbost et al.,

1993). Stanniocalcin, a peptide hormone produced by the corpuscles of Stannius, does not act directly on apical calcium channels, but rather initiates a second messenger cascade involving cyclic AMP that results in regulation of voltage-independent sites of Ca^{2+} influx. It has been long known that removal of the corpuscles of Stannius results in significant hypercalcemia in teleost fish (Pang and Pang, 1986). In the present study, the hypocalcemic effects of stanniocalcin induction reduced both Ca^{2+} influx and Pb accumulation (Fig. 3-9a, b). This provides further evidence that Pb uptake is by the same mechanism as Ca^{2+} uptake. These results are similar to those reported by Hogstrand et al. (1996), where both Ca^{2+} and Zn influx rates were reduced by injection of Ca^{2+}.

The other significant process in the transepithelial uptake of Ca^{2+} is the movement across the baso-lateral membrane against an electrochemical gradient via a high affinity Ca^{2+}-ATPase mechanism (Perry and Flik, 1988). The importance of this enzyme to calcium transport makes it a vulnerable target for Ca^{2+} antagonists such as Cd, which following sufficient accumulation within the chloride cell, inhibits Ca^{2+}-ATPase activity contributing to disturbances in Ca^{2+} homeostasis (Verbost et al., 1989). The present study has shown that prolonged Pb exposure results in significant inhibition of high affinity Ca^{2+}-ATPase activity (Fig. 3-10a). This reduction in activity occurred after 24 - 96 h of Pb exposure, suggesting that similar to Cd, Pb must accumulate in sufficient amounts at the gill to have a negative effect on Ca^{2+}-ATPase activity (Fig. 3-10c). Accumulation at the gill was significantly elevated after 24 h and 96 h of Pb exposure (Fig. 3-10b), the same time points where substantial inhibition of enzyme activity was observed. This mechanism of Ca^{2+} homeostasis disruption is also similar to that proposed for Zn (Hogstrand et al., 1996). With

increased amounts of branchial Pb accumulation following prolonged exposure a slower, non-competitive component to disruption of Ca^{2+} homeostasis would occur. This would ultimately have an effect on J_{max}, decreasing the overall efficiency of the Ca^{2+} transport system.

Ca^{2+} efflux was much less sensitive to Pb than Ca^{2+} influx (Table 3-2). This is evidenced by the absence of efflux stimulation at a waterborne Pb concentration (6.3 μM) that significantly reduced Ca^{2+} influx (Rogers et al., 2003) and is at least four times higher than the minimum concentration required to elicit Ca^{2+} influx inhibition. These findings are consistent with those for Cd (Verbost et al., 1987; Reid and McDonald, 1988). Verbost et al. (1987) found the effect of Cd on Ca^{2+} efflux was insignificant with the exception of measurements made at the highest Cd concentration. Reid and McDonald (1988) found no effect of Cd on branchial Ca^{2+} efflux at circumneutral pH and at moderately acidic pH (4.8). While significant stimulation of efflux by Pb was not observed a slight, non-significant increase in efflux rate did occur after 48 h of Pb exposure. This could reflect a secondary effect of Pb on the integrity of paracellular routes through which Ca^{2+} efflux is thought to occur (Perry and Flik, 1988).

Conclusions

To our knowledge, this study is the first to present direct evidence that the uptake of waterborne Pb by freshwater adapted rainbow trout is by the same mechanism as Ca^{2+}. This involves competitive inhibition of apical entry at lanthanum-sensitive Ca^{2+} channels and interference with the function of the ATP-driven baso-lateral Ca^{2+} pump. Similarities are evident between Pb and other known

calcium antagonists such as Cd, Zn, and Co, suggesting avenues for further research that include characterization of the interaction between Pb and Ca^{2+}-ATPase as well as the intracellular handling of Pb by Ca^{2+} transport proteins such as calmodulin.

This study could have potential implications for a predictive modelling approach such as the Biotic Ligand Model (BLM) (McGeer et al., 2000; Paquin et al., 2000; DiToro et al., 2001), which is based on a toxic metal binding to the gills in competition with protective ions in the water column (Playle et al., 1993; Playle, 1998; McGeer et al., 2000; Di Toro et al., 2001). Currently, binding models for Pb are being developed (Macdonald et al., 2002), and based on their predictions and the physiological evidence presented in this study, it appears that Pb is indeed capable of out-competing Ca^{2+} for specific transport sites at the freshwater fish gill.

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Fig. 3-1

a) Calcium influx rates in juvenile rainbow trout in City of Hamilton dechlorinated tap-water in control conditions or water with 2.3 ± 0.1 μM dissolved Pb (grey bars) after exposure for 0, 12, or 24 h b) Calcium influx rates in control water (black bars) or water with 1.4 ± 0.2 μM dissolved Pb after exposure for 0, 12, or 24 h. Means ±1 SEM (n = 8 throughout). Asterisk "*" indicates significant difference ($p<0.05$; two-tailed Student's t-test) from corresponding control means.

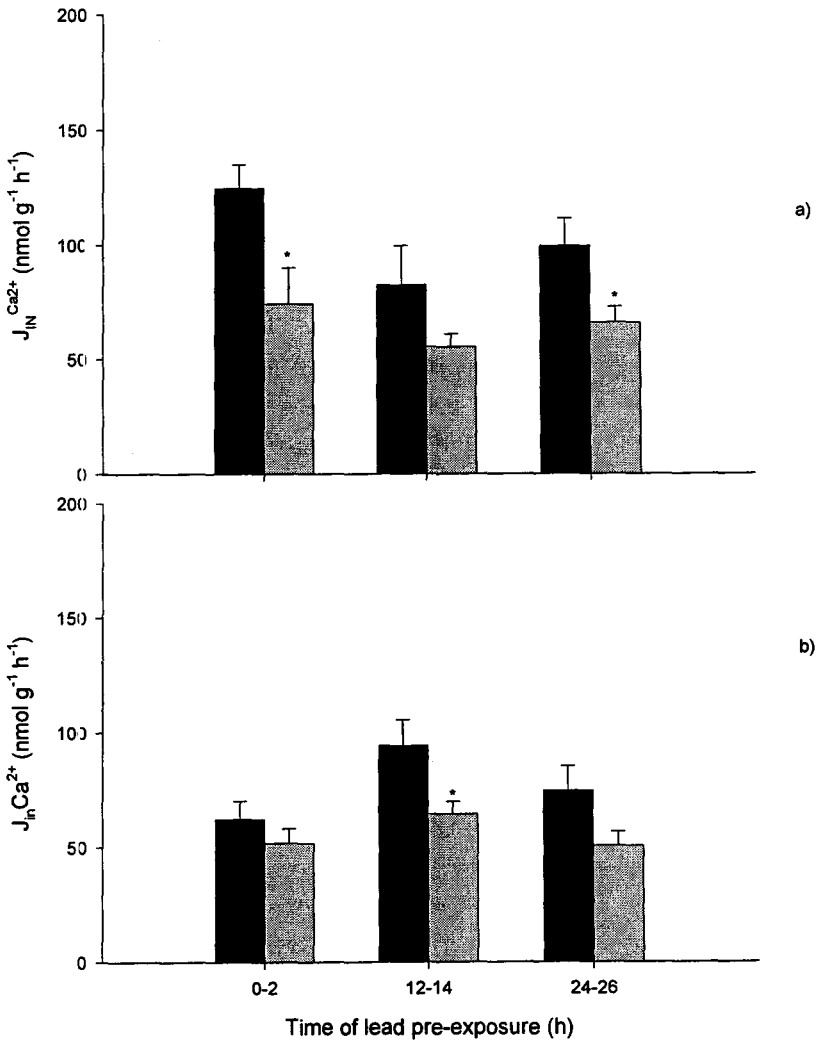

200
150
100
50
0
J_{IN}^{Ca2+} (nmol g^{-1} h^{-1})
a)
200
150
100
50
0
$J_{in}Ca^{2+}$ (nmol g^{-1} h^{-1})
b)
0-2
12-14
24-26
Time of lead pre-exposure (h)

Fig. 3-2

Unidirectional branchial calcium influx rates in juvenile rainbow trout at various waterborne calcium concentrations in synthetic water for four different lead concentrations. Control fish are shown as black circles. Fish exposed to 0.48μM dissolved Pb are shown as white triangles, 2.4 μM Pb as white circles, and 4.8 μM Pb as white squares. Means ± 1 SEM (n = 7 per treatment).

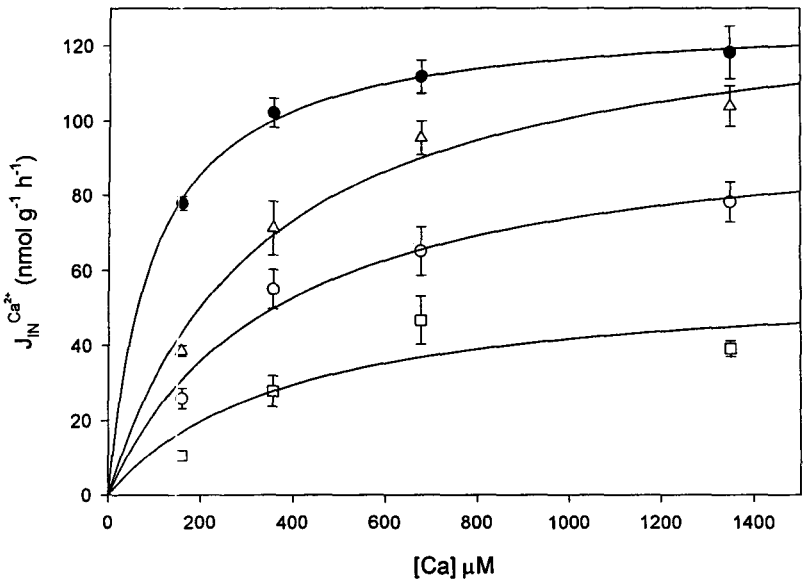
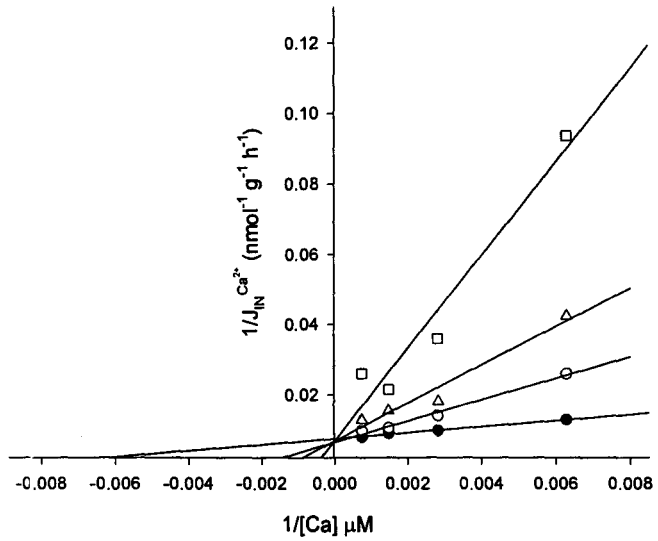

120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
[Ca] µM
$J_{IN}^{Ca^{2+}}$ (nmol g^{-1} h^{-1})

Fig. 3-3

Analytical plots for data presented in Fig. 2 using a) Michaelis-Menten analyses by Lineweaver-Burk regression (double reciprocal plot) illustrating the competitive relationship between lead and calcium uptake in the rainbow trout, and b) regression analyses of apparent K_m / J_{max} versus waterborne lead concentration (using apparent Km and Jmax values presented in Table 1. The regression equation was $y = 2.38x + 1.12$ ($R^2 = 0.95$). The inhibitor constant ($-K_{i,[Pb]}$ = x intercept of the regression line) was 0.48 μM Pb.

a)


0.12
0.10
0.08
0.06
0.04
0.02
$1/J_{IN}^{Ca^{2+}}$ (nmol^{-1} g^{-1} h^{-1})
-0.008
-0.006
-0.004
-0.002
0.000
0.002
0.004
0.006
0.008
1/[Ca] μM

b)

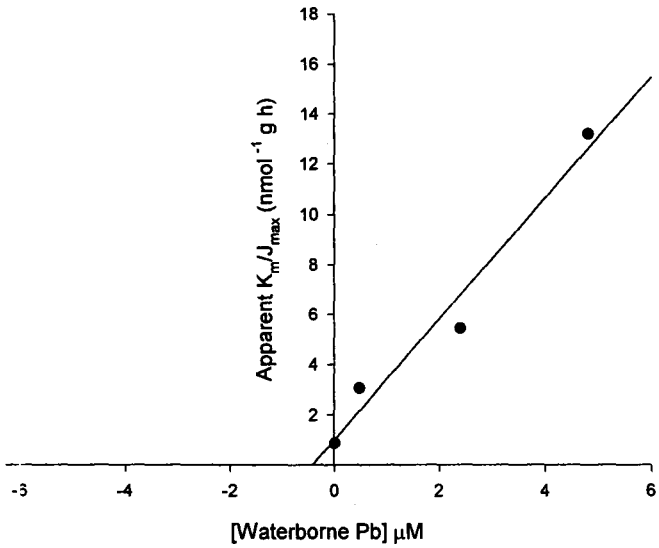

18
16
14
12
10
8
6
4
2
Apparent K_m/J_{max} (nmol^{-1} g h)
-5
-4
-2
0
2
4
6
[Waterborne Pb] μM

Fig. 3-4

Measurements of branchial lead accumulation in juvenile rainbow trout at various waterborne calcium concentrations in synthetic water for four different lead concentrations. Means ± 1 SEM (n = 7 per treatment).

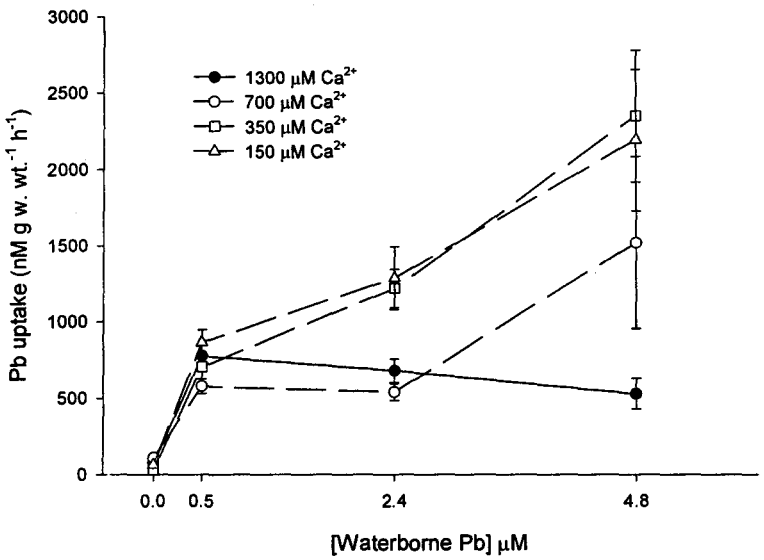

3000
2500
2000
1500
1000
500
0
Pb uptake (nM g w. wt.$^{-1}$ h^{-1})
1300 µM Ca^{2+}
700 µM Ca^{2+}
350 µM Ca^{2+}
150 µM Ca^{2+}
0.0
0.5
2.4
4.8
[Waterborne Pb] µM

Fig. 3-5

The effect of waterborne La^{3+} on a) calcium uptake in juvenile rainbow trout exposed to control conditions (black bars) or 1 μM La^{3+} (grey bars) in synthetically modified water (600 μM Ca^{2+}), and b) branchial lead accumulation in juvenile rainbow trout exposed to control conditions (black circle) or a series of waterborne La^{3+} concentrations (white circles). Data are expressed as mean± 1 SEM (n = 7). Asterisk "*" indicates significant difference ($p<0.05$; two-tailed Student's t-test) from corresponding control mean.

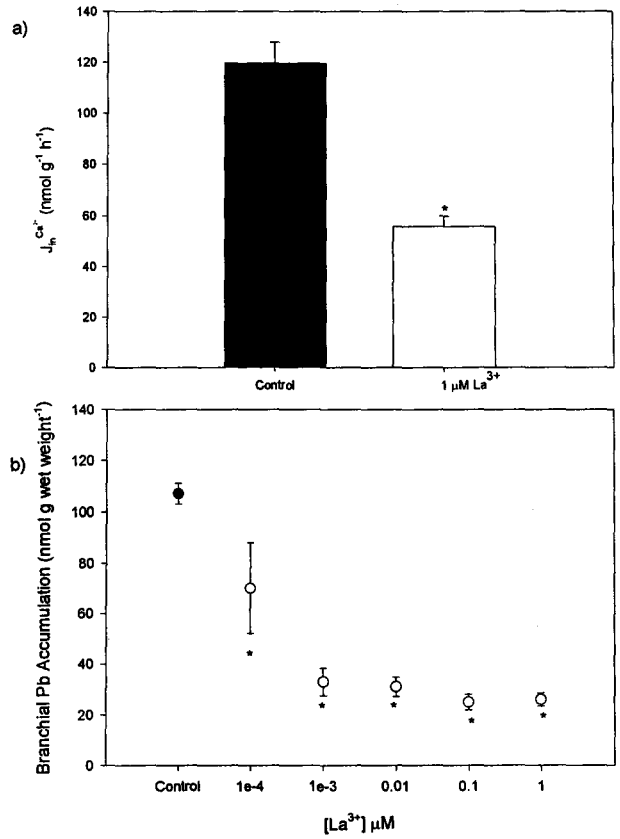

a)
140
120
100
80
60
40
20
0
$J_{in}^{Ca^{2+}}$ (nmol g^{-1} h^{-1})
*
Control
1 µM La^{3+}
b)
Branchial Pb Accumulation (nmol g wet weight^{-1})
140
120
100
80
60
40
20
0
*
*
*
*
*
Control
1e-4
1e-3
0.01
0.1
1
[La^{3+}] µM

Fig. 3-6

The use of Cd as a voltage-independent Ca^{2+} channel blocker: effects on a) calcium uptake in juvenile rainbow trout exposed to control conditions (black bars) or 1 μM Cd (grey bars) in synthetically modified water (600 μM Ca^{2+}), b) branchial Pb accumulation in juvenile rainbow trout exposed to control conditions (black circle) or waterborne Cd concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 μM Cd (white circles). Data are expressed as mean ± 1 SEM (n = 7). Asterisk "*" indicates significant difference ($p<0.05$; two-tailed Student's t-test) from corresponding control mean.

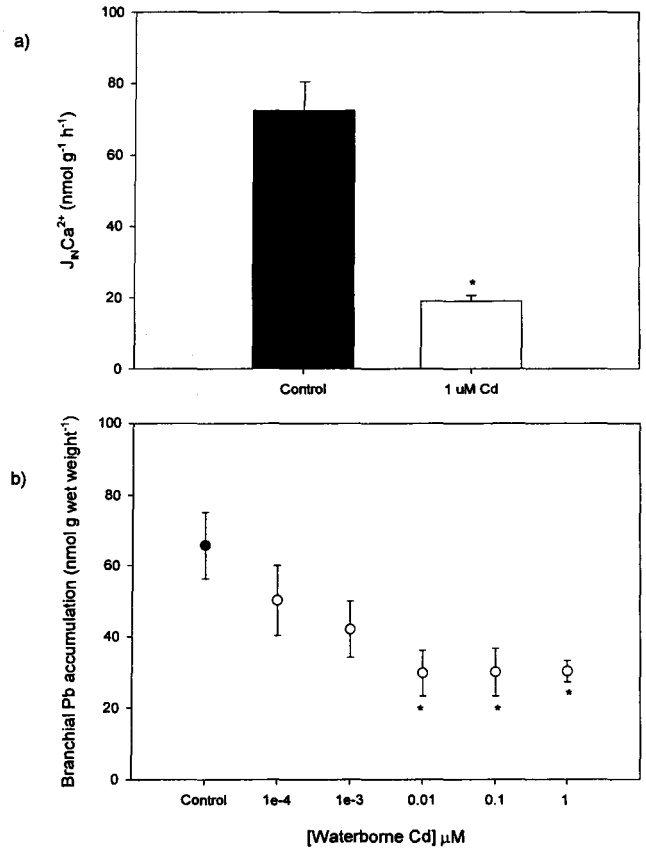

a)
100
80
60
40
20
0
$J_{in}Ca^{2+}$ (nmol g^{-1} h^{-1})
*
Control
1 uM Cd
b)
100
80
60
40
20
0
Branchial Pb accumulation (nmol g wet weight^{-1})
*
*
*
Control
1e-4
1e-3
0.01
0.1
1
[Waterborne Cd] μM

Fig. 3-7

The use of Zn as a voltage-independent Ca^{2+} channel blocker: effects on a) calcium uptake in juvenile rainbow trout exposed to control conditions (black bars) or 100 μM Zn (grey bars) in synthetically modified water (600 μM Ca^{2+}), and b) branchial Pb accumulation in trout exposed to control conditions (black circle) and Zn concentrations of 0.01, 0.1, 1, 10, and 100 μM Zn (white circles). Data are expressed as mean ± 1 SEM (n = 7). Asterisk "*" indicates significant difference ($p<0.05$; two-tailed Student's t-test) from corresponding control mean.

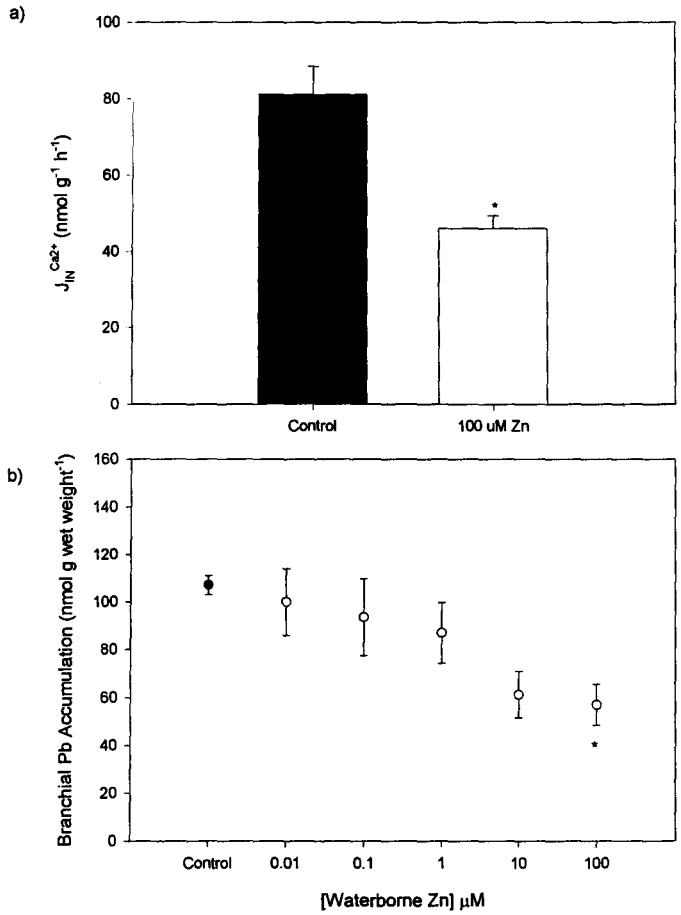
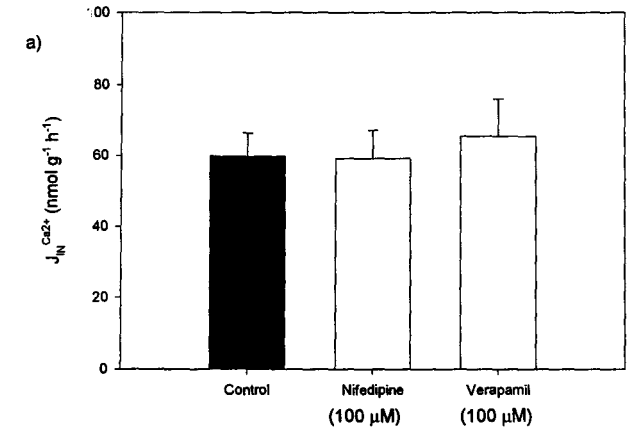
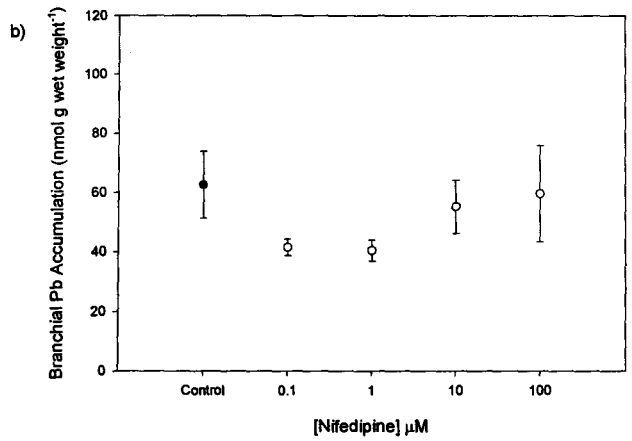

a)
100
80
60
40
20
0
J_{IN}^{Ca2+} (nmol g^{-1} h^{-1})
*
Control
100 uM Zn
b)
160
140
120
100
80
60
40
20
0
Branchial Pb Accumulation (nmol g wet weight^{-1})
*
Control
0.01
0.1
1
10
100
[Waterborne Zn] µM

Fig. 3-8

a) The effects of the voltage-dependent Ca^{2+} channel blockers nifedipine and verapamil on calcium uptake in juvenile rainbow trout exposed to control conditions (black bars) or 100 μM nifepine and 100 μM verapamil (white bars) in synthetically modified water (600 μM Ca^{2+}). b) The effect of nifedipine on branchial Pb accumulation in juvenile trout exposed to control conditions (black circle) or 0.1, 1, 10 and 100 μM nifedipine (white circles). c) The effect of verapamil on branchial Pb accumulation in juvenile trout exposed to control conditions (black circle) or 0.1, 1, 10, and 100 μM verapamil (white circles). Data are presented as mean± 1 SEM (n = 7 per treatment).


a)
100
80
60
40
20
0
J_{IN}^{Ca2+} (nmol g^{-1} h^{-1})
Control
Nifedipine
(100 μM)
Verapamil
(100 μM)


b)
120
100
80
60
40
20
0
Branchial Pb Accumulation (nmol g wet weight^{-1})
Control
0.1
1
10
100
[Nifedipine] μM

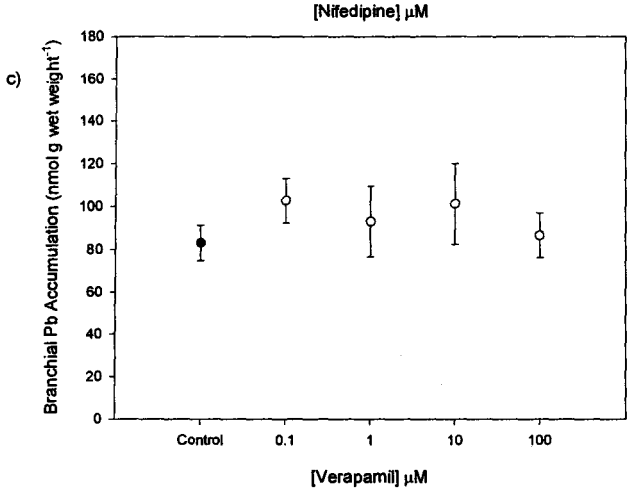
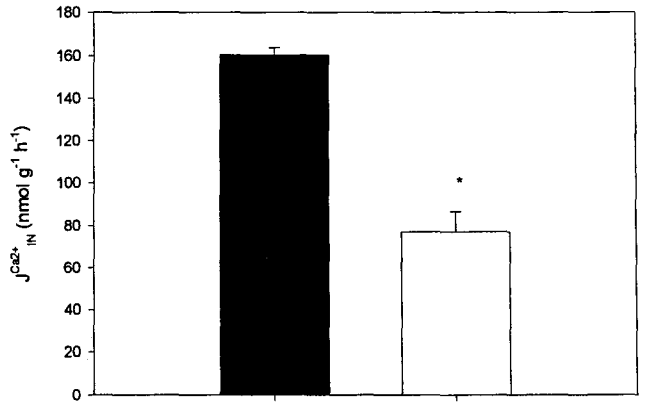

c)
180
160
140
120
100
80
60
40
20
0
Branchial Pb Accumulation (nmol g wet weight^{-1})
Control
0.1
1
10
100
[Verapamil] μM

Fig. 3-9

The effect of $CaCl_2$ injection on a) Ca^{2+} influx in juvenile rainbow trout in synthetically modified water (600 μM Ca^{2+}), and b) branchial Pb accumulation in juvenile rainbow trout. Data are expressed as mean± 1 SEM (n = 7 per treatment). Asterisk "*" indicates significant difference ($p<0.05$; two-tailed Student's t-test) from corresponding control mean.

a)


180
160
140
120
100
80
60
40
20
0
J^{Ca2+}_{IN} (nmol g^{-1} h^{-1})
*

b)

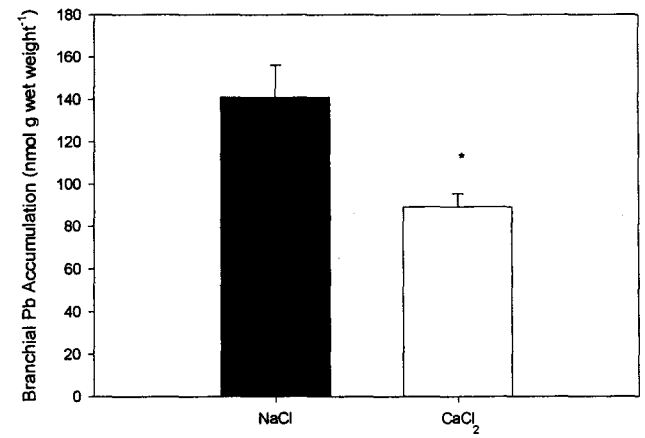

180
160
140
120
100
80
60
40
20
0
Branchial Pb Accumulation (nmol g wet weight^{-1})
*
NaCl
$CaCl_2$

Fig. 3-10

a) Time course analysis of branchial high affinity Ca^{2+}-ATPase activity in adult rainbow trout, b) time course of branchial Pb accumulation, and c) plot of high-affinity Ca^{2+}-ATPase activity versus branchial Pb accumulation in rainbow trout exposed to control conditions and to 5.5 ± 0.4 μM dissolved Pb for 3 h, 24 h, and 96 h. Data are expressed as mean ± 1 SEM (N = 6). Asterisk "*" indicates significant difference ($p<0.05$; One-way ANOVA with Dunnett's post-hoc multiple comparison) from control sampling mean.

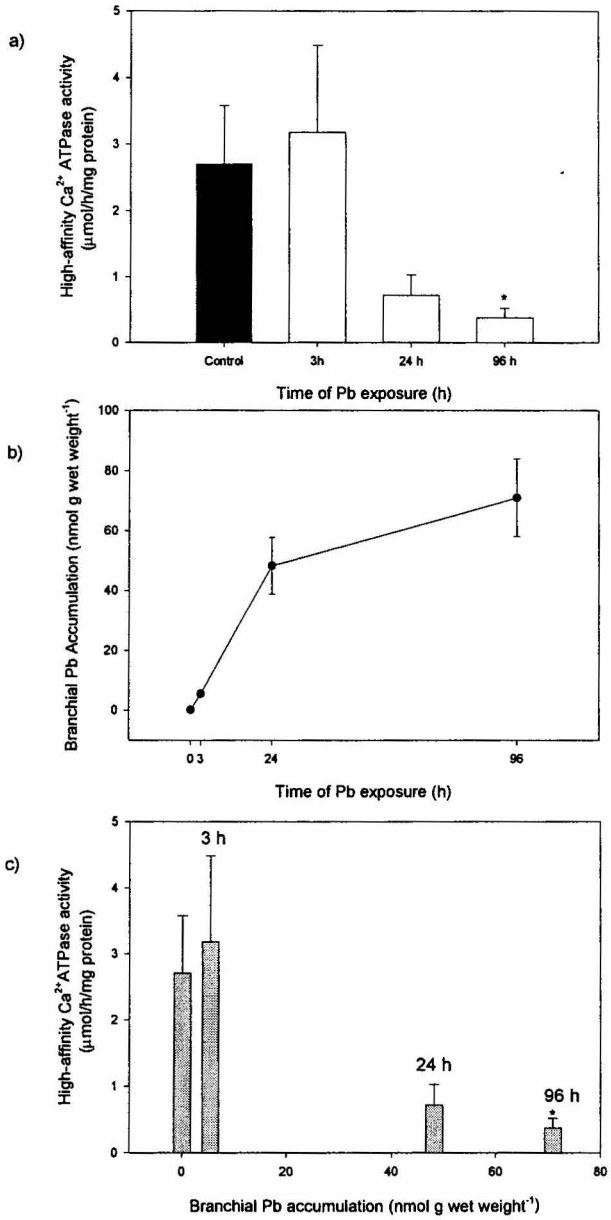

a)
High-affinity Ca2+ ATPase activity (µmol/h/mg protein)
Control
3h
24 h
96 h
Time of Pb exposure (h)
b)
Branchial Pb Accumulation (nmol g wet weight-1)
0 3
24
96
Time of Pb exposure (h)
c)
High-affinity Ca2+ATPase activity (µmol/h/mg protein)
3 h
24 h
96 h
Branchial Pb accumulation (nmol g wet weight-1)

Table 3-1

J_{max} and apparent K_m for unidirectional branchial calcium influx rates in juvenile rainbow trout at various waterborne lead concentrations. Asterisk "*" indicates significant difference ($p<0.05$) from control mean. Mean ± 1 SEM (n = 7).

Waterborne Pb (μM)	J_{max}(nmol g^{-1} h^{-1})	Apparent K_m(μM)
Control	135.1 ± 7.6	116.2 ± 2.7
0.48	156.3 ± 12.5	475.6 ± 66.9 *
2.4	147.1± 20.8	798.7 ± 58.4 *
4.8	144.9 ± 25.2	1911.7 ± 121.8 *

Table 3-2

Unidirectional calcium efflux rates in juvenile rainbow trout in City of Hamilton dechlorinated tap-water in control water or after exposure to 6.3 ± 0.14 μM dissolved Pb for 0, 3, 27, or 51 h. Means± 1 SEM (n = 7) There are no significant differences.

Time of Pb exposure (h)	J_{out}^{Ca2+} (nmol $g^{-1}h^{-1}$)
Control (0h)	32.8± 4.6
0 - 3	39.6± 7.2
24 - 27	37.1 ±4.0
48 – 51	48.6 ±8.8

CHAPTER 4

SUMMARY OF RESULTS AND CONCLUSIONS

The mechanism of acute Pb toxicity in the rainbow trout

As outlined in Chapter One, the teleost gill is a multi-purpose organ involved in gas exchange, acid/base balance, osmotic and ionoregulation, and waste excretion. Being in direct contact with the aquatic environment, the gill is susceptible to toxicants in the water column, which may include metal contaminants. Our understanding of the physiological consequences of waterborne metal exposure begins with the identification of toxic sites where metal-gill binding will occur and potentially cause physiological disruption. In recent years, our understanding of the acute toxic mechanism of numerous metals and characterization of key toxic binding sites has increased; however, until recently, the acute toxic mechanism for Pb in freshwater teleosts was poorly understood.

Present studies have shown that Pb accumulates in multiple biological tissues in the fish, however, the most significant accumulation occurs at the gill. Once at the gill, the physiological impact of Pb does not include disruption of respiratory function or acid/base balance, but is primarily ionoregulatory in nature. Contrast to metal toxicants such as aluminum or nickel, Pb did not negatively impact respiratory parameters. Additionally, acid/base status remained stable in both control and experimental groups. Though Pb exposure did not alter respiratory or acid/base balance, ionoregulation was significantly disrupted as evidenced by declines in plasma Ca^{2+}, Na^{+}, and Cl^{-} concentrations in experimental fish compared to controls. This was consistent with the inhibition of Ca^{2+}, Na^{+}, and Cl^{-} influx observed in the

presence of waterborne Pb and suggests that the acute toxic mechanism for Pb in the rainbow trout is ionoregulatory disruption.

Mechanism of Pb uptake: interplay with calcium transport

To our knowledge, the research presented in Chapter Three was the first to provide direct evidence that Pb shares, at least in part, a similar uptake pathway as that for Ca^{2+}. Through a series of Ca^{2+} influx measurements in the presence and absence of Pb, it was shown that Pb does inhibit Ca^{2+} influx, and that this interaction appears to be mostly competitive in nature. Additionally, Pb uptake by the gills was inhibited by voltage-independent Ca^{2+} channel blockers such as lanthanum, cadmium, and zinc but not by blockers of L-type Ca^{2+} channels, verapamil and nifedipine. While Pb-gill binding appeared to be primarily by competitive interaction at apical, voltage-independent Ca^{2+} channels, other steps of calcium transport were also impacted such as transport across the baso-lateral membrane by high-affinity Ca^{2+}-ATPase. This inhibition correlates well with branchial Pb accumulation and suggests that prolonged Pb exposure results in both a competitive and non-competitive disruption of overall Ca^{2+} handling and homeostasis.

Implications for predictive modelling: suggestions for further research

The research presented in Chapters Two and Three has important implications for the use of predictive modelling approaches like the biotic ligand model. The construction of metal-gill binding models requires an understanding of the acute toxic mechanism of a waterborne metal and the key active sites of toxicity. Therefore, directions for future research on Pb toxicity in fish should encompass further characterization of Pb binding, including interaction with the mechanisms of Na^+ and Cl^- transport and homeostasis, and accumulation/effect relationships at regulatory

organs such as the gill, kidney and liver. Studies involving chronic Pb exposure are also of importance in terms of Pb-gill binding models. Again, accumulation/effect relationships at chronic, low-level Pb concentrations would be of interest, as well as consideration of possible Pb uptake via the dietary pathway. In conjunction with the present research, these areas of study would contribute greatly to our understanding of the physiological consequences of Pb exposure in the freshwater teleost fish.