THE GENOMIC AND PHYSIOLOGICAL EFFECTS OF WATERBORNE COPPER EXPOSURE IN ZEBRAFISH, *DANIO RERIO*

By

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ABSTRACT

Little is known regarding the impact excessive waterborne metals, like copper (Cu), have on tropical freshwater species. Zebrafish (*Danio rerio*) only recently became popular as a tool for environmental monitoring, due to their fully sequenced genome. Despite this, little was known regarding the ionoregulatory physiology of zebrafish. I first examined the impact softwater acclimation has on the ionoregulatory capacity of zebrafish, and identified a high degree of phenotypic plasticity associated with changes in both gene and protein expression, which highlighted the need for proper experimental design for studies involving pre-acclimation to softwater.

I then examined the acute and chronic effects of waterborne Cu exposure under the influence of both softwater conditions and the addition of protective ions (Na^+ & Ca^{2+}). On an acute timeframe, I found that Na^+ provided a greater protective effect than Ca^{2+} in reducing Cu uptake, which was mimicked during chronic exposure, although the transcriptional effects were not as clear cut. I found that although Na^+ and Ca^{2+} provided protective effects from Cu accumulation, there were still tissue specific and global changes at the transcript level, as determined by microarray analysis. Furthemore, this set of experiments identifed the metal- and stress- induced transcriptional effects due to Cu exposure, which is key to identifying gene endpoints of chronic Cu exposure.

A final experiment went further and looked at the effects of mixed metal contamination, examining chronic waterborne Cu exposure with interactive effects of elevated dietary Fe on gene expression and tissue metal accumulation. Not only did this experiment highlight that gene expression is not necessarily reflected in protein abundance/activity, but also demonstrated that high dietary Fe can significantly alter the transcriptional expression pattern of Cu transporters in the gill, liver, and gastrointestinal tract. This thesis has made significant steps in identifying viable gene endpoints of Cu toxicity.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format approved by McMaster University and with the recommendation of the supervisory committee. The thesis consists of seven chapters. Chapter one comprises an introduction and summary of the major findings and significance of the research conducted. Chapters 2-6 comprise discrete manuscripts published, accepted for publication, or in preparation for submission to peer-reviewed scientific journals. Finally, Chapter 7 summarizes the consequences of waterborne copper exposure in zebrafish and establishes directions for future research.

Chapter 1: **General Introduction and major findings.**

Chapter 2: **Gill membrane remodelling with softwater acclimation in zebrafish (*Danio rerio*).**

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Chapter 6:		**Dietary iron alters waterborne-copper induced gene expression in softwater acclimated zebrafish (*Danio rerio*).**
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Chapter 7:		**General summary and major conclusions.**
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FIGURE LEGEND

TABLE LEGEND

CHAPTER 1

GENERAL INTRODUCTION

The world's population is expected to increase by approximately 41% to 9.5 billion by the year 2050, and 80% of this growth is projected to arise from developing countries in Africa, Asia, and Latin America (Population Reference Bureau (PRB) 2008). In 2008, India and China comprised the majority of the population growth from 1950, with a combined total of 37% (PRB 2008). Associated with such rapid expansion in population size and density in urban and rural areas are increased anthropogenic sources of contaminants stemming from agricultural runoff, industrial effluents, and greatly increased mining activity. India, with a current population of 1.14 billion people, has heavily relied on natural resources to boost its economic prowess, particularly related in the mining sector (copper, tin, zinc, etc), with little respect for the environmental impact (Deshpande & Shekdar 2005). Likewise, China, having the world's largest population (1.32 billion people), has implemented a capitalist development program to ensure a foothold as one of the world's largest economies. This entails massive development of the rural industrial sector with the resulting pollution causing major detriment to air and water quality (Tilt & Pichu, 2007). All one needs to remember are the 2008 Beijing Olympics and the deteriorating air quality due to massive urban and rural industrial factories surrounding the city. However, efforts in these and other industrialized nations are underway to improve the environmental and ecological impacts of rapid industrialization in order to make these practices sustainable (Deshpande & Shekdar 2005, Tilt & Pichu, 2007). Congruent with this is a need to asses the damage associated with environmental contaminants in freshwater and marine systems. Of particular interest is the effect of metal contaminants, on both an acute and chronic time frame, on an array of aquatic species. The focus of this thesis is to examine the physiological and genomic impacts of waterborne Cu on a tropical model species, the zebrafish (*Danio rerio*), which is endemic to Southeast Asia, having originated in the Indian sub-continent (Talwar & Jhingran 1991).

Traditional Ambient Water Quality Criteria (AWQC) have been questioned for their inability to accurately predict the impact of metal pollutants in the environment. This is because the bioavailability and reactivity of metals in aquatic environments are affected by any accompanying water chemistry. Many naturally occurring anions in the water complex metal ions, changing them to metal species which are unavailable to the reactive surfaces of organisms and many naturally occurring cations compete with metal ions for binding sites on these reactive surfaces. However, the Biotic Ligand Model (BLM) is a predictive tool that does take into account both the metal species and other ions present when examining toxicological effects (Paquin et al 2002). In brief, BLM-based predictions of potential toxicity consider the water chemistry of a particular source and assume that waterborne metals bind to an organism, particularly the gill, which results in an impairment of normal biological function. This metal binding directly correlates to the

level of acute toxicity. Moreover, this method can be used for an indirect evaluation of toxicological properties of natural bodies of water. To date, BLMs to predict "acute" toxicity (i.e. mortality occurring within 48-96 h) have been developed for a number of metals (e.g. McGeer et al., 2000; Paquin et al., 2002; Niyogi & Wood 2004). One application of the BLM in particular, to predict the acute toxicity of Cu, has been implemented as the legal basis for AWQC regulations on Cu in the United States (U.S. EPA, 2007). However, this BLM predicts only acute Cu toxicity, and its outputs are then factored by arbitrary coefficients (the "acute-to- chronic ratio") to derive AWQC to protect against "chronic" Cu toxicity (i.e. deleterious effects occurring over a significant portion of the lifespan of an organism) (U.S. EPA, 2007). The current push in other jurisdictions is to extend the BLM so as to directly predict chronic toxicity. This will be essential for the full adoption of the BLM approach in Canada and the European Union where guidelines are designed to provide lifetime protection.

Although temperate countries have initiated the implementation of a freshwater BLM, acceptance of the BLM in tropical countries is dependent on data acquired from endemic tropical species, of which only a few are currently being studied, such as tropical algae (Wilde et al 2006). Zebrafish are an ideal model for assessment and implementation of the BLM due to their prevalence in tropical ecosystems that have the potential for heavy contamination, particularly in the Indian sub-continent, to which they are endemic. Furthermore, due to their known softwater tolerance (Krisnaswami & Sarin 1984, Talwar & Jhingran 1991), well characterized reproduction & life cycle (Briggs 2002), and fully sequenced and accessible genome (www.ensembl.org/Danio_rerio), they make the ideal candidate for the assessment of physiological and transcriptional endpoints of chronic metal contaminant studies.

In order to predict chronic metal toxicity, we have to first ascertain what the biological endpoints are. Traditionally, these have been relatively coarse indicators - chronic effects on mortality (that occurring beyond 48-96 h), growth, and reproduction; the few chronic BLMs which are currently available are centered around Daphnia studies and based on these metrics (DeSchamphelaere & Janssen 2004a, 2004b, 2004c) In this thesis, I have taken a more sensitive approach by examining the physiological and gene transcriptional impacts related to acute and chronic waterborne Cu exposure. Such effects may not be immediately discernable to the naked eye, but slight perturbations in transcription and translation may have far reaching effects on growth, reproduction, morbidity and mortality, and therefore ultimately on fitness. Figure 1.1 depicts the goal(s) of this thesis, and although simple in description, the implementation of these goals is extremely complex. In short, after a given insult (i.e. metal exposure) in animals that do not succumb to the exposure, there is an initial gene-level response associated with increased or decreased transcription of genes which facilitate survival in such a situation (Fig 1.1). Based on a central dogma of biology, that is the one-gene-one-product tenet, this should relate to a functional change in protein abundance, which in turn will have survival implications for the individual and further consequences for the local population as a whole, which in essence defines chronic toxicity (Fig 1.1). This gross simplification implies that based on a transcription and protein responses, we should be able to predict costs at both an individual and population level, hence impose strict limits on

contaminants to avoid population level declines. However, the complex nature of biological systems, pertaining to exogenous and endogenous signals, makes interpretation of gene and physiological endpoints difficult, as is demonstrated in Chapters 3 through 6. Here, I examine the acute and chronic effects of Cu exposure in both lone exposures and in combination with other competitive ions. This is essential in identifying not only the responses due to Cu alone, but to determine if these changes persist under the influence of well known protective ions, such as Na^+ and Ca^{2+} (Pagenkopf 1983, Playle et al 1992, 1993). By taking this approach, I have designed experiments to test whether physiological and transcriptional endpoints are viable markers to incorporate into a BLM approach.

Copper and Copper Uptake

The reason why Cu is so interesting to study is that although toxic at high levels, Cu is an essential micronutrient for all higher plant and animal life. Naturally occurring levels of Cu found in lakes and rivers range from 0.2 to 30 μg/l, whereas areas under anthropogenic influence (mining, industrial discharge, etc) have shown levels ranging from 100 to 200,000 μg/l in heavily mined areas (Hem 1989, Robins 1997). Zebrafish, a species endemic to the Indian subcontinent, have been found in waters containing Cu at a level 15 times those dictated by Indian environmental protection rules (Rawat 2003). Further interest in Cu biology stems from human research into diseases related to Cu homeostasis, such as Menkes and Wilsons diseases, in which there is either a Cu deficiency or Cu excess, respectively, primarily due to mutations in Cu transporting mechanisms (Lutsenko 2008, Bertini & Rosato 2008). Although relevant studies for mechanisms of Cu uptake in humans involve the use of model mammalian species such as rats, mice, and sheep (Li et al 1991, Theophilos 1996, Haywood 2001), zebrafish are becoming a model organism for studying small molecules (i.e ions, pharmacologically active compounds) that affect Cu homeostasis, and for gene screening to identify Cu regulators (Mendelsohn et al 2006). In this light, I took a similar approach and used zebrafish as a model for Cu uptake, homeostasis, and toxicity.

Unlike terrestrial animals, aquatic organisms, such as teleosts, have two uptake pathways for Cu: the gill and gut (Clearwater et al 2002, Grosell & Wood 2002, Kamunde et al 2002, Bury et al 2003). However, Cu uptake in fish has not yet been completely characterized; the majority of our knowledge of cellular Cu uptake and transport stems from research based on mammalian and yeast cell studies (Dancis et al 1994, Lee et al 2001, 2002, Eisses & Kaplan 2005). Here, I present a generalized model of Cu uptake in the fish gill based on known pathways from multiple species (Figure 1.2). Waterborne Cu can be taken up via the apical Cu specific transporter CTR-1. CTR-1 is a high-affinity copper transporter first identified in yeast and is used as the means of essential copper uptake (Dancis et al 1994) and further characterized as a means to mediate Cu uptake with high affinity and specificity in both human and mouse cells (Lee et al 2001, 2002, Eisses & Kaplan 2005). In yeast cell studies, this transporter has demonstrated the potential for uptake of either Cu^{2+} or Cu^{1+}, the later requiring metallo-reductases on the surface of the cell, which have been shown to enhance Cu uptake in Cu deficient environments (Hasset & Kosman 1994, Georgatsou 1997). Furthermore, Lee et al (2002) effectively demonstrated that monovalent silver blocks Cu uptake, implying that

Cu is taken up in the monovalent form, at least in humans. Dissolved in water, the predominant form for Cu is Cu^{2+}, which suggests there are reductases located on the surface of gill epithelium to reduce Cu^{2+} to Cu^{1+}, thereby enhancing Cu uptake, although this enzyme has yet been detected in fish gill. CTR-1 is ubiquitously expressed in all tissues in both mammals and other vertebrates including zebrafish. The CTR-1 gene in zebrafish was first cloned and characterized in studies focusing on normal embryonic development (Mackenzie et al 2004).

Other steps in cellular Cu transport have also been characterized. Once in the cytosol, Cu binds to several cytosolic Cu chaperones, however in fish, these mechanisms are poorly understood. In mammals, cytosolic Cu can bind to COX17, antioxidant protein 1 (ATOX1), or Ccs which is a Cu delivery chaperone for Cu/Zn-superoxide dismutase (Wong et al 2000, Hamza et al 2003, Takahashi et al 2002). In fish, little is known regarding the specific action of these proteins, and of the genes involved, only COX17 and Ccs have been cloned in teleosts (*Danio rerio*-Pubmed accession #NM_001004652 & *Salmo salar* - Pubmed accession #NM_001140314, respectively). The putative action of these molecules in fish may be inferred from mammalian and yeast studies which have shown that COX17 is a metal chaperone that transports Cu to the mitochondrial intermembrane space. There it delivers Cu to cytochrome C oxidase (COX), the terminal enzyme of the electron transport chain, where Cu is required for proper assembly (Tzagoloff & Dieckmann 1990, Glerum et al 1996, Horng et al 2004). ATOX1 transfers Cu to the trans-Golgi network, where another Cu transporter, ATP7b, often called Wilsons Cu-ATPase is localized which incorporates Cu into ceruloplasmin (Hamza et al 2001). Additionally, Ccs delivers Cu to Cu/Zn superoxide dismutase for its proper function (Wong et al 2000). Any unbound, or free cytosolic Cu, will become bound to either metallothioneins or stored in vesicular Cu pools, thereby preventing the formation of reactive oxygen species (Kagi & Schaffer 1988).

Basolateral transport of Cu is regulated by two Cu-ATPases in humans, ATP7a (coded by the Menkes gene) & ATP7b (coded by the Wilsons gene). However, in zebrafish, only one Cu-ATPase has been identified to date, which appears to be homologous to ATP7a and presumably functions in the same capacity. In humans, both Cu-ATPases function in a similar manner, although it is their tissue distribution that defines their characteristics, where ATP7a is predominant in all tissues, particularly in the intestine for Cu uptake, whereas ATP7b is only found in the liver for excretion into the bile (La Fontaine & Mercer 2007). Effectively, Cu-ATPases assist in the transfer of Cu from the Golgi network to be incorporated into Cu-dependent proteins such as ceruloplasmin, the major vehicle for Cu transport through the circulation and the enzyme lysyl oxidase, which aids in cross-linking collagen and elastin fibers. ATP7b also facilitates Cu secretion from the liver into the bile so it can be excreted when in excess. Knowing the potential transport pathway for normal Cu homeostasis allowed for the design of experiments that focused on genes (CTR-1, COX17, ATP7a) involved in transport mechanisms, evidence of which is presented in Chapters 4 & 6.

This summary has presented only the pathway for specific Cu uptake; however, Cu can out-compete other cations for their specific uptake pathways, and rapidly accumulate within tissues (Fig 1.2). The discovery of these alternate pathways arose from

experiments that involved trout exposed to Cu in softwater conditions, where there is little competition from other ions (Pagenkopf 1983, Playle et al 1992, 1993). By adding specific cations to the external medium, specific/shared uptake channels could be identified. Current theory suggests that Cu toxicity in fish occurs mainly at the gills, which initially involves Cu outcompeting Na^+ at apical Na^+ channels normally used for Na^+ uptake from the external water, such as the H^+-ATPase coupled Na^+ channel (Grosell & Wood 2002, Wood 2001). However, this does not necessarily imply Na^+ pathways are routes for toxicity, as current evidence suggests there are Na-sensitive pathways that have low affinities for Cu, such as the putative epithelial Na channel (ENaC; Bury & Wood 1999, Grosell & Wood 2002). Ultimately excessive Cu entering the cell inhibits Na^+K^+-ATPase (NKA) on the basolateral membrane, thereby disrupting normal active Na^+ uptake. It has been shown that increased waterborne Na^+ levels reduced Cu uptake, again due to competition of metal uptake with Na^+ (Lauren & McDonald 1987, Playle et al 1992, 1993, Grosell & Wood 2002). Further evidence suggests that Cu uptake is also modulated by Ca^{2+}, as elevated ambient Ca^{2+} levels inhibit Cu accumulation, although this effect is probably associated with Ca^{2+} reducing membrane permeability and stabilizing tight junctions (Hunn 1985, Playle 1993, Spry & Wiener 1991). Although epithelial calcium channels (ECaC) exist on the gill epithelium (Shahsavarani & Perry 2006), there is little evidence to suggest that Cu can traverse through this pathway, and it is more probable that Ca^{2+} blocks non-specific Cu binding sites, reducing Cu permeability (Verbost et al 1987, 1989). A final pathway for involvement in Cu uptake, although with moderate evidence, is transport via the divalent metal transporter, DMT-1. Although characterized as a Fe transporter, DMT-1 appears to function as a carrier for most divalent metal ions across the apical surface of the cell, and has recently been implicated in the dietary uptake of Cu and Zn in the rainbow trout intestine (Gunshin et al 1997, Savigni & Morgan 1998, Nadella et al 2007, Ojo et al 2008).

In summary, Cu has multiple potential pathways for absorption, and presents a daunting task to assess all means of apical Cu uptake. Nevertheless, this thesis is constructed around experiments designed to address not only the presence of these uptake pathways in softwater medium alone (Chapter 2), but also determine the functional response of these pathways under increased ambient Cu concentrations (Chapters 4 & 6).

Effects of Excessive Copper

The gills of freshwater fish are the target site for most environmental toxicants at acute levels. High levels of waterborne Cu are known to inhibit branchial Na^+K^+-ATPase (Li et al 1996). This leads to an impairment of ion uptake at the gills, inducing a solute accumulation (particularly increased Na^+) within the epithelial cells, with a resulting influx of water (Sola et al 1995). Moreover, with the loss of ionoregulatory control, there is an efflux of electrolytes from the blood across the gill epithelium, with a consequent cardiovascular collapse and death (Wilson & Taylor 1993, Nussey et al 1995). Certainly, these are biologically relevant endpoints for acute toxicity, yet these same endpoints are not useful in determining chronic metal toxicity or to set criteria for lifetime protection.

Unlike the rapidly pathological responses to acute exposure, chronic elevations in metals may result in physiological changes and altered functions in a wide range of body

systems within fish. Overall, sub-lethal aqueous concentrations of Cu tend to accumulate in the liver and kidneys of teleost fish (McGeer et al 2000). Additionally, due to the sub-lethal concentration of Cu, fish have more time to respond to this aquatic insult, and are also able to excrete excess metal through the liver and kidney (Grosell et al 1997; 1998, 2001). There is a wide variety of physiological responses that occur when fish are exposed to Cu including altered cellularity at the gills (Berntssen et al 1999), changes in ionoregulatory physiology (Lauren & McDonald 1987), and alteration in mitochondrial respiration and reactive oxygen production (Britton 1996; Pourahmad & O'Brien 2000). In general, these physiological responses can be attributed to alterations in both the general stress response and oxidative stress response systems.

The general stress response system of fish has been well characterized, and comprises two major response systems: (1) the adrenergic system that regulates circulating levels of adrenaline and noradrenaline, which can increase ventilation, mobilize substrates for aerobic metabolism, and increase cardiac output (Handy 2003); (2) the hypothalamic-pituitary-interrenal axis (HPI-axis) that regulates the release of cortisol, which can stimulate ion uptake at the gills (Shrimpton et al 1995). The resulting cortisol increase is modulated by Cu at various levels of the HPI-axis, in which Cu can interrupt serotonergic tone in the hypothalamus (Saucier & Astic 1995), upregulate dehydrogenases (5,3 β-hydroxysteroid dehydrogenase) involved in the synthesis of cortisol from cortisol precursors (Solaiman et al 2001), and possibly result in direct stimulation of interrenal cells to release cortisol due to the kidney being a site of Cu accumulation (Handy et al 1999, McGeer et al 2000). It is plausible that many of the physiological responses to Cu may be due in part to the elevated circulating cortisol levels which can alter enzyme activity, protein synthesis , and effect transcriptional expression via glucocorticoid response elements (GREs; Mancera & McCormick 1998; Solaiman et al 2001; Sumpter 1997; Smith et al 2001). Many of theses physiological changes are beneficial in protecting an organism against an oncoming threat, although chronic stress is known to be deleterious (Pottinger et al 1992). The activation of the HPI-axis and adrenergic system are referred to as a general stress response, since their actions can be elicited by numerous stressful factors such as overcrowding, changes in salinity, or pollution (McDonald & Milligan 1997). Cu is known to stimulate both of these stress response systems in fish, which may explain the common physiological changes involved in chronic metal exposure (Elling et al 1999; Pelgrom at al 1995). However, another Cu-induced stress response may be the result of increased reactive oxygen species.

Reactive oxygen species (ROS) and oxidative stress are associated with a wide range of age-related pathologies. More importantly, in mammals, increased ROS production is directly related to chronic Cu exposure (Britton 1996). Precise mechanisms of mitochondrial ROS production *in vivo* are unclear, yet it is presumed that mitochondria are predisposed to ROS production when they are respiring, yet not producing ATP (state IV respiration; Brand et al 2004; Green et al. 2004). ROS comprise a group of oxygen-derived molecules, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and peroxynitrite ($ONOO^-$) (James et al. 2005). Cu is known to readily catalyze reactions that produce hydroxyl radicals through the Fenton and Haber-Weiss reactions (Halliwell & Gutteridge 1990):

rxn1: $Cu^{+} + H_2O_2 \rightarrow HO^{\cdot} + HO^{-} + Cu^{2+}$

rxn 2: $O_2^{\cdot -} + Cu^{+2} \rightarrow Cu^{+} + O_2$

Additionally, Cu can also increase ROS by decreasing the cellular pools of glutathione, a substrate of several enzymes that removes ROS (Mattie & Freedman 2004). The ROS produced are responsible for lipid peroxidation, oxidation of proteins, and DNA or RNA cleavage (Halliwell & Gutterridge 1990; Imlay & Linn 1988). However, the cytotoxic effects of ROS are limited by the actions of ROS scavenger enzymes, such as superoxide dismutase, which converts superoxides to hydrogen peroxide, and catalase that in turn converts hydrogen peroxide to water and oxygen. Therefore, oxidative stress can be measured directly by the production of ROS (measured in cell cultures), or more easily measured from tissue samples by the presence of lipid peroxidation and oxidation of protein by-products (malondialdehyde and protein carbonyls, respectively), or by the presence of compensatory increases in reactive oxygen scavenger enzymes. Although it is well established that Cu, and other metal contaminants such as iron, can induce oxidative damage, little attention has been directed towards establishing this as an important physiological endpoint associated with chronic waterborne Cu exposure, and this thesis examines the potential to incorporate oxidative response into a BLM approach.

Associated with changes in the general and oxidative stress response pathways is an increase in gene expression of a variety of responsive genes that help maintain metal homeostasis and protect cellular components from oxidative and metal reactive damage (Zhu & Thiele 1996). Such responsive genes include metallothionein (metal storage protein), heat shock proteins (protein chaperones), and ROS scavenger enzymes, such as superoxide dismutase (Roesijadi 1992, Kiang & Tsokos 1998, Scandalios 2005). Gene expression is predominantly regulated by receptors or transcription factors that bind to response elements in the promoter region of specific genes, and either repress or induce gene expression. However any changes in gene expression may not necessarily be reflected in changes in protein abundance or activity. As cortisol increases due to a general stress response, it acts on target cells by binding to glucocorticoid receptors found within the cytosol, which are then translocated into the nucleus and bind to glucocorticoid response elements (GRE), and with associated interaction with transcription factors results in an increase or repression of gene transcription. Likewise, there is a Cu induced increase in ROS production, and ROS are known to activate various kinase pathways (i.e. mitogen-activated protein kinase), which in turn phosphorylate and activate metal transcription factors which bind to metal response elements (Kyriakis & Avruch 1996, Giedroc et al 2001, Chen et al 2004, Mattie & Freedman 2004). Therefore the cellular response and resulting changes in gene expression to excessive metals are regulated by both metal- and general stress responsive pathways. Moreover, it is unclear whether Cu can directly stimulate gene expression, and transcription regulation associated with excessive Cu may be related to indirect metabolic responses associated with stress. In summary, both acute and chronic exposure to Cu induces varied responses in physiology and gene expression, and this thesis exploits these endpoints for potential use in chronic BLM modelling.

Toxicogenomics

With the rapid expansion of capabilities in molecular biology, researchers have been able to accumulate banks of data with detailed description of the genomes of multiple species, including, humans, mice, drosophila, and of particular interest to my thesis, zebrafish. This progress has expanded human knowledge in the field of genomics, which studies the structure, sequence, and function of genes to determine how they interact and influence biological and cellular pathways (Pennie et al 2001, Thomas et al 2002). By connecting conventional toxicology with the field of genomics, scientists have developed the emerging and popular field of toxicogenomics, which examines the complex interactions of the genome associated with adverse biological and cellular effects stemming from environmental stressors, such pharmaceuticals, organic pollutants, and metals. Combing results from the other –omic fields of proteomics and metabolomics, we can fill in the gaps left by genomics to capture a picture of gene-environment interactions and the resulting disease and dysfunction. What has accelerated the field of toxicogenomics is the advent of DNA/oligonucleotide microarray platforms, which can rapidly asses the transcription expression of thousands of gene in a particular tissue/cell type under a given exposure regime. Results from microarray based experiments are analysed and interpreted by bioinformatic techniques which can rapidly relay information about particular genes, pathways, or predicted diseases, using knowledge stored in databases (Nuwaysir et al 1999).

Essentially, there are two approaches associated with toxicogenomics: investigative/mechanistic or predictive. An investigative approach focuses more on the biological and biochemical response to a particular toxin or threat, whereas a predictive approach identifies substances to be potentially toxic (Cunningham & Lehman-McKeeman 2005). In this thesis, I have taken a mechanistic approach to Cu exposure in zebrafish. In either case, the value of a toxicogenomic approach stems from the expressional profile associated with a given contaminant exposure, which can then be identified as a fingerprint to said contaminant. However, the complex and interactive nature of multiple contaminants ensures difficultly in interpretation of data, and therefore reduces specific fingerprints into more generalized categories of chronic toxicity. By using this approach, I hope to gain insight into the specific effects of Cu, and determine if endpoints associated with lone Cu exposure are persistent during supplementation of competitive ions. The information generated can be used to develop a chronic BLM based upon physiological and transcriptional endpoints of Cu exposure.

Objectives

The overall objective of this thesis is to examine the changes in gene expression of metal responsive systems of zebrafish exposed, both acutely and chronically, to sublethal, environmentally realistic Cu concentrations. These first steps will provide insight into the modifications in gene expression in relation to metal exposure. These can then be related to specific changes to biochemical pathways that will yield biological endpoints of chronic toxicity. The intent behind this project is to incorporate new toxicogenomic methods into the development of biotic ligand models for chronic metal exposure.

Copper was chosen for this experiment for several reasons. Firstly, Cu, in excess, can be extremely toxic in the aquatic environment; hence it's tight regulation by Environment Canada and the EPA in the United States. Therefore it is essential to determine biological endpoints of toxicity to maintain accurate levels of regulation. Furthermore, since Cu is an essential micronutrient, we can not only gather insight into the mechanisms of adverse toxicity, but also understand biological responses involved in normal physiological function. The chronic toxic effects of Cu were tested using the zebrafish model, a tropical species that is tolerant of softwater, and more importantly, a model organism with an available and comprehensive genomic database. Thus, the specific objectives of this thesis are to:

1) Examine the physiological and transcriptional consequences of softwater acclimation in zebrafish, and assess the viability of zebrafish as a model for metal exposure studies.

2) Examine the acute and chronic effects of Cu exposure under soft-water conditions to assess the physiological and transcriptional endpoints associated solely with various concentrations of excess waterborne Cu.

3) Examine the acute and chronic effects of Cu exposure supplemented with waterborne protective ions (Na^+ & Ca^{2+}) and assess the physiological and transcriptional endpoints in comparison with lone Cu exposure.

4) Examine the chronic effects of Cu exposure when the fish are supplemented with a dietary competitive ion (Fe), so as to determine the interactive effects of mixed metal contamination.

5) Compare and contrast all experiments in an effort to identify specific physiological and transcriptional endpoints of chronic Cu exposure, and make inferences on the reliability of results for use in the development of a tropical chronic BLM

Chapter Summary

Although zebrafish are recognized to be very softwater tolerant (Talwar & Jhingran 1991), at the onset of this thesis, little was known regarding the response of this species simply to altered water chemistry, particularly in an ion-poor environment such as soft-water. This has significant relevance considering that a majority of metal toxicological studies, including all of the following chapters, are preceded by soft-water acclimation (Pagenkopf 1983, Playle et al 1992, 1993). **Chapter 2** examines the gene expression and phenotypic responses associated with acclimation to an ion-poor medium. This was a crucial preliminary step in identifying a link between gill remodelling and ion homeostasis.

Chapter 3 was the initial step in identifying the physiological and gene expression endpoints associated with a moderate and high level of Cu exposure in soft-

water acclimated zebrafish over a period of 21 days. Here I employed a custom-designed 16.5 k oligonucleotide microarray chip in an attempt to not only identify the global response in gene expression associated with Cu exposure, but also distinguish the metal-specific and stress-specific changes. Using bioinformatics analysis, this chapter associates Cu exposure predominately with a general stress response with significant ties to cortisol-induced changes in gene expression.

Chapters 4 and 5 evaluated whether a basic tenet of the BLM approach, the protection against Cu toxicity by competing waterborne cations, could be seen using internal Cu accumulation, physiological changes, oxidative damage, and alterations in gene-expression as endpoints. Specifically, these chapters assessed the effects of elevations in either waterborne Na^+ or Ca^{2+} concentrations on an acute (**Chapter 4**) and chronic timeframe (**Chapter 5**). **Chapter 4** took a more specific approach at identifying gene expression endpoints as they related to oxidative stress and metal transport by examining only a handful of genes through quantitative PCR. **Chapter 5** examined the broad impacts related to gene expression by use of a commercially purchased microarray chip from Affymetrix®. On an acute scale, it appears as though there is a protective effect associated with increased concentrations of Ca^{2+} and Na^+ (i.e. reduced Cu load, reduced oxidative damage, altered transcriptional profile), however on a chronic scale, it appears as though only Na^+ is effective at reducing Cu load within given tissues, as other markers did not fluctuate (i.e. oxidative damage, transcription profile). These studies recognize the importance of examining multiple water chemistries to elucidate the transcriptional and physiological effects of Cu in zebrafish.

The final research **Chapter (6)** examines the transcriptional profile of Cu transporters in soft-water acclimated zebrafish chronically exposed to waterborne Cu and supplemented with a high Fe diet. The goal was to determine the interactive effects of mixed metal contamination. This study shows that high dietary Fe can significantly alter the gene expression pattern of Cu transporters at the level of the gill, liver, and gastrointestinal tract. The study also provides evidence that transcriptional responses do not directly reflect changes in protein activity, and highlights the importance of functional studies in future research due to this disconnect between transcript and protein. This thesis concludes with **Chapter 7**, which is a general discussion of significant findings, perspectives, and potential routes of future research. Furthermore, there is an examination of potential endpoints and their viability to be incorporated into a tropical chronic BLM for Cu.

Fig 1.1
Graphic demonstrating the underlying approach of this thesis in determining the gene endpoints of chronic Cu toxicity in zebrafish. Simply stated, upon exposure to a given insult, there is a transcriptional response, which perpetuates a cascade of protein/peptide responses, which in turn has consequences at the level of the individual and population as a whole.

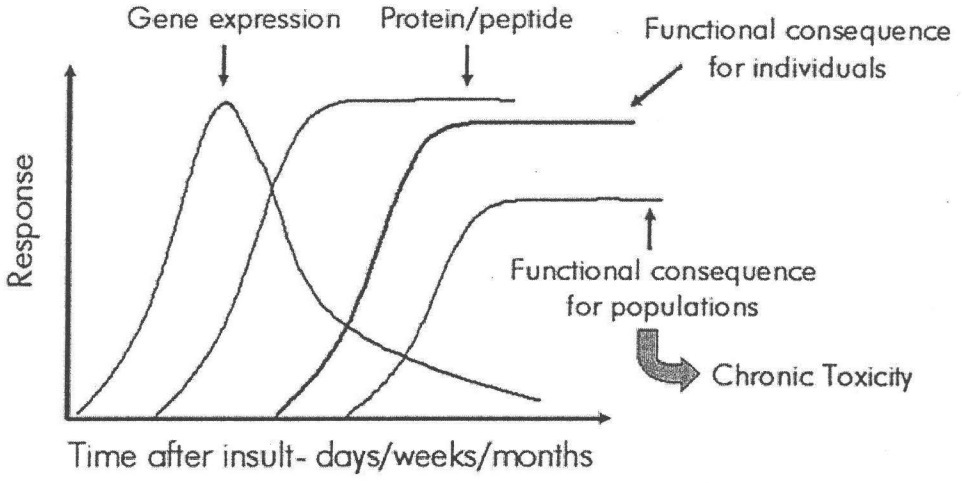
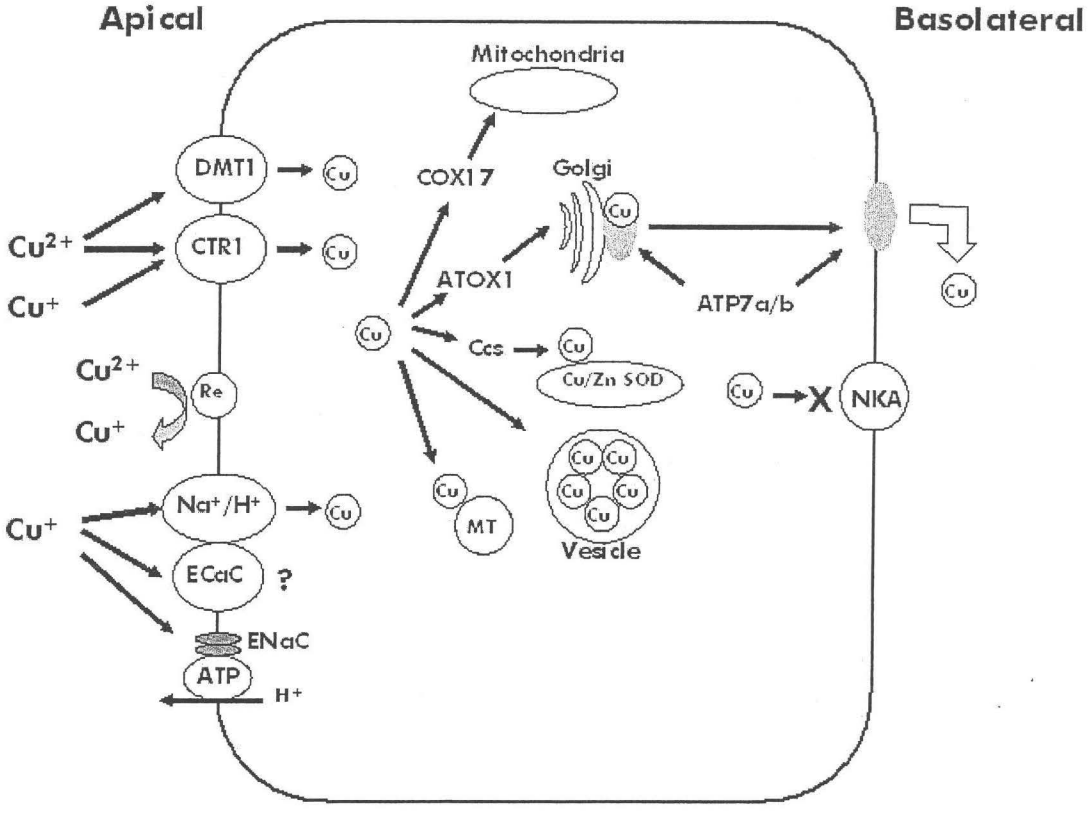

Gene expression
Protein/peptide
Functional consequence for individuals
Response
Functional consequence for populations
Chronic Toxicity
Time after insult- days/weeks/months

Fig 1.2
Generalized cellular model of Cu uptake, storage, and transport pathways. Cupric ions can be taken up via divalent metal transporter 1(DMT-1) and copper transporter 1(CTR-1), whereas cuprous ions reduced by metallo-reductases (Re) can be taken up by CTR-1, Na^+/H^+-Exchanger (Na^+/H^+), or the putative epithelial Na channel (ENaC), facilitated by H^+-ATPase. Potentially Cu ions can traverse through epithelial calcium channels (ECaC), however, there is currently no evidence to support this. Cytosolic Cu is bound by 3 probable Cu chaperones: COX17, which shuttles Cu to the mitochondria for assembly into COX; Ccs, which chaperones Cu to Cu/Zn superoxide dismutase; and ATOX1 which shuttles Cu to the trans-Golgi network to ATP7a or ATP7b, depending on the tissue type. Free cytosolic Cu is bound either by metallothioneins (MT) or stored in vesicular pools. ATP7a/b transports Cu to either Cu- dependent enzymes/proteins, or facilitates basolateral excretion. There is the potential for excessive cytosolic Cu to bind and inhibit Na^+K^+-ATPase (NKA) on the basolateral membrane, disrupting Na homeostasis.


Apical
Basolateral
Mitochondria
DMT1
CTR1
Cu2+
Cu+
Cu2+
Cu+
Re
Cu+
Na+/H+
ECaC
?
ENaC
ATP
H+
COX17
Golgi
ATOX1
ATP7a/b
Ccs
Cu/Zn SOD
MT
Vesicle
NKA
Cu

CHAPTER 2

GILL MEMBRANE REMODELLING WITH SOFTWATER ACCLIMATION IN ZEBRAFISH (*DANIO RERIO*).

Abstract

Little is known regarding the ionoregulatory abilities of zebrafish exposed to softwater despite the popularity of this model organism for physiology and aquatic toxicology. We examined genomic and nongenomic changes to gills of zebrafish as they were progressively acclimated from moderately hard freshwater to typical softwater over 7 days and held in softwater for another 7 days. Gills were sampled daily and mRNA expression levels of gill Na^+/K^+ATPase (NKA) α1a subunit, epithelium calcium channel (ECaC), carbonic anhydrase-1 & 2 (CA-1; CA-2), Na^+, H^+-exchanger (NHE-2), V-type proton (H^+)-ATPase and copper transport protein (CTR-1) were quantified by real-time PCR. Changes in enzyme activities of gill NKA were determined and protein levels of NKA and ECaC were quantified by Western blotting. Levels of mRNA for ECaC increased 4-fold after day 6, with an associated increase in ECaC protein levels after 1 week in softwater. CA-1 and CA-2 exhibited a 1.5- and 6-fold increase in gene expression on day 6 and 5, respectively. Likewise, there was a 5 fold increase in NHE-2 expression after day 6. Suprisingly, CTR-1 mRNA showed a large transient increase (over 3-fold) on day 6 while H^+-ATPase mRNA did not change. These data demonstrate a high degree of phenotypic plasticity in zebrafish gills exposed to an ion-poor environment. This not only enhances our understanding of ionoregulatory processes in fish but also highlights the need for proper experimental design for studies involving pre-acclimation to softwater (e.g. metal toxicity).

Introduction

The zebrafish has a long history as a model organism in the study of developmental biology, but only recently has it gained acclaim for examining vertebrate physiology (e.g. Briggs, 2002). Despite its many advantages as a model organism, little is known regarding the physiology or ability of zebrafish to tolerate environmental stress. Although growing in popularity for both physiological (e.g. Barrioneuvo & Burggren 1999) and especially aquatic toxicology studies (Neumann & Galvez 2002), few studies have examined the responses of zebrafish to different water chemistries. This has significant relevance to toxicology studies in particular where softwater acclimation usually precedes toxic exposures.

Teleosts living in freshwater must constantly battle the loss of ions from the body to the surrounding environment. Many studies have examined changes in the mechanisms of gill transport that deal with salinity changes accompanying sea-water to fresh-water transfer, including recent studies examining gene expression (e.g. Richards et al., 2003; Scott et al., 2005). However, ion loss is further exacerbated in a softwater situation where ion concentrations for Na^+ and Ca^{2+} can be 10 times less than measured in typical freshwater (Na^+ & Ca^{2+} ~ 1mM, Mg^{2+} 0.4 mM), necessitating alterations in osmoregulatory physiology to maintain ion homeostasis in tolerant species. To maintain a high internal concentration of salts in an ion-deficient medium, teleosts must employ enzymes, such as basolateral NKA, apical Na^+, H^+-exchanger, and a (V-type) H^+-ATPase to enhance Na^+ uptake at the gills (McCormick, 1995; Lin & Randall, 1991, 1993; Lin et al., 1994; Scott et al., 2005). Additionally, Ca^{2+} uptake occurs through epithelial calcium channels (ECaC) in the apical membrane, and through Ca^{2+}-ATPases or Na^+/Ca^{2+} exchangers in the basolateral membrane (Flik et al., 1984; Verbost et al., 1994). Anions like Cl^- appear to be taken up at the gill by an apical Cl^-/HCO_3^- exchanger assisted by cytoplasmic carbonic anhydrase catalyzing the conversion of CO_2 to HCO_3^- and a proton (Wilson et al., 2000; Perry, 1997; Perry et al., 2003a).

Zebrafish in the wild are found throughout the Indian subcontinent where they face a wide variety of environmental water chemistries, and are known to be a softwater-tolerant species (Krisnaswami & Sarin, 1984; Talwar & Jhingran, 1991). Their tolerance to ion poor water, comprehensive genomic database, amenability to large scale screening and reverse genetics makes the zebrafish an ideal model to investigate gill remodeling and ionregulatory physiology. These same reasons have also made them an increasingly popular model for aquatic toxicology. Surprisingly, very little is known regarding zebrafish osmoregulatory physiology. To date, only one other study has examined the effects of softwater acclimation on zebrafish gill physiology and none have looked at responses at the genomic level (Boisen et al., 2003). Zebrafish are able to maintain their plasma and whole body ion composition in the face of exposure to extremely low ambient ion concentrations in softwater (Boisen et al., 2003). This seems to be accomplished partly through a rapid change in Na^+ and Cl^- transport affinity and the regulation of transport processes. Carbonic anhydrase activity seems to play a critical role in Cl^- uptake, as demonstrated by Boisen et al. (2003). When CA activity was inhibited, there was a complete blockade of Cl^- uptake. However, there is evidence that teleosts in freshwater employ apical epithelial Na^+ channels, and/or Na^+/H^+ exchangers, in

association with cytoplasmic carbonic anhydrase for Na^+ uptake (Boisen et al., 2003; Perry et al., 2003a, 2003b; Scott et al., 2005). In addition, studies on rainbow trout demonstrate that Ca^{2+} transport capacity is increased under conditions of low ambient Ca^{2+} concentrations (Perry & Wood, 1985). This suggests that softwater acclimation is accompanied by gill remodeling at the level of membrane transporters and enzymes. However, to date there are limited data on the inducibility of gene expression for these components of zebrafish gill. It has been shown that salinity transfer (freshwater → seawater/ seawater → freshwater) induces changes in gene expression for gill ion transporters in other fish species (Richards et al., 2003; Scott et al., 2005, respectively). This suggests that transcriptional regulation of gill ion transporters may occur with softwater acclimation in zebrafish. Although the change in 'salinity' is relatively small compared to seawater/freshwater acclimation, softwater acclimation represents an environmentally relevant stressor for freshwater fish.

The main goals of this study were to determine during a progressive 7 day softwater acclimation: 1) the temporal pattern of gene expression for key ion channels, transporters, and enzymes in zebrafish gills, 2) whole body changes of ion levels and 3) the effect of acclimation on changes in protein expression and enzyme activities for selected membrane components. These data will help identify the molecular changes involved in gill remodeling and ion homeostasis in zebrafish. They will also provide valuable information for the design of experiments where softwater acclimation precedes treatment. Aquatic toxicology studies on waterborne metal exposures use a pre-acclimation to softwater to eliminate the confounding effects of protective ions (e.g. Kjoss et al., 2005; Morgan et al., 2004). Any changes in gill phenotype that result from this pre-acclimation must be accounted for when interpreting metal-induced gene expression changes.

Materials and Methods

Animals

Zebrafish (*Danio rerio*) were purchased from a local pet supply store (PetsMart, Canada) and housed in two 40L-aquaria in dechlorinated Hamilton tap water, which is considered moderately hard (Na^+ 927 ± 16 μM, Ca^{2+} 946 ± 11 μM, Mg^{2+} 422 ± 17 μM, Cu^{2+} 2.1 ± 0.7 μg/L, pH 8.3 pH 8.3), maintained at 28°C (hardwater). The fish were allowed 1 week to acclimate to the new tanks before experimentation. Tanks were aerated, and filtered with Aqua Clear 150 aquarium filters (Hagen, Montreal, Canada). Fish were fed daily with a commercial tropical fish food (Topfin, Phoenix, AZ) and maintained on a 12-h light, 12-h dark photoperiod regime. All procedures used were approved by the McMaster University Animal Research Ethics Board, and conform to the principles of the Canadian Council for Animal Care.

Experimental Design

Over a period of 7 days, hardwater was progressively removed and replaced with ion-poor reverse osmosis water (approximately 15-20% daily, over a period of 15 minutes), until ion levels in the water were reduced to Na^+ 115 ± 3 μM, Ca^{2+} 51 ± 1 μM, Mg^{2+} 26 ± 1 μM, Cu^{2+} 1.8 ± 0.5 μg/L; pH 6.8. At 10:00am daily, a sample of zebrafish

were removed from both tanks, terminally anesthetized in buffered tricaine methanesulfonate (MS-222, Sigma, St Louis, MO) and weighed. A control group was kept in hardwater throughout and sampled simultaneously. The gills were removed and either immediately frozen in liquid N_2 for mRNA extraction and western blotting, or immersed in 100 µL ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) for analysis of Na^+K^+ATPase (NKA) activity. Whole zebrafish were also weighed and frozen in liquid N_2 for analysis of body ion concentrations. Additionally, water samples were taken daily for analysis of pH and ion composition.

Water and Whole Body Ion Composition

Whole zebrafish were first digested in 5 volumes of 1N HNO_3 for 48 h at 60°C. Both whole body and water ion composition were measured by flame atomic absorption (Spectra AA 220FS, Varian, Palo Alto, CA) after 1:10 dilutions were made with 1% HNO_3 (Na^+) or 0.5% $LaCl_3$/1% HNO_3 (Ca^{2+}, Mg^{2+}), and verified using certified Na^+, Ca^{2+}, Mg^{2+} standards (1 mg/L diluted in 1% HNO_3 or 0.5% $LaCl_3$/1% HNO_3; Fisher Scientific).

Na^+/K^+-ATPase activity

Gill NKA activity was determined using the microassay method of McCormick (1993). Both NKA activity and Bradford protein assays (Bio-Rad, Hercules, CA) were run in 96-well format on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA).

Gill carbonic anhydrase activity

CA activity in the gill was measured using the electrometric pH method (Henry, 1991). Whole gill arches were homogenized in reaction buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris, pH 7.4), briefly centrifuged, and the supernatant was diluted 25-fold and assayed for CA activity using 6 ml of reaction buffer at 4°C, and 200 µl of CO_2-saturated distilled water to initiate the reaction. The reaction velocity was measured over a 0.15 unit pH change and the final result was a measure of the observed rate minus the uncatalyzed rate. The pH of the reaction medium was measured by a GK2401C pH electrode (Radiometer, London, ON) connected to a PHM84 pH meter (Radiometer), and recorded by data acquisition software (Biopac with AcqKnowledge v 3.7.3). The protein content of the supernatant was measured using the BCA protein assay.

Quantification of mRNA by real-time RT-PCR

Total RNA from the gill tissue was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by UV spectrophotometry at 260 nm. First strand cDNA was synthesized from 1 µg of total RNA treated with DNase I (Invitrogen, Calsbad, CA) and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase (Invitrogen, Calsbad, CA). mRNA expression was quantified in duplicate on a Stratatgene MX3000P real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each

reaction contained 12.5 µl SYBR green mix, 1 µL of each forward and reverse primer (0.5 uM), 5.5 µL RNase/DNase free H_2O, and 5 µL cDNA template. Cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec. This was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock gill cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the house-keeping gene EF1α, which did not change over the course of the experiment. Both water and non-reverse transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer Express (Table 2.1; Applied Biosystems, Foster City, CA). Target genes of interest for this study were carbonic anhydrase-1 (plasma; CA-1), carbonic anhydrase-2 (cytoplasmic; CA-2) , copper transporter 1 (CTR-1), epithelial calcium channel (ECaC), elongation factor-1 alpha (EF1a), H^+-ATPase, Na^+, H^+-exchanger 2 (NHE2), and NKA (α1a). Genes were normalized to EF1a, and each value was expressed as a percentage of the hardwater control, which allowed for the calculation of SEM.

Western Blots

Whole gill arches were homogenized in buffer (100 mM imidazole, 5mM EDTA, 200 mM sucrose, and 0.1% deoxycholate, pH 7.6), and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was collected and diluted to 30 µg of protein in 4× loading buffer (48 mM Tris-HCl pH 6.8, 4% glycerol, 3.2% SDS, 600 mM β-mercaptoethanol, 1.6% bromophenol blue). Samples were denatured in boiling water for 5 minutes, and loaded onto a 7.5% SDS-polyacrylamide gel. Samples were electrophoresed for 1h at 150V. Samples were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Mississauga, ON), and blots were incubated overnight at 4°C in 5% skim-milk + PBST (10mM phosphate buffer, 0.09% NaCl, 0.05% Tween-20, pH 7.5). Blots were washed 3× 5min in PBST and incubated at room temperature with the primary antibody diluted in 3% skim-milk + PBST. Primary antibodies included rainbow trout ECaC (1:1000); NKA (chicken; 1:500) & tubulin as a loading control (chick retina; 6G7, 1:500). Tubulin was used due to availability and good reactivity with zebrafish tissues. Membranes were washed 3 × 5min in PBST, and incubated for 1 h at room temperature with an HRP-conjugated anti-rabbit IgG (for ECaC, 1:25,000; PerkinElmer, Boston MA) or anti-mouse IgG (for NKA & tubulin, 1:50,000). After 3x5min washes with PBST, proteins were visualized with a Western Lightning chemiluminescence kit, following the manufacturer's protocol (PerkinElmer). Blots and band density analysis were completed on a ChemiImager (AlphaInnotech Corporation, San Leandro, CA), which used pixel density to quantify band intensity. Bands were normalized to to tubulin, and expressed as a ratio of the hardwater control.

Statistical Analysis

Statistical analysis was performed using Sigma Stat (SPSS Inc, Chicago, MI). All data have been expressed as a mean +/- SEM. A one-way ANOVA and a Dunnett's test was used to test for significance relative to the control values, and a Students t-test was used to compare between treatments and controls at any given time point ($P<0.05$).

Results

Whole body ion content

Despite the drop in ion concentration from hardwater to softwater (Fig 2.1), there were no significant differences found in whole body ions. Na, Ca, and Mg values fluctuated little from average values of 48.42±7.61, 29.47±4.43, and 20.67±1.87 mmol kg^{-1}, respectively (Fig 2.2).

NKA and CA activity

Values of gill NKA activity in hardwater-acclimated controls were 2.17±0.38 μmol ADP/mg protein/hour, and the activity increased ~2.5 fold to a maximum activity of 5.66±0.55 μmol ADP mg $protein^{-1}$ $hour^{-1}$ after 3 days of progressive water ion depletion (Fig 2.3). The NKA activity decreased after 3 days, to values not significantly different than hardwater-acclimated controls (Fig 2.3).

We measured gill CA activity on whole gill arches from fish exposed to hardwater, and fish exposed to softwater acclimation on day 7 , and after a further 1 week in softwater. Initial gill CA activity was 865±73 μmol CO_2 $mg^{-1}min^{-1}$ in hardwater, which increased slightly to 1231±211 μmol CO_2 mg^{-1} min^{-1} at day 7 and 934±151 μmol CO_2 mg^{-1} min^{-1} after 1 week in typical softwater. These changes were not significant.

Gene expression profile

Although NKA enzyme activity increased significantly during the first 3 days of softwater acclimation, there was no significant change in the mRNA expression of the gill NKA isoform α1a gene over the entire 7 day period (Fig 2.4). Similarly, there were no significant changes in H^+-ATPase mRNA expression over the duration of the experiment (Fig 2.5). We did see significant changes in the mRNA expression of the ECaC gene (Fig 2.6), which corresponded with decreasing waterborne Ca^{2+} concentrations (Fig 2.1). After 6 days of progressive ion depletion, there was a dramatic 4-fold increase in gill ECaC mRNA, which attenuated only slightly over days 7-14 and remained significantly elevated up to the end of the experiment (Fig 2.6). Likewise, we saw a significant increase in the mRNA expression of CA-1 and CA-2 within the gill after day 6 and 5, respectively (Fig 2.7a,b). It should be noted that the magnitude of the gene expression increased varied between CA-1 and CA-2, (1.5 fold versus 6 fold, respectively). Furthermore, we saw a significant increase in NHE-2 gene expression after day 6, which remained elevated after 7 days in softwater (Fig 2.8). Interestingly, there were changes in the mRNA expression of the Cu transport protein CTR-1, with rapid peak in expression after 6 days of ion depletion (Fig 2.9). This was an ion-specific effect since waterborne copper analysis indicates that copper levels did not change throughout the course of the softwater acclimation (1.93±0.07 μg L^{-1}).

Western Blot Analysis

We examined the protein expression levels of gill NKA and gill ECaC to elucidate the effect of gene expression on protein levels in the gills. There were no significant changes in gill NKA protein levels over the course of the experiment (Fig 2.10), despite the initial changes in enzyme activity (Fig 2.3). Similar to ECaC mRNA changes, which peaked early in the acclimation period (Fig 2.6), there was a progressive increase in ECaC protein expression, which increased 3-fold at day 14 (Fig 2.11).

Discussion

This study demonstrates that zebrafish gills display a high degree of phenotypic plasticity to environmental challenge. Zebrafish respond to softwater acclimation with the induction of gene expression and protein expression for key ion channels (such as ECaC). They also invoke the upregulation of NKA activity possibly through covalent modulation or translocation of the existing protein. These represent potential mechanisms which allow zebrafish to maintain ion homeostasis in an extremely low ion environment.

The osmoregulatory response of zebrafish to a step-wise acclimation to softwater over a period of 7 days was assessed by examining both changes in known ion responsive enzymes (NKA, H^+-ATPase, CA) and ion transport proteins, ECaC and NKA. This is an important factor to consider especially in the emerging field of toxicogenomics since there is the need to initially test metals in softwater where effects are not confounded by competing cations and complexing anions (Bury & Wood, 1999; Grosell & Wood, 2002; Morgan et al., 2004). Although this represents a limited number of total gill genes and proteins that may respond, they highlight the flexibility of this organ to environmental changes. Future studies can expand on the short list of genes examined here with the use of commercially produced microarrays. Other environmental stressors, such as heat or waterborne-metal stresses, are known to induce a number of genes and proteins in both the goby (*Gillichthys mirabilis*) and rainbow trout (*Oncorhynchus mykiss*), including genes involved in chloride and calcium transport, and NHE proteins (Buckley et al. 2006; Hogstrand et al. 2002, respectively). We demonstrate here that care is needed in the design of toxicity experiments since softwater acclimation alone can induce significant changes in gene and protein expression. Indeed changes in environmental ion concentrations resulted in changes in mRNA for an important metal transport protein (CTR-1; Fig2.8).

This is the first study to examine gill NKA enzyme activity in zebrafish and the changes in expression during softwater acclimation. Enzyme activities initially rise upon softwater acclimation, and are elevated after 1 day of progressive softwater acclimation (Fig 2.3). We found there were no significant changes in the gene expression of the NKA isoform of the catalytic subunit α1a (Fig 2.4), shown to be upregulated during sea water to freshwater acclimation in other species (Richards et al., 2003). However, this is not surprising, since the magnitude in the change of salinity is only from 0.1 ppm to 0.01 ppm. We also saw no change in total protein expression (Fig 2.9) using an antibody which recognizes all α subunits isoforms. These data suggest that NKA activity is not regulated by transcriptional means, but rather is modified at the protein level in response

to softwater acclimation (Fig 2.9). It also suggests that unlike the situation seen in trout with respect to changes in salinity (Richards et al., 2003), zebrafish do not switch α1a and b subunit isoforms of NKA to alter enzyme kinetics with changes in water hardness. Various hormones modulate NKA activity, and short-term regulation can have direct effects on the kinetics of enzyme activity or the translocation of NKA pumps between the plasma membrane and the intracellular stores (see review by Therien & Blostein, 2000). This rapid response in NKA may explain how zebrafish are able to maintain ion balance even in the first days of acclimation before any changes in gene expression have occurred. In addition, the V-type H^+ATPase may play a role in Na^+ uptake by modifying the electrochemical gradient of the apical membrane by extruding H^+ ions, which favors the absorption of Na^+ (Lin & Randall, 1991, 1993; Lin et al., 1994). We initially hypothesized there would be a change in the expression of the H^+-ATPase mRNA upon softwater acclimation. However, there were no significant changes in expression (Fig 2.5). Moreover, Boisen et al. (2003) did not see any changes in H^+-ATPase protein abundance after softwater exposure in zebrafish. This does not necessarily downplay the importance of H^+-ATPase in maintaining ion homeostasis in zebrafish exposed to softwater, since H^+-ATPase kinetics can increase without changes in protein abundance or location (Gluck et al., 1992). Conversely, we did see a 5-fold increase in NHE-2 gene expression after day 6 (Fig 2.8). NHE proteins have been characterized in the gills of many freshwater species of fish (Wilson et al., 2000; Edwards et al., 2002), and their potential role in Na^+ uptake in the gill has been proposed (Scott et al., 2005). Certainly, NHE-2 may play an essential role in the uptake of Na^+ from the water into the gill cell. However, NHE-2 has yet to be localized in the gill of the zebrafish.

During softwater acclimation, we saw a significant elevation in the gene expression of CA-1 and CA-2 in the gills of zebrafish after days 6 and 5, respectively (Fig 2.7a, b). However, CA-2 had a greater magnitude of gene expression than CA-1. When the CA isozymes were compared to the red blood cell (RBC) (accession # AY307082) and cytoplasmic (accession # AY514870) isozymes of trout CA, we found that CA-1 shared a 78% homology with the RBC isozyme, and CA-2 shared a 68% homology with the cytoplasmic isozyme. Due to the small size of the gill samples, and the inability to flush out all of the contaminating blood, it was necessary to measure both CA isozyme expression levels to determine if there were any contaminating effects of blood, and to determine if an ion-poor medium contributed to increased cytoplasmic CA expression. We did see a significant 6-fold increase in CA-2 after day 6, indicating that cytoplasmic CA may aid in ion-uptake. Despite this increase in gene expression, we did not see a significant elevation in CA enzyme activity on these days, although there was a slight but non-significant elevation on day 7 of acclimation. Although this represents the combined activity of both CA isoforms, the cytoplasmic activity is expected to be threefold higher per unit gill protein (Morgan et al., 2004). Cytoplasmic CA can provide bicarbonate for the Cl^-/HCO_3^- exchanger on the apical surface of the gill and aid in Cl^- uptake (Goss & Wood, 1991). Generally there is evidence to support the role of cytoplasmic CA in ion uptake (Boisen et al., 2003; Chang & Hwang, 2004). Furthermore, CA may provide H^+ ions for an apical Na^+, H^+-exchanger (Scott et al., 2005), which would allow for the passage of Na^+ ions into the gill of the zebrafish.

Aside from diet, a major source of Ca^{2+} for teleost fish comes from the surrounding medium. Ca^{2+} from the environment is taken up by epithelial calcium channels and transported across the basolateral membrane via Ca^{2+}-ATPases or Na^{+}/Ca^{2+} exchangers (Flik et al., 1984; Verbost et al., 1994). Therefore, it is likely that changes in environmental Ca^{2+} levels will result in significant changes in ECaC to maintain adequate Ca^{2+} uptake. After day 6 (80% softwater), there was 3.5 fold increase in the gene expression of ECaC, which remained elevated for the remainder of the experiment (Fig 2.6). This coincided with a corresponding increase in the protein level, with a 3-fold increase after a 1-week acclimation in softwater (Fig 2.11; Ca^{2+} 51 ± 1.0 μM) demonstrating that ECaC is primarily transcriptionally regulated under these conditions. Similarly, Pan et al. (2005) demonstrated an increase in the expression of gill and skin ECaC in zebrafish embryos after exposure to 20 μM Ca^{2+}, and localized the ECaC to the mitochondrial rich cells. In addition, Shahsavarani & Perry (2006) showed significant increases in ECaC gene expression (10-fold) and protein expression (2.5-fold) after a 5-day exposure of rainbow trout to low Ca^{2+} (20-30μM). Combined, these results indicate that ECaC plays a key role in Ca^{2+} uptake from the surrounding medium, although how the cells sense Ca^{2+} concentrations is not fully understood. Potentially, the presence of a Ca^{2+} receptor might mediate changes in ECaC expression (Radman et al., 2002). There is a need to further examine changes in plasma ion levels and key ionoregulatory hormones that may affect changes in gill epithelial structure and ionoregulatory capacity during softwater acclimation.

The primary goal of this study was to examine the effects of softwater acclimation on the ionoregulatory capabilities of a tropical, softwater-tolerant fish. In addition zebrafish are becoming an important model in vertebrate physiology and aquatic toxicology, due to their publicly available genome and their ability to tolerate softwater environments. To this end, we examined a recently identified copper transport protein (CTR-1) found in the gill epithelium of zebrafish (Mackenzie et al., 2004). Since copper is an essential micronutrient obtained from the environment, we predicted that soft-water acclimation might affect CTR-1 expression. The mRNA expression for this transporter spiked (3-fold increase) after 6 days of softwater acclimation but declined to baseline one day later (Fig 2.9) despite constant environmental copper content. This result suggests that the timing of softwater acclimation might be crucial in studies which require a pre-acclimation period (e.g. metal toxicology). Despite the transient change in CTR-1 expression, zebrafish in softwater may have an increased sensitivity to copper, or to other metals, since many metals have shared pathways with Na^{+} and Ca^{2+} transport across the apical surface of the gill (Bury & Wood, 1999; Grosell & Wood, 2002; Morgan et al., 2004).

The results of this study indicate a high degree of phenotypic plasticity in zebrafish gills during softwater acclimation to maintain ion homeostasis. Zebrafish gills respond to softwater acclimation through genomic (ECaC, CA-2) and nongenomic changes (NKA) in ionoregulatory machinery. These represent potential mechanisms which allow zebrafish to tightly regulate whole-body ion levels, although further work is required to establish causations between these changes and ion transport needs to be established. The ability to apply molecular techniques such as reverse genetics to the

zebrafish model will allow for direct connections to be made between gill remodelling and ion homeostasis.

Table 2.1: Forward (F) and reverse (R) primers used for real-time PCR.

Gene	Accession #	Primer	Amplicon
CA-1	NM131110	F - ccttgctgtggttggagttt R - acactgatcggctccttcaa	211
CA-2	NM199215	F - tggacatacctgggctctct R - agtggctgaggaggacgata	179
ECaC	AY383562	F - acttggtcaaccgcagaaag R - cagattccacttgagcgtga	197
EF1α	NM131263	F - gtgctgtgctgattgttgct R - tgtatgcgctgacttccttg	201
H^+-ATPase	NM182878	F- gtatgtagacagacagctgc R- atcagcgtgatctttgcgg	120
NKA α1a	AF286372	F - gccccaaaaagagaggaaac R - tgcccttggactttgacttc	201
NHE-2	XM686272	F - ccactcgacccttctttgaa R - gcagatggcaaatagggaga	104
CTR-1	AY077715	F - cgtgttcttgttggctgtgt R - ccaccacttggatgatgtga	209

Fig 2.1

Measured water pH and concentrations of Na^+, Ca^{2+}, and Mg^{2+} (mean ± SEM) during acclimation of zebrafish from hardwater to softwater, over a period of 7 days, with a further acclimation for 7 days (n=2).

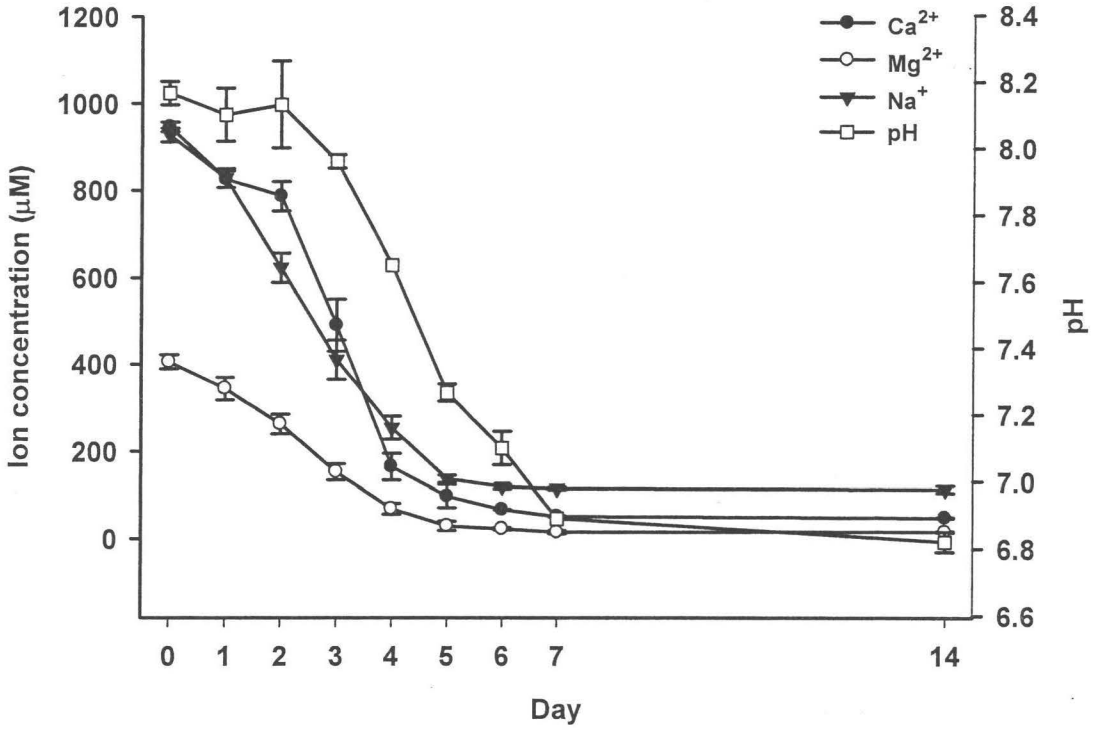

Ion concentration (μM)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 14
Day
Ca2+
Mg2+
Na+
pH
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
pH

Fig 2.2

Whole body concentrations of Na^+, Ca^{2+}, and Mg^{2+} (mean ± SEM) measured from zebrafish exposed to a progressive softwater transfer over 7 days, and acclimation in softwater for an additional 7 days. (n=6).

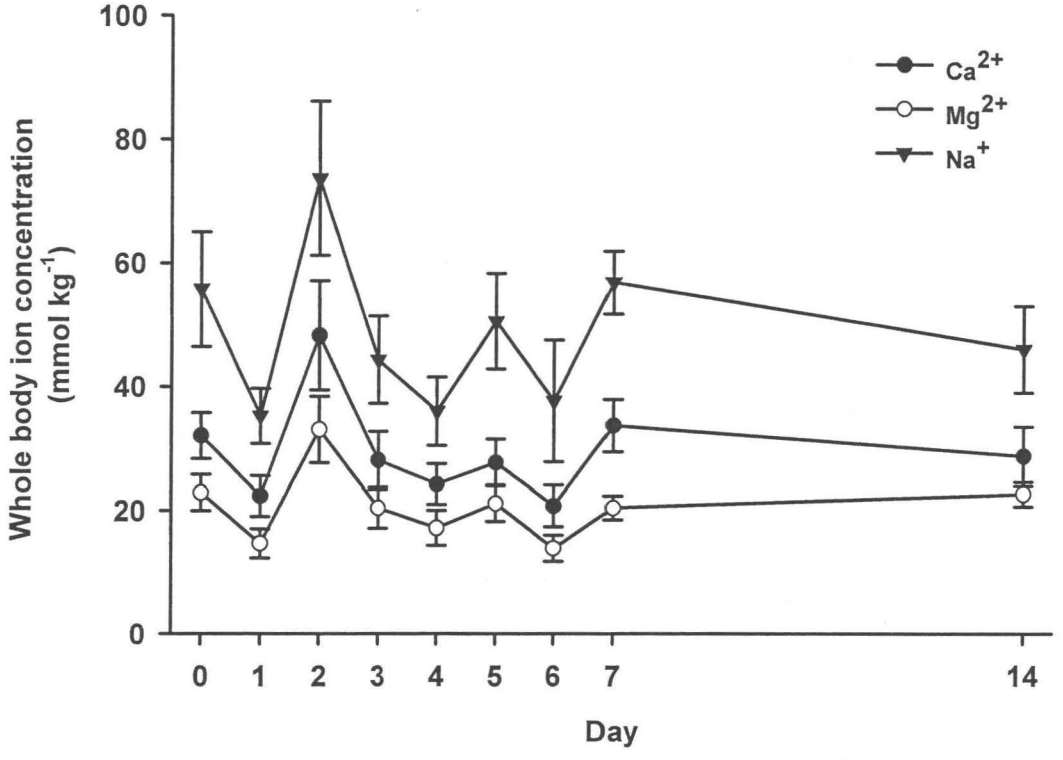

Whole body ion concentration (mmol kg-1)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
14
Day
Ca2+
Mg2+
Na+

Fig 2.3

Changes in gill Na^+K^+ATPase activity in zebrafish during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. * Indicates significant difference from the day 0 control value as determined by one-way ANOVA and by Dunnett's test. δ Indicates a significant difference at a given time between hardwater and softwater treatments as determined by Student's t-test (n=6-8, $P<0.05$).

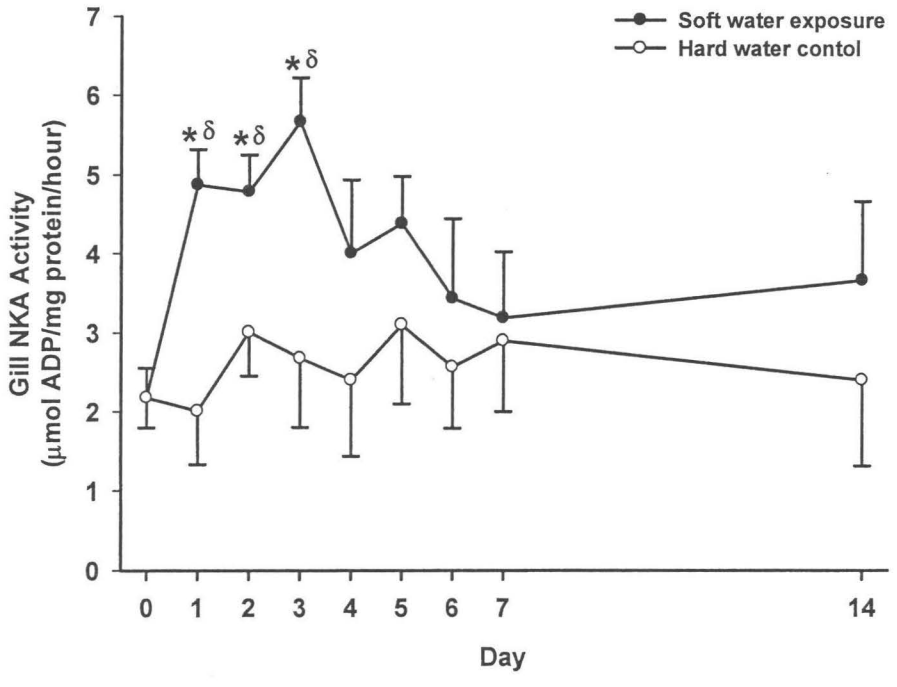

Soft water exposure
Hard water contol
Gill NKA Activity
(µmol ADP/mg protein/hour)
*δ
*δ
*δ
0
1
2
3
4
5
6
7
14
Day

Fig 2.4

mRNA expression of the Na^+K^+ATPase alpha isoform 1 (α1a) gene in the zebrafish gill during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Values are represented as means ± SEM, and based as a percentage of the control (100%). There were no significant differences between controls or treatments. (n=5-6, $P<0.05$).

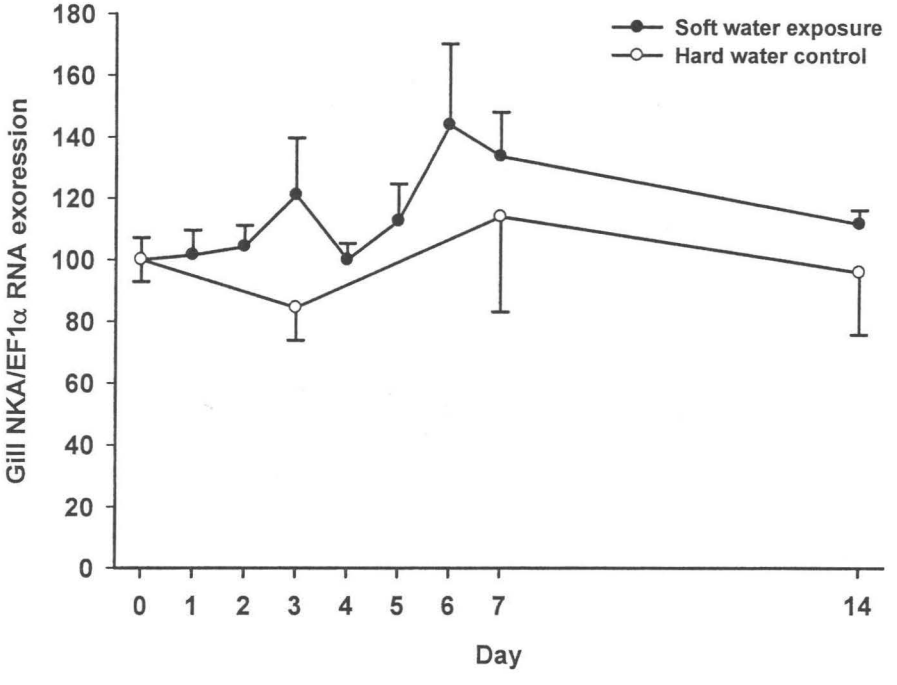

Soft water exposure
Hard water control
Gill NKA/EF1α RNA exoression
180
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 14
Day

Fig 2.5

mRNA expression of the H^+-ATPase gene in the zebrafish gill during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Values are represented as means ± SEM, and based as a percentage of the control (100%). There were no significant differences between controls or treatments. (n=5-6, $P<0.05$).

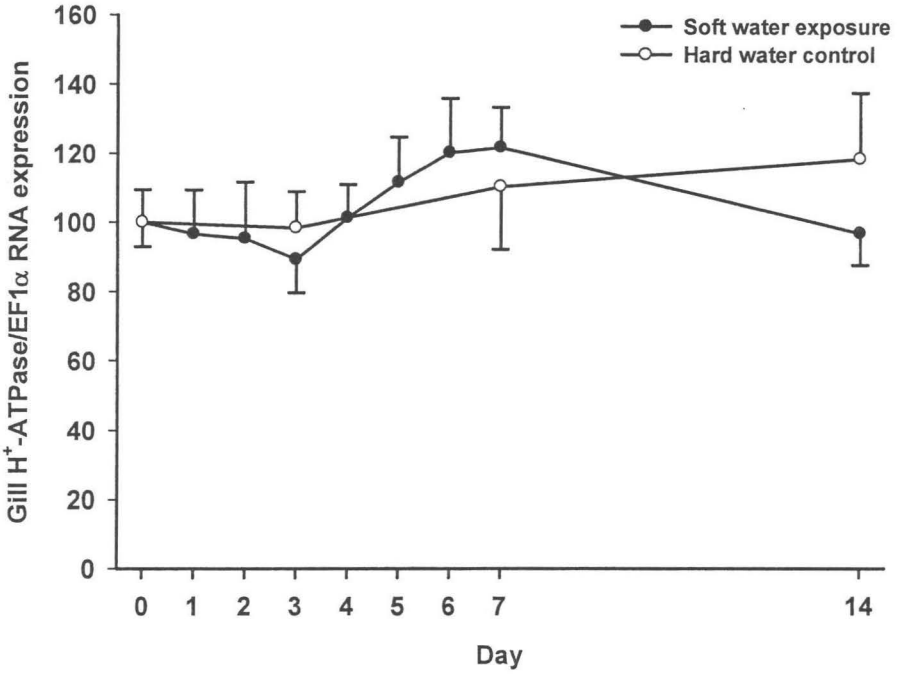

Soft water exposure
Hard water control
Gill H+-ATPase/EF1α RNA expression
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 14
Day

Fig 2.6

mRNA expression of the epithelial calcium channel (ECaC) gene in the zebrafish gill during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Values are represented as means ± SEM and based as a percentage of the control (100%). * Indicates significant difference from the day 0 control value as determined by one-way ANOVA followed by a Dunnett's test. δ Indicates a significant difference at a given time between hardwater and softwater treatments as determined by Student's t-test (n=5-6, $P<0.05$).

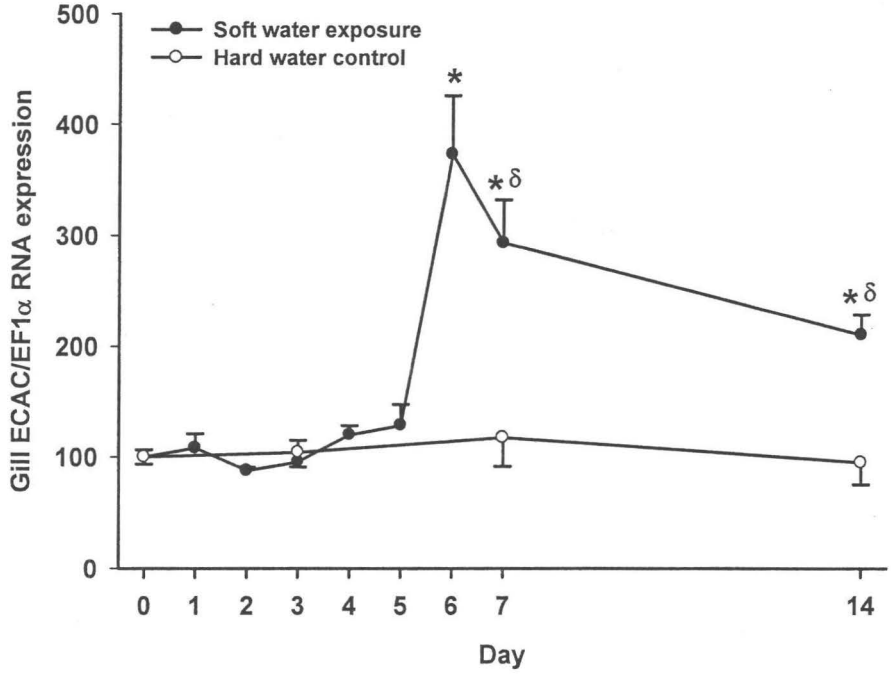
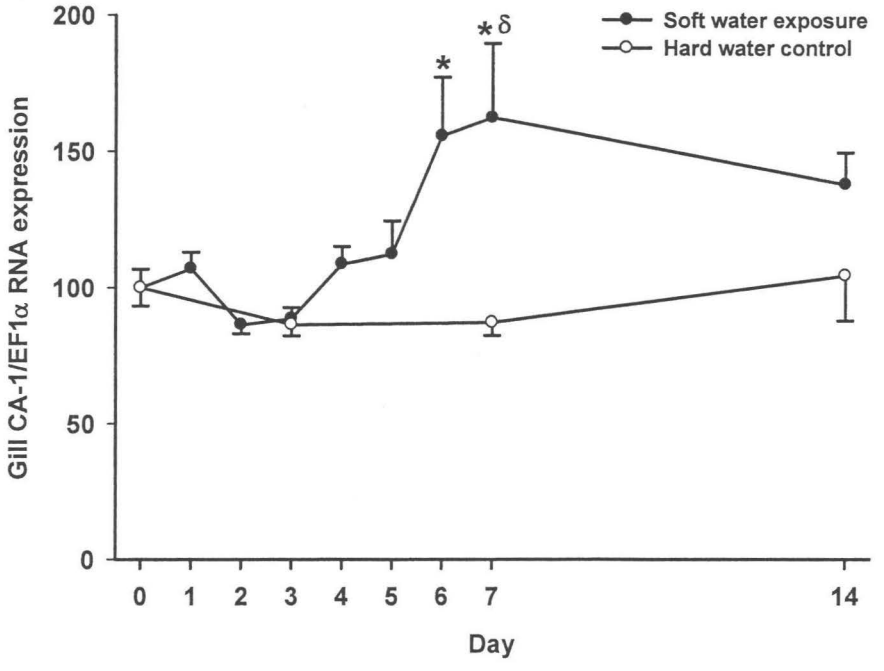

Soft water exposure
Hard water control
Gill ECAC/EF1α RNA expression
500
400
300
200
100
0
0 1 2 3 4 5 6 7 14
Day
*
*δ
*δ

Fig 2.7

mRNA expression of the carbonic anhydrase 1 (CA-1; **A**) and carbonic anhydrase 2 (CA-2; **B**) genes in the zebrafish gill during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Values are represented as means ± SEM, and based as a percentage of the control (100%). * Indicates significant difference from the day 0 control value as determined by one-way ANOVA followed by a Dunnett's test. δ Indicates a significant difference at a given time between hardwater and softwater treatments as determined by Student's t-test (n=5-6, $P<0.05$).

A


Soft water exposure
Hard water control
Gill CA-1/EF1α RNA expression
200
150
100
50
0
*
*δ
0 1 2 3 4 5 6 7 14
Day

B

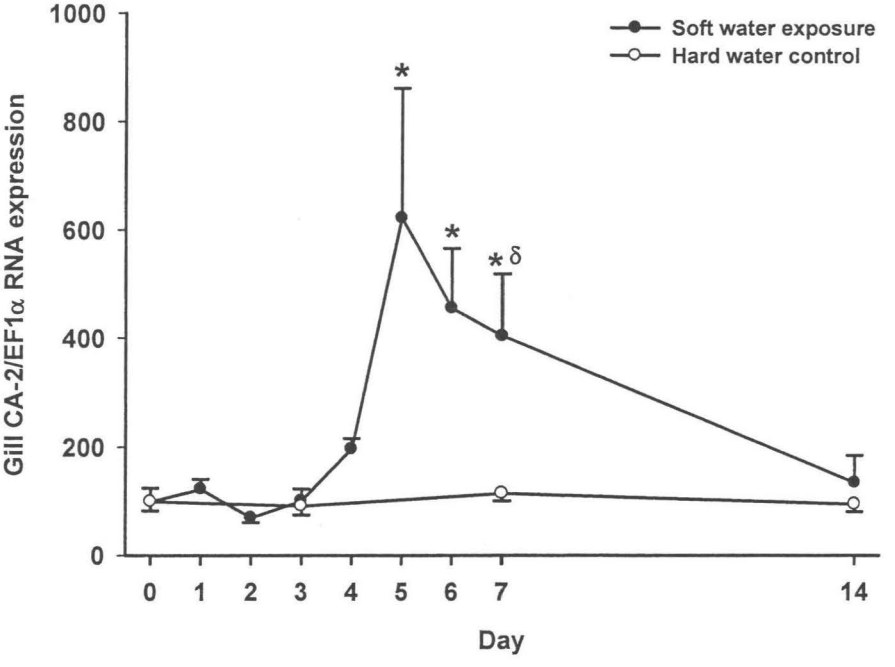

Soft water exposure
Hard water control
Gill CA-2/EF1α RNA expression
1000
800
600
400
200
0
*
*
*δ
0 1 2 3 4 5 6 7 14
Day

Fig 2.8

mRNA expression of the Na^+, H^+-exchanger 2 (NHE-2) gene in the zebrafish gill during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Values are represented as means ± SEM, and based as a percentage of the control (100%). * Indicates significant difference from the day 0 control value as determined by one-way ANOVA followed by a Dunnett's test. (n=5-6, $P<0.05$).

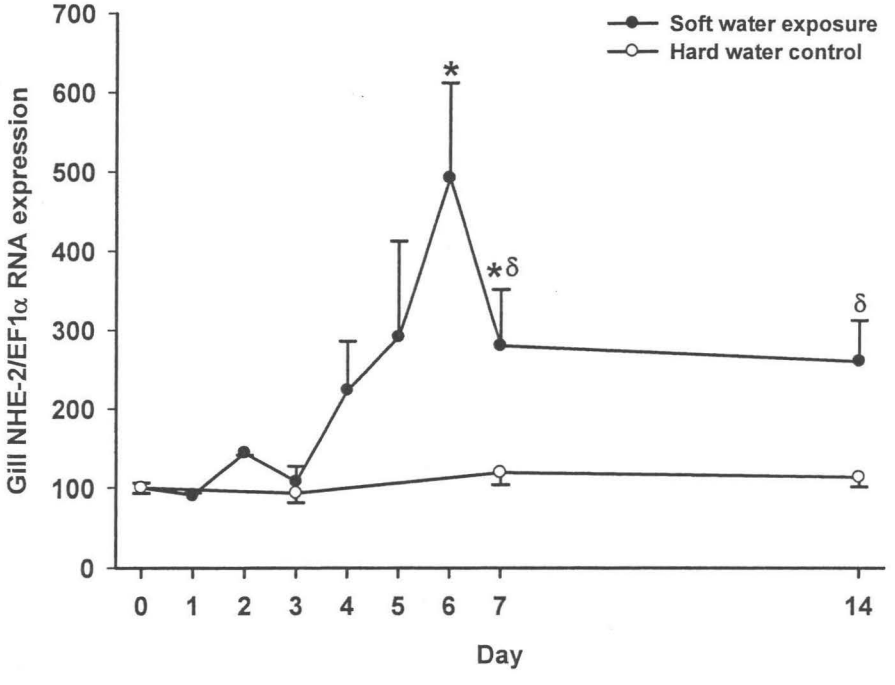

Soft water exposure
Hard water control
Gill NHE-2/EF1α RNA expression
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 14
Day
*
*δ
δ

Fig 2.9

mRNA expression of the copper transporter 1 (CTR-1) gene in the zebrafish gill during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Values are represented as means ± SEM, and based as a percentage of the control (100%). * Indicates significant difference from the day 0 control value as determined by one-way ANOVA followed by a Dunnett's test. (n=5-6, $P<0.05$).

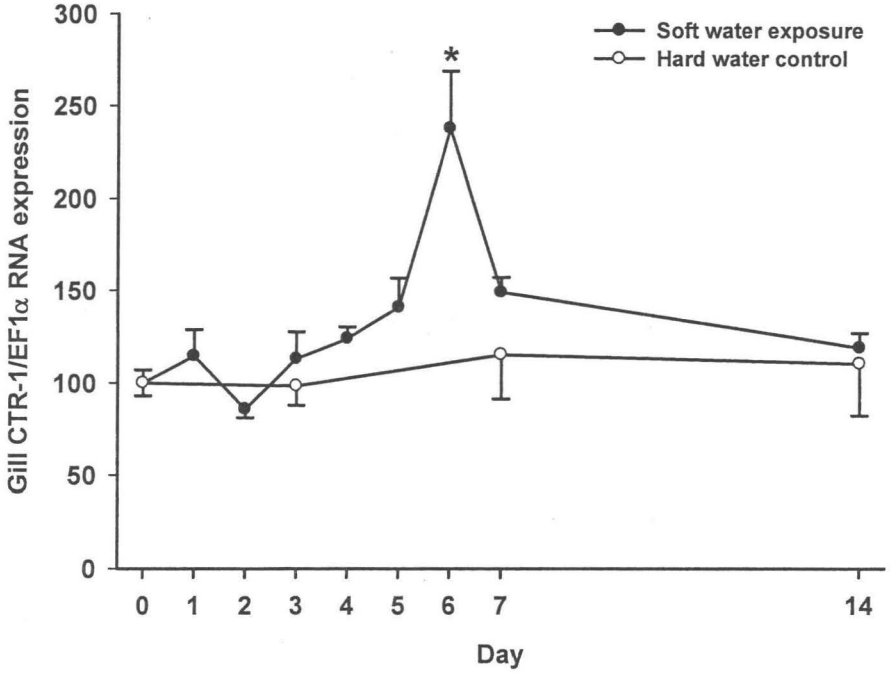
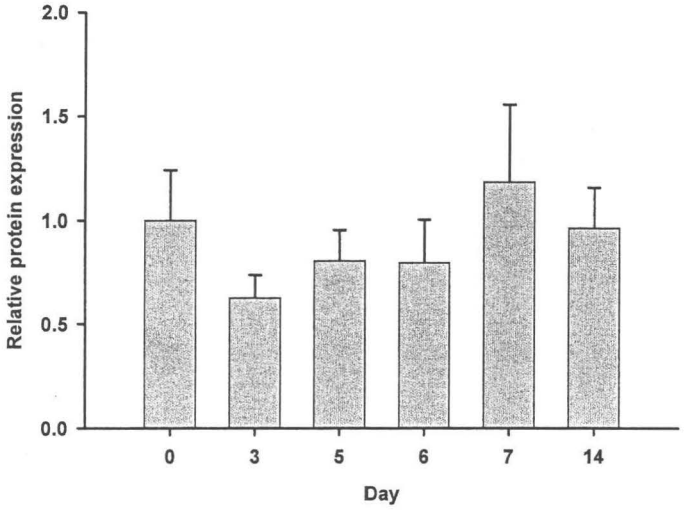

Soft water exposure
Hard water control
*
Gill CTR-1/EF1α RNA expression
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 14
Day

Fig 2.10

(**A**) Relative protein expression of gill NKA, and (**B**) representative Western blot of gill NKA in zebrafish during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Tubulin was also measured to account for differences in protein loading (n=4-5). There were no significant differences in (**A**). Values are represented as means ± SEM, and based as ratio of the control.

A


2.0
1.5
1.0
0.5
0.0
Relative protein expression
0
3
5
6
7
14
Day

B

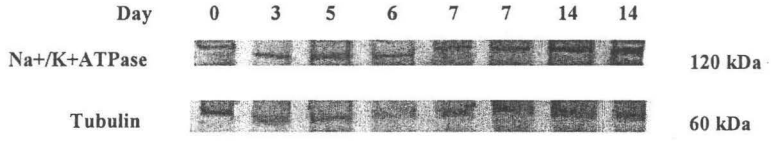
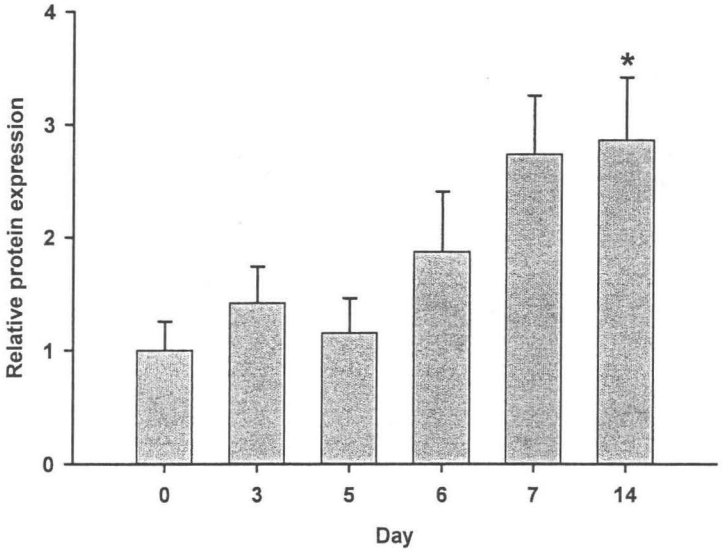

Day
0
3
5
6
7
7
14
14
Na+/K+ATPase
120 kDa
Tubulin
60 kDa

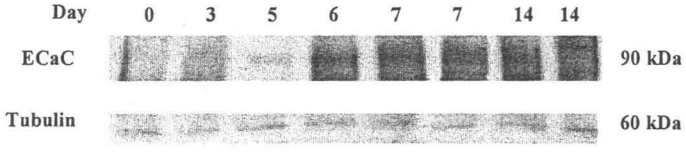
Fig 2.11

(**A**) Relative protein expression of gill ECaC, and (**B**) representative Western blot of gill ECaC in zebrafish during a progressive 7 day softwater acclimation experiment, and acclimation to softwater for an additional 7 days. Tubulin was also measured to account for differences in protein loading. In (**A**), * indicates significant difference from the initial hardwater value (day 0) as determined by one-way ANOVA followed by a Dunnett's test (n=4-5, $P<0.05$). Values are represented as means ± SEM, and based as ratio of the control.

A


Relative protein expression
4
3
2
1
0
*
0
3
5
6
7
14
Day

B


Day
0
3
5
6
7
7
14
14
ECaC
90 kDa
Tubulin
60 kDa

CHAPTER 3

GENE EXPRESSION ENDPOINTS FOLLOWING CHRONIC WATERBORNE COPPER EXPOSURE IN A GENOMIC MODEL ORGANISM, THE ZEBRAFISH, *DANIO RERIO*

Abstract

Although copper (Cu) is an essential micronutrient for all organisms, waterborne Cu in excess poses a significant threat to fish from the cellular to population level. We examined the physiological and gene expression endpoints that chronic waterborne Cu exposure (21 d) imposes on soft-water acclimated zebrafish at two environmentally relevant concentrations: 8 μg/L (moderate) and 15 μg/L (high). Using a 16,730 65-mer oligonucleotide customized zebrafish microarray chip related to metal metabolism and toxicity to assess the transcriptomic response, we found that 573 genes in the liver responded significantly to copper exposure. These clustered into 3 distinct patterns of expression. There was distinct up- regulation of a majority of these genes under moderate Cu exposure, and a significant down-regulation under high Cu exposure. Microarray results were validated by qPCR of 8 genes; 2 genes, metallothionein 2 (mt2) and Na^+K^+ATPase 1a1 (atp1a1), displayed increased expression under both Cu exposures, indicative of potential genetic endpoints of Cu toxicity, whereas the remaining 6 genes demonstrated opposing effects at each Cu exposure. Na^+K^+ATPase enzyme activity decreased during Cu exposure, which may be linked to Cu's competitive effects with Na^+. Whole-body cortisol levels were significantly increased in Cu-exposed fish, which prompted an analysis of the promoter region for glucocorticoid (GRE) and metal (MRE) response elements to dissociate metal- and stress-specific gene responses. Of the genes significantly regulated, 30% contained only a GRE sequence, whereas 2.5% contained only a consensus MRE. This study identifies direct and indirect effects of Cu exposure which is key to identifying genetic endpoints of chronic toxicity.

Craig, P.M., Hogstrand C., Wood, C.M., and McClelland, G.B. 2009. Submitted to *Physiological Genomics* May/2009.

Introduction

It has become commonplace to use microarrays to identify the effects of environmental stressors on both human and non-human organisms in an attempt to ascertain the global genetic response to a particular threat (Rouse et al 2007, Auslander et al 2008, Moretti et al 2008). When used effectively, scientists can elucidate major and minor pathways that define how the environment influences an organism, and the enormous amount of data generated can be amalgamated into databases for use in legislative and environmental protective standards. With anthropogenic contamination exceeding environmental remediation, genetic markers of environmentally pollution are essential to identify detrimental effects to populations, where phenotypic responses such as reduced reproductive capacity or altered morphology are not immediately visible (Williams et al 2007; Tanguay & Reimers 2008).

The response of organisms to environmental stressors often reveals a biphasic response, as first identified by Selye (1936), and more recently confirmed through meta-analysis of dose-response curve studies (Calabrese 2005). A biphasic pattern generally indicates that an organism is displaying a possible phenotypic response to a mild chronic threat, whereas a more severe, chronic threat will have extreme negative effects that can ultimately result in death and population decline. Despite this known phenomenon, chronic effects at low versus high doses of particular toxicants have been little studied. The primary objective of the present study was to examine the differential genetic expression between exposure to moderate or high chronic waterborne doses of copper (Cu) in zebrafish, both within an environmentally relevant range.

Zebrafish, having clear developmental stages and a fully sequenced, publicly available genome (Ensembl Zebrafish Genome Server, www.ensembl.org/Danio_rerio), are a model species for the examination of toxic effects of waterborne contaminants. Zebrafish are particularly useful in the study of metal contaminants, due to their softwater tolerance, which is key when looking at metal effects alone without the interference from competitive cations (Playle et al, 1992;1993; Neumann & Galvez 2002; Craig et al 2007a;b). Cu, an essential micronutrient, is taken up from the environment through either the gills or the gut via ingestion of food. Although essential, Cu in excess is known to have a variety of detrimental effects impacting ionoregulation in the shorter term, and growth in the longer term, and can play a role in endocrine disruption (Dethloff et al 1999; Zahner et al 2006; Mohanty et al 2009). Furthermore, excessive Cu can cause a cascade of cellular damage through Fenton-like reactions that produce excessive amounts of reactive oxygen species (ROS). These in turn can attack proteins, lipids, and DNA if not kept in balance via ROS scavenging enzymes, such as (Cu/Zn) superoxide dismutase and catalase (Di Giulio et al 1989; Craig et al 2007b). Given such a broad range of possible effects of Cu, microarrays may offer an ideal tool to identify specific targets of chronic Cu toxicity at the level of gene transcription. To date, no study has examined the chronic impact of varying doses of waterborne Cu using a microarray platform as an approach to determine the gene expression profile of Cu toxicity in zebrafish.

Cu, in similar fashion to many other environmental toxicants, elicits a general stress response, as indicated by increased circulating cortisol levels (see review Handy 2003). The resulting cortisol increase is modulated by Cu at various levels of the

hypothalamus-pituitary-interrenal axis (HPI-axis), in which Cu can interrupt serotonergic tone in the hypothalamus (Saucier & Astic 1995), upregulate dehydrogenases (5,3 β-hydroxysteroid dehydrogenase) involved in the synthesis of cortisol from cortisol precursors (Solaiman et al 2001), and possibly result in direct stimulation of interrenal cells to release cortisol due to renal Cu accumulation (Handy et al 2002, McGeer et al 2000, Gangon et al 2006) In fact, many of the physiological responses to Cu may be due in part to the elevated circulating cortisol levels which can alter enzyme activity and protein synthesis (Mancera & McCormick 1998; Solaiman et al 2001; Sumpter 1997; Smith et al 2001). Many of these physiological changes are beneficial in protecting an organism towards an oncoming threat, although chronic stress is known to be deleterious (Pottinger et al 1992).

Cortisol acts on target cells by binding to receptors on the cell surface, which are then translocated into the nucleus and bind to glucocorticoid response elements (GRE). Along with the associated interaction with transcription factors, this results in an increase or repression of gene transcription. A consensus GRE sequence (5'-GGTCANNNTGTTCT-3') was first identified in mammalian systems, and is responsive to glucocorticoid stimulation (Karin et al 1984; Kelly et al 1997). Other consensus sequences, including a mineralocorticoid response element have been identified in various non-mammalian species (e.g. *Carassius cuvieri*; Ren et al 2006) with response to cortisol stimulation. A second objective of this study was to identify the genes up- or down-regulated in association with chronic Cu exposure that contain a consensus GRE sequence that is likely responsive to cortisol stimulation.

A general stress response is not the only gene expression response elicited by excessive metal exposure; there may be also a direct metal response. Cu is known to stimulate metal-regulatory transcription factor 1 (MTFI), which in turn activates the DNA-binding region to specifically bind metal response elements (MRE), containing a consensus sequence of 5'-TGCRCNC-3'. This initiates a transcriptional response (Heuchel et al 1994, Selvaraj et al 2005). Very recently, Hogstrand et al (2008) performed an analysis of MREs in the promoter region of zebrafish genes significantly altered by zinc exposure, and found over 44% of significantly regulated genes contained one or more putative MREs. This analysis was based on results obtained from using the same custom designed zebrafish arrays we have used in this study. Therefore, our third objective was to identify gene transcription responses to Cu associated with potential MRE activation. This study therefore investigates both the direct and indirect effects of Cu exposure, through promoter region analysis of both GRE and MRE motifs. In effect, we aimed to identify and isolate genetic endpoints solely related to chronic Cu stress, as opposed to combinational endpoints of chronic stress and metal exposure.

The present study employs a custom designed 16,399 65-mer oligonucleotide zebrafish microarray chip with an additional 331 customised oligonucleotides related to metal metabolism and toxicity. By exposing softwater-acclimated zebrafish to chronic waterborne Cu levels for a period of 21 days, we were able to assess the transcriptomic changes associated with 2 levels of waterborne Cu exposure; a moderate (~8μg/L) and high (~15μg/L) Cu dose. Naturally occurring levels of Cu found in lakes and rivers range from 0.2 to 30 μg/l, while areas influenced by anthropogenic sources (e.g., mining,

industrial discharge, passage though Cu pipes) can exhibit levels ranging from 100 to 200,000 µg/l in heavily mined areas (Hem 1989, Robins et al 1997,). Significantly regulated genes were further analyzed using web-based bioinformatics tools to ascertain whether the physiological and genetic changes due to excessive waterborne Cu were due to metal effects, a general stress response, or a combination of these two pathways. Although not the first study to investigate a biphasic response to contaminants (see review of Calabrese 2009), this study does provide a message of caution when interpreting results based on single contaminant exposure in whole organisms, in that responses may only in part be due to the specific treatment, and further pathway analysis is required.

Methods & Materials

Animals

Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local pet fish distributor (DAP International, Canada) and acclimated to soft-water (Na^+ 75.5 ± 3.6 µM, Ca^{2+} 49.2 ± 3.7 µM, Mg^{2+} 20.5 ± 1.5 µM, total Cu 0.044 ± 0.001 µM or 2.8 ± 0.1 µg/L; pH 6.9) over a 7 day period in an aerated 40-L aquarium as described previously (Craig et al 2007a). The hardness of this water, expressed as $CaCO_3$ equivalents, was approximately 8 mg/L. After acclimation, zebrafish were housed in multiple 3-L self-cleaning tanks racked in a soft-water re-circulating stand-alone filtration system (AHAB, Aquatic Habitats, Apopka, Fl). Fish were fed daily with a commercial tropical fish food (Topfin, Phoenix, AZ: Ion content: 8.3 ± 0.2 mg Cu · kg^{-1} food , 147.0 ± 2.3 mmol Na · kg^{-1} food, 191.0 ± 6.2 mmol Ca · kg^{-1} food, 45.4 ± 0.9 mmol Mg · kg^{-1} food) and maintained on a 12-h light, 12-h dark photoperiod regime. Zebrafish were fasted for 24h prior to the beginning of experimentation. All procedures used were approved by the McMaster University Animal Research Ethics Board, and conform to the principles of the Canadian Council for Animal Care.

Experimental Protocol

Zebrafish (n=120; 40/treatment) were weighed and placed in 8-L aerated, flow-through tanks served with a softwater flow of 25 ml/min. Mariotte bottles were used to dose tanks with Cu (concentrated Cu solution made from $CuSO_4$ dissolved in 0.05% HNO_3) to 3 treatment regimes of either control (background Cu levels of 1.8 ± 0.3 ug/L), low Cu (8.0 ± 0.4 ug/L), and high Cu (14.4 ± 0.6 ug/L). The dilute nitric acid had no measurable impact on water pH. Fish were fed 2% body weight once per day of the commercial tropical fish food. Tanks were monitored daily for mortality and cleaned of any food or waste that had accumulated. Each day, a 10-ml water sample was taken from each tank, filtered though a 0.45µm filtration disc (Pall Corporation, East Hills, NY), added to a plastic vial containing 100µl HNO_3 and kept at 4°C for analysis of ion content and Cu concentration. At the end of the exposure period, fish were quickly euthanized by cephalic concussion and sampled for gill, liver, and gut, which were immediately frozen in liquid N_2 for further analysis of Cu burden and gene expression. Whole fish were also snap-frozen in liquid N_2 for analysis of whole body cortisol concentration.

Water and tissue ion & Cu levels

All tissues were first digested in 1ml of 1N HNO_3 for 48 h at 60°C. Tissue digests were diluted 10× and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z, Varian, Palo Alto, CA) using an appropriately diluted commercial Cu standard (40 µg/L, Fisher Scientific, Ottawa, ON). Water samples were measured undiluted. Both tissue and water ion composition were measured by flame atomic absorption spectroscopy (Spectra AA 220FS, Varian, Palo Alto, CA) after 10× dilutions were made with 1% HNO_3 (Na^+) or 0.5% $LaCl_3$/1% HNO_3 (Mg^{2+}, Ca^{2+}), and verified using certified Na^+, Mg^{2+}, and Ca^{2+} standards (1 mg/L diluted in 1% HNO_3 or 0.5% $LaCl_3$/1% HNO_3; Fisher Scientific).

Whole body cortisol extraction and measurement

Whole body cortisol was extracted based on combining methods described by Ramsey *et al* (2006) and Sink *et al* (2007). In brief, whole frozen zebrafish were weighed, cut into small pieces, and placed in a 15 x 85mm screw cap test tube. Three ml of de-ionized H_2O was added and the mixture was homogenized on ice for 45sec using a PowerGen homogenizer (Fisher Scientific). The homogenizer was rinsed with 1ml diethyl ether (Sigma, St. Louis, MO). To the mixture, 200µl of food-grade vegetable oil per gram body weight was added prior to the addition of another 7ml of diethyl ether. The samples were vortexed vigorously for 30sec, then centrifuged at 2,700rpm for 3min to separate the aqueous and ether (cortisol containing) phases. Tubes were snap-frozen in liquid nitrogen for 30sec, and the ether phase was poured into a new test tube. This extraction was repeated again, and ether was collected in a tube which was evaporated under a gentle stream of nitrogen for 30min. The remaining oil + cortisol mixture was stored at 4°C for no more than 24h prior to measuring cortisol.

A competitive cortisol RIA assay was purchased from MP Biomedicals (Solon, OH) to quantify cortisol in all of the fish extracts. Ten µl samples were measured in duplicate following the manufacturer's instructions. As suggested by Sink et al. (2007), the vegetable oil used to boost extract volume was tested by RIA to ensure that no cortisol was present. Additional spiked samples were extracted with known cortisol concentrations (50, 25, 12.5 ng/ml), which yielded greater than a 90% recovery. Linearity and parallelism were tested using serial dilutions (0, 25, 50, 75% dilutions) of extracted samples, which gave an $R^2 = 0.92$. With the use of pooled samples, intra- and inter-assay variability was 6.8% (n=9) and 9.7% (n=9), respectively.

Na^+/K^+-ATPase activity

Tissue Na^+/K^+-ATPase (NKA) activity was determined using the microassay method of McCormick (1993). All liver samples were homogenized in SEI buffer (150mM sucrose, 10mM EDTA, 50 mM imidazole, pH 7.3) containing 0.3% Na deoxycholic acid. Both NKA activity and Bradford protein assays (Bio-Rad, Hercules, CA) were run in 96-well format on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA).

Zebrafish oligonucelotide arrays

The zebrafish oligonucleotides arrays were spotted on UltraGAPS™ Coated Slides (Corning Life Sciences, Promega), using a Qarray2 robot (Genetix Ltd) in the Genomics Centre, King's College London, UK. The 16,399 65-mer oligonucleotides were designed and synthesised by Compugen and Sigma Genesys as a Zebrafish OligoLibrary ready set, which represent 15,806 LEADST clusters plus 171 controls (http://www.labonweb.com/chips/libraries.html). In addition, 331 customised oligonucleotides were added to the array set, and the array design was submitted to public archive ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/). Also included within the array set were 23 controls from Amersham Lucidea Universal ScorecardTM (Amersham Biosciences, GE Healthcare), which were used to perform a quality control (QC) test.

Reporter annotation was adopted from that by Zheng et al. (2008). For this re-annotation, the oligonucleotide reporters were mapped at the sequence level (using megablast allowing 2 bp mismatches) to Unigene and Ensembl (mapped and predicted), and this allowed implied linkage to ZFIN, GO and human orthologues. Because there is a shortage of functional information for zebrafish genes, and zebrafish share a similar gene set as humans, the functional analysis of genes of interest was performed using corresponding human orthologues (with sequence similarity higher than 45%) . These were extracted from NCBI HomoloGene database using Unigene IDs.

RNA extraction, cDNA production, and Cy3 & Cy5 coupling

Frozen liver tissue (10-20 mg tissue) was homogenized in 800µl Trizol reagent (Invitrogen, Carlsbad, CA), incubated at room temperature, mixed with 200 µL chloroform, incubated for a further 3 min, then centrifuged at 12,000g for 15min at 4°C. The aqueous phase was removed and further purified using the QIAgen RNeasy kit (Mississauga, Ont, Canada), following the manufacturer's instructions. A DNase I digest was performed on the column as outlined in the QIAgen protocol to remove any contaminating genomic DNA. RNA concentration was determined on a ND-1000 Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA) to ensure that the concentration was greater than 0.625µg/µl and that there was no contamination based on A260/A280. Amino-allyl cDNA was synthesized (2 tubes: one for each Cy dye) by the addition of 10 µg total RNA, 0.5 µl of both Oligo-dT (3µg/µl; Invitrogen) and random hexamers (3µg/µl; Invitrogen), and the volume topped-up to 17µl with DEPC H_2O. The mix was incubated at 70°C for 15 min, snap cooled, and pulse spun. A master mix of 6µl 5x first strand buffer, 3µl 0.1M DTT, 0.6µl 50x dNTP (no aa-dUTP), 0.9µl 10mM aa-dUTP, 2µl Superscript II RT, and 0.5µl RNase OUT was added to each tube for a final volume of 30µl, and was incubated at 42°C for 3 h. The RNA was then hydrolysed with 10µl 1M NaOH and incubated for 15 min at 65°C. The pH was then neutralized with 10µl 1M HCl. cDNA was purified by the addition of 40µl H_2O, 10µl 3M NaOAc pH5.2, and 300µl of 100% EtOH and allowed to precipitate overnight at -20°C. Samples were spun at 12,000rpm for 20min and washed twice with 75% ethanol before continuing. The aa-cDNA was coupled to Cy3 and Cy5 dyes (PA23001 and PA25001, Amersham, GE Healthcare, Piscataway, NJ) in separate tubes by resuspending the air-dried aa-cDNA pellet in 10µl fresh 0.1M $NaHCO_3$ pH 9.0 and added to each ready-made dye aliquot.

Samples were incubated in the dark at room temperature for 1 h with shaking at 15min intervals. Ester-dye coupled aa-cDNA was further purified using QIAquick PCR purification columns (Qiagen) as per the manufacturers' instructions, and was eluted in 35µl of PCR quality H_2O. Incorporation of dye molecules into aa-cDNA was measured on the Nanodrop spectrophotometer, ensuring an incorporation rate of 30 ([cDNA in ng/µl]/[CyDye in pmol/µl] *3.45) and that a concentration greater than 70 pmol of dye was achieved. A final volume of 20µl (70pmol of each dye + H_2O) was used to hybridize to the microarray slides.

Microarray hybridization

Prehybridization solution (5xSSC, 0.1% SDS, 1%BSA) was pre-warmed to 42°C, and microarray slides were immersedfor 60min. Slides were washed 2x in 0.1x SSC for 30sec, and slides were dried by centrifugation at 1,600rpm for 2min. Arrays were kept dust free and immediately used for hybridization, 20µl of fresh 2x hybridization solution (50% formamide, 10x SSC, 0.2%SDS, 0.2mg/ml salmon sperm) was combined with the 20µl of combined Cy3 and Cy5 aa-cDNA samples from above, heated to 95°C for 5min, and pipetted onto the microarray slides. A glass cover slip was placed on top to ensure even distribution of the hybridization sample. The slides were placed in a closed humidified chamber containing 3x SSC, and allowed to hybridize overnight in the dark at 42°C. Coverslips were removed and slides serially washed in a progressively diluted solution of 2xSSC and 0.1% SDS until a final rinse in H_2O (5min each wash with vigorous shaking). After the final wash, slides were dried by centrifugation and stored in light-protected slide boxes until ready to scan.

Microarray scanning and analysis

Hybridized slides were scanned at 5µm with a GenePix 4100A microarray scanner (Axon instruments, Redwood City, Ca, USA). Standard parameters in the GenePix Pro 6.0 software were modified to optimize cut-off signal values while retaining the maximum number of informative spots. Signal data was exported to GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA, USA) software package for Lowess signal normalization after the subtraction of the median background. A total of four slides per treatment were used (4 biological replicates, 12 slides total). The p-value cut-off was $\alpha 0.01$ for the ANOVA and post-hoc Tukey's tests, and the Benjamini and Hochberg multiple corrections was used, for a resulting 0.05 false discovery rate (FDR). A minimal change threshold set to 1.5 was employed to maximize the detection of significant genes in a situation of high biological variability (outbred fish). Significant genes were mapped to their respective human orthologue and the list was submitted to the Database for Annotated, Visual, and Integrative Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp) for ontological analysis of the significantly overrepresentation of GO biological (general descriptive function) and molecular (detailed descriptive process) terminology. Genes were further clustered into similar patterns of expression based on treatment using the K-mean Pearson centered algorithm found within the Genespring software package. We conducted a GRE and MRE analysis of the promoter region of up- and down-regulated Cu responsive genes. Using the

zebrafish Ensembl ID, we were able to extract a 2KB region upstream of the start codon using the BioMart tool from the Ensembl zebrafish Genome server Zv7. Using consensus sequence matrices found in NUBIScan software (http://www.nubiscan.unibas.ch/; Podvinec et al 2002), we analysed the 2kb promoter region for significant GRE hits. There were a total of 257 genes that contained at least 1 consensus GRE, and significant hits were ranked based on the number of hits and the proximity to the start codon to maximize the likelihood that the gene, in part, is regulated by glucocorticoids. Sequences were also searched for the MRE core consensus, 5'-TGCRCNC-3', as well as other MREs contained within the Transfac database (2007) using GCG version 11.1 (Accelrys) software. A total of 99 sequences contained at least one consensus MRE, and significant hits were ranked in the same fashion as GRE sequences. Unique and overlapping genes were submitted to DAVID for ontological analysis of their respective molecular functions. Complete results of this microarray experiment were submitted to the public archive ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) in accordance with Microarray Gene Expression Data Society (MGED) recommendations.

Microarray validation via qPCR

Microarray results were validated by real-time quantification of eight genes of interest plus a house-keeping gene (primer sequences found in Table 3.1): Na^+K^+ATPase isoform 1a (atp1a1), elongation factor 1 alpha (ef1α), estrogen receptor 1 (esr1), heat shock transcription factor 2 (hsf2), heat shock protein 60 (hsp60), heat shock protein 90a (hsp90α), metallothionein 2 (mt2), vascular endothelial growth factor a (vegfα), and the zinc transporter, zip1 (slc39a1). First strand cDNA was synthesized from 1 μg of liver total RNA (from above) treated with DNase I (Invitrogen, Calsbad, CA) and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase (Invitrogen, Calsbad, CA). mRNA expression was quantified in duplicate on a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 μl SYBR green mix, 1 μL of each forward and reverse primer (5 μM), 5.5 μL RNase/DNase free H_2O, and 5 μL of 5x diluted cDNA template. Cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15 sec, 60°C for 45sec, 72°C for 30 sec. This was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock liver cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the house-keeping gene ef1α which did not change over the course of the experiment. Gene expression data was calculated using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001). Both DNase- and RNase-free water and non-reverse transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA).

Statistical Analysis

Statistical analysis for Cu load, whole body cortisol, and qPCR validation was performed using Sigma Stat (SPSS Inc, Chicago, MI). In particular, a one-way ANOVA and post-hoc Tukey's test were used to test for pairwise significance of waterborne Cu levels, Cu tissue load, whole-body cortisol, NKA activity, and atp1a1 gene expression. Additionally, least squares regression analysis was used for qPCR validation ($p<0.05$). qPCR data was transformed to $\log_2$ values to match values obtained from the microarray experiment. All data have been expressed as a mean ± SEM.

Results

Over the 21 day exposure period, we maintained a constant softwater (average hardness amongst treatments: 8.9 ± 0.2 mg/L $CaCO_3$) environment throughout all tanks, and only the Cu concentrations were significantly different between control, low, and high Cu treatments (Table 3.2). There were no mortalities in the control and moderate exposure tanks; however, there was a 36% mortality rate in the high Cu exposure, although mortality occurred only within the first 4 days of the experiment. Tissue Cu load was consistent with previous experiments, with significant Cu accumulation in the gills and liver in the high Cu exposure, yet no clear elevation under moderate Cu exposure (Fig 3.1). There was also a distinct accumulation of Cu in the gut under both moderate and high Cu exposure (Fig 3.1).

There were significant ~2.75 and 3.25-fold increases in whole body cortisol levels under moderate and high Cu exposure, respectively, indicative of a generalized stress response (Fig 3.2). An enzyme known to be responsive to both Cu and elevated cortisol Na^+K^+ATPase (NKA) was assessed in the liver tissue for changes in both enzymatic activity and transcription of one of its primary subunits isoform 1a (atp1a1). We saw a distinct 40-50% reduction of activity under both moderate and high Cu concentrations (Fig 3.3). However, there were significantly elevated transcript expression levels (~ 2.5 fold) under the same conditions (Fig 3.3). The significant increase in atp1a1 mRNA is also validated on the microarray (See Supplemental Table A.1a and Table 3.2).

Microarrays were validated for 8 genes of interest (see Methods) under the two exposure conditions by real-time qPCR, and there was a significant, positive correlation ($R^2=0.787$; $p<0.001$) between the $\log_2$ transformed expression found on the arrays compared that of the qPCR measurements (Fig 3.4).

After normalization and statistical analysis, there was a total of 573 genes that had significant up or down changes in gene expression when compared to the control arrays, representing a 3.4% change in gene expression across the entire array. GO biological function analysis revealed predominant transcription regulation activity, with a strong significance related to negative transcription activity, and these ontological categories contained a number of transcription factors relating to cellular damage and carcinogenesis (i.e. zinc finger protein 281; Kruppel-like factor 12; Fig 3.5A). Also interesting is the over-representation of genes found within the apoptosis category related to cellular damage (i.e.DAXX protein; p53, MAPKKK; Fig 3.5A). GO molecular analysis further indicated a high degree of transcription regulation due to a significant overrepresentation of genes in the transcription factor category, indicating gene expression regulation (Fig

3.5B). Also highlighted in the molecular category is the alteration in antioxidant activity, signified by changes in gene expression of glutathione transferase and gluthathione peroxidase (Fig 3.5B; Supplemental Table A.1). Cluster analysis revealed 3 distinct clusters based on the expression profile across the treatments, containing 231, 201, and 141 significantly expressed genes (Fig 3.6A,B,C, respectively). The interesting aspect of this cluster analysis is the opposing effects of treatment on expression profile, where there is little similarity in up- and down-regulated genes when comparing moderate and high Cu treatments (Fig 3.6; Supplemental Table A.1 A,B,C). The clusters are essentially broken down into magnitude of opposing response, with few exceptions: Cluster 1 represents a strong increase in expression at a moderate Cu exposure, whereas there was a moderate decrease in expression under high Cu conditions (Fig 3.6A). Cluster 2 indicates a weak increase in expression at moderate levels, and a weak decrease in expression at high Cu levels (Fig 3.6B). Finally, Cluster 3 depicts a strong decrease in expression at a moderate Cu exposure, and a weak decrease in expression at high Cu concentrations (Fig 3.6C).

GRE analysis using NUBIscan v2.0 revealed that over 44% of significantly expressed genes (up or down) contained at least 1 consensus GRE in the 2KB promoter region directly upstream of the start codon (Supplemental Table A.2). Additional MRE analysis (GCG version 11.1, Accelrys) indicated that 17% of significantly expressed genes contained at least one consensus motif (Supplemental Table A.3). Further analysis isolated the shared and unique sequences: containing only GREs, only MREs, or containing both MRE and GRE motifs in the promoter region (Fig 3.7, Supplemental Table A.2 & A.3). A total of 172 (30%) genes contained only the GRE motif, and could further be broken down into GO molecular process categories of ribonucleotide binding (i.e. serum/glucocorticoid regulated kinase, MAPK4, protein kinase c; $p<0.01$), ATP Binding (ATPase1a1, HSP70, lysosomal H^+-ATPase; $p<0.01$), and pyrophosphatase activity (i.e ATP synthase lipid-binding protein; $p<0.05$). Further clusters of low significance ($p<0.1$) indicated some potential relationship to ion transport and binding (Fig 3.7, Supplemental Table A.3). There were 14 (out of 573 or 2.5%) genes that were unique in containing only an MRE motif, which were over-represented in the category of metal binding (e.g. mt2, $p<0.05$) and non-significantly represented in the cellular transport (e.g. VEGFa, $p<0.1$) GO category (Fig 3.7, Supplemental Table A.3). Genes that shared both a GRE and MRE consensus motif totalled 85 (15%), and were overrepresented in the GO molecular categories of GTP binding (e.g. Rho family GTPase 3, $p<0.05$), phosphoprotein (e.g. protein tyrosine phosphatase 3, $p<0.05$), transcription activity (e.g. HOXB1, $p<0.05$), and GTPase activity ($p<0.05$; Fig 3.7, Supplemental Tables A.2 and A.3). There were also categories of ribonucleotide- and ion-binding of low significance ($p<0.1$; Fig 3.7, Supplemental Tables A.2 and A.3).

Discussion

Toxicogenomics is a booming discipline, and much effort is currently directed towards its application as an environmental monitoring tool to identify patterns of gene expression that signify ecotoxicological impacts from pollutants. Microarrays have the ability to highlight alterations in previously unsuspected target pathways which can be

beneficial or detrimental to a particular species faced with a given threat. In this study, we have demonstrated a biphasic response associated with long-term waterborne Cu exposure over a period of 21 days. As the dose of Cu was altered between moderate and high levels (both well within an environmentally relevant range), respective patterns of expression changed as well. There was distinct up- regulation of a majority of genes under moderate Cu exposure, and predominantly a significant down-regulation of genes under high Cu exposure (Fig 3.5; Supplemental Table A.1). A cursory glance at these results might suggest that Cu has a significant role in modulating multiple pathways, however this is not necessarily the case. As Cu is seen to stimulate a systemic stress response, as indicated by increased whole-body cortisol (Fig 3.2), we can hypothesize that cortisol, in turn, may act globally on many tissues in either a beneficial or deleterious manner, presumably through glucocorticoid receptors, which can activate/inhibit gene transcription through GREs. Note that there were no differences in whole-body cortisol levels between moderate and high Cu treatment (Fig 3.2) groups, yet moderate Cu exposure resulted in liver Cu burdens intermediate between control and high exposure (Fig 3.1). Therefore we can refine our hypothesis with the idea that although cortisol plays a significant role in gene expression, Cu modulates this expression depending on the exposure level. After analysis for GREs in significantly regulated genes, we found 44% of significantly regulated genes contained at least one consensus GRE, and 30% of the genes contained only a GRE motif (i.e. no MRE; Supplemental Table A.2). Furthermore, we found that 17% of regulated genes contained a consensus MRE motif, with 2.5% containing only an MRE motif (i.e. no GRE, Supplemental Table A.3), indicating Cu may play a direct role in gene regulation. Therefore, Cu can have potential direct and indirect effects on gene expression, although it appears as though the indirect effects regulate gene expression to a much greater degree than the direct effects. We also provide evidence that gene expression is not always correlated with protein expression and function. NKA, a protein known to react to both Cu and cortisol (Gagnon 2006, Atli & Canli 2007, Ojima et al 2009) , was assessed for both activity and expression of its primary subunit (atp1a1), demonstrating a dichotomy in which changes in activity and expression are not congruent (Fig 3.3). Overall, this study effectively identifies the gene expression response to waterborne Cu exposure in softwater-acclimated zebrafish, and highlights a handful of the complex homeostatic pathways involved.

Waterborne Cu uptake

Primary uptake pathways of trace metals, such as Cu, are usually through the diet, however, in aquatic organisms Cu is also known to be taken up from the water (Clearwater et al 2002; Grosell and Wood 2002; Kamunde et al 2002). Cu homeostasis is tightly regulated, and excessive amounts of Cu are accumulated within the liver and excreted via the bile (Grosell et al. 1998, 2001). Indicative of this, we see a significant increase in the Cu concentration in the liver of zebrafish exposed to high Cu concentrations (Fig 3.1). As the liver is the primary Cu sequestering organ, we expect Cu regulated gene expression to be highest in this tissue.

As metals accumulate within cells above ambient levels, mechanisms of cellular protection are invoked, in particular metal chaperone proteins, such as metallothioneins,

are increased to reduce the amount of free Cu within a cell (Kagi & Schaffer 1988, Hogstrand et al 1991). Isolated mouse hepatocytes demonstrated saturation kinetics associated with high external Cu, which indicates there is a maximum capacity inherent with increased Cu exposure (McArdle et al 1990). Excess hepatic Cu in the moderate exposure group (where there was no significant elevation) was probably sequestered and excreted, as indicated by substantially elevated Cu levels within the gut, whereas excess Cu in the high treatment group (where there was a highly significant elevation) was probably not effectively sequestered and excreted, as indicated by the lower Cu burden in the gut (Fig 3.1). Indeed cellular damage associated with excessive free Cu ions may have impeded efficient Cu excretion; earlier we reported extensive acute oxidative damage in the liver associated with this level of Cu exposure (Craig et al 2007b). Cu transport and excretion is regulated by a suite of accessory cofactors and two Cu-ATPases (ATP7a (Menkes gene); ATP7b (Wilson gene) in humans (Lutsenko et al 2008), although to our knowledge, zebrafish appear to have only one Cu-ATPase (atp7a, Craig et al 2009). Deficiencies in ATP7b in humans result in an accumulation of Cu within liver hepatocytes, resulting in decreased biliary concentrations of Cu, and increased ROS damage within the cell (Prohaska 2008). We speculate that with only 1 functional Cu transporter, efficiency in Cu excretion is lower in zebrafish and only a minor increase beyond a set Cu threshold may induce cellular damage and a negative transcriptional response, as discussed subsequently. Further experimentation into metallothionein induction, Cu-ATPase function, and biliary Cu excretion in zebrafish is needed to test these ideas.

Microarray validation

Although phenotypic responses may not be identifiable, we know that genome-wide changes in expression are commonplace with environmental stressors (Gracey et al 2004, Ju et al 2007), and waterborne Cu exposure in this regard is no different (Osun-Jimenez et al 2009). In this study, we used a unique 16,399 65-mer oligonucleotide microarray chip to study the genotypic changes associated with two distinct waterborne Cu concentrations. Congruent with current microarray experiments (i.e. Boswell et al 2009, Osuna-Jimenez et al 2009), validation by real-time qPCR was performed on 8 identifiable genes of particular interest to metal exposure studies. This validation exercise indicated that gene expression results from the microarrays were in parallel with quantitative expression experiments (Fig 3.4).

The validation exercise also confirmed the biphasic response associated with the two distinct levels of waterborne Cu exposure. From our qPCR results, all but two genes (atp1a1 and mt2) had opposing responses in expression at the two Cu concentrations; (Table 3.3; Supplemental Table A.1). We have previously demonstrated that mt2 is a Cu-responsive isoform of metallothionein in zebrafish (Craig et al 2009), and it appears that independent of Cu exposure level, the mt2 gene is upregulated. However, in mammalian studies, Cu induction of metallothioneins (mts) is only detectable under high doses (Mercer 1997). In fact zinc (Zn) levels, rather than Cu levels, in the liver are thought to be extremely important in the synthesis and degradation of mts (Bremner 1980). Mobilization of free Zn induces metal-regulatory transcription factor 1 (MTF-1) to bind

to metal response elements that promote the transcription of mts in both trout and zebrafish (Dalton et al 2000, Hogstrand et al 2008). Despite Zn induction of mts, Cu has a higher avidity to mt than Zn and can outcompete Zn for mt-binding sites (Bremner and Beattie 1995, Bremner 1998). Notably, the Zn uptake transporter ZIP1 (slc39a1) is down-regulated under moderate Cu exposure, which either decreases competitive effects, or signifies a shared uptake pathway, which in turn would allow for increased detoxification of excess Cu. Conversely, we see a distinct increase in ZIP1 expression under high Cu levels, which may reflect increased mt transcription (i.e. increased mt2 expresson) via increased Zn levels, and/or indicate Zn deficiency under excess Cu. This presents a potential interesting mode of interaction between two competitive ions, and warrants further investigation.

Similarly to mt2, atp1a1, which is the catalytic subunit for the NKA enzyme (Table 3.3; Supplemental Table A.1), showed an increase in gene expression under both levels of Cu exposure (Fig 3.3). Cu is known to inhibit NKA enzymatic activity in the gills of both the juvenile tilapia and rainbow trout (Lauren and McDonald, 1987; Wu et al 2008, Gagnon et al 2006), and zebrafish liver tissue follows the same pattern (Fig 3.3). Potentially, zebrafish are up-regulating atp1a1 in effort to combat the inhibitory effects of excessive Cu accumulation, although further investigation is required. These results conceivably identify 2 genetic markers of chronic Cu toxicity (atp1a1 and mt2), which are independent of exposure level.

In proteins, metals can serve essential purposes, such as stabilization of protein structure and participation in catalytic events. However, in excess, metals can non-specifically bind and interfere with essential receptors, promote interferences by inappropriate metal substitutions, and induce the production of reactive oxygen species (ROS). Divalent metals, such as Cu, can also bind and activate estrogenic receptors and increase the risk of endocrine-related diseases (Martin et al 2003). Under conditions of moderate Cu exposure, we found a significant up-regulation of the esr1 gene, whereas there was a significant down-regulation in high Cu exposure (Table 3.3; Supplemental Table A.1), indicative of an endocrine response, although further responses in this pathway were not investigated.

Cu-induced reactive oxygen species (ROS) may cause lipid, protein, and DNA damage (Di Guilio et al 1989; Craig et al., 2007b). Heat shock proteins are also known to be responsive to increased metal accumulation and ROS (Yu et al 2006). Upon exposure to moderate Cu, we saw increased expression of hsp60 and its respective transcription factor hsf2, although we saw a decrease in another heat shock protein, hsp90a (Table 3.3; Supplemental Table A.1). Distinctively, the opposite effects were seen under conditions of high Cu exposure (Table 3.3; Supplemental Table A.1). hsp90a has been implicated in the apoptosis pathway in humans (Wu et al 2002; Perotti et al 2008). Furthermore, both Cu and ROS have been shown to induce apoptosis (Raes et al 1999; Simon et al 2000). Therefore, under conditions of continued influx of Cu, particularly at high levels, apoptosis may be occurring at an accelerated rate resulting in tissue damage and organ shut-down, which was reflected in the significant mortality rate during the first few days of exposure in zebrafish exposed to high waterborne Cu. Tissue damage is also highlighted by the increased expression of vegfα under high Cu exposure, since Cu-

induced oxidative damage has been shown to promote angiogenesis in human keratinocyte cell lines (Sen et al. 2002). Apoptosis was also identified as an overrepresented GO biological process category, and will be further discussed.

Ontological analysis of microarrays

Statistical analysis revealed that only 3.4% of the quantifiable genome exhibited changes in gene expression greater than 1.5 fold. After submission to DAVID (Denis et al. 2003; Huang et al. 2009) for ontological analysis, we found that the predominant changes are related to transcriptional regulation, due to the significant over-representation in those GO categories (Fig 3.5). This is not surprising as excessive Cu can result in a cascade of excessive cellular damage, which may stimulate transcription of genes involved in various defence systems. Of particular note, is the significant ($p<0.001$, Fig 3.5A) overrepresentation in the negative regulation category, which can be defined as any process that prevents or reduces the frequency of reactions and pathways in a given cell/organism. Genes found within this category are often associated with apoptosis. Examples are zinc finger protein 281 which is phosphorylated during DNA damage (Matsuoka et al 2007). This also belongs to the Kruppel-like zinc finger protein family (i.e. Kruppel-like factor 12 (klf12)) which is important as regulators of gene expression during development and carcinogenesis (Black et al 2001). Additional factors include the aforementioned vegfα which is associated with angiogenesis during oxidative damage (Sen et al 2002), and the mapkkk, daxx, and p53 associated proteins that are all involved in signalling pathways which can either prevent or induce oxidative stress related apoptosis (Ohiro et al 2003; Matsuura et al 2001). To a lesser extent, we saw some significant changes in genes associated with ion regulation and homeostasis (i.e. atp1a1 and slc39a1; see above), including changes in membrane Ca^{2+}-ATPase transport, which may be linked to competitive effects on Ca^{2+} uptake associated with excessive Cu (Craig et al 2007b). An exhaustive description of genes can be relayed, yet the general consensus is that the predominant response of liver tissue to chronic waterborne Cu exposure in zebrafish seems to be an induction of transcription factors associated with increased oxidative stress pathways, backed by the over-represented antioxidant category ($p<0.01$; Fig 3.5), in accord with our earlier findings of severe oxidative stress during acute waterborne Cu exposure (Craig et al., 2007b). Here we see induction of several antioxidant related proteins (i.e. glutathione peroxidase 2; peroxiredoxin 5) which are known to aid in antioxidant defence (Singh et al 2006; Wang et al 2002).

What is most interesting about this study is the biphasic response associated with a moderate and high dose of Cu, as we see both the magnitude and direction of gene expression differ at a given exposure (Fig 3.6, Supplemental Table A.1). Although cluster analysis revealed differences in magnitude of gene expression, caution needs to be exercised as the magnitude of the gene expression response does not always confer an equal response in protein expression or activity, as seen in this and previous experiments (Fig 3.3; Craig et al 2009). Knowledge of the transcriptomic response to chronic Cu toxicity in zebrafish is essential for identification of genetic endpoints, and particularly useful if the dose-response is not linear. Although we have used only 2 exposure levels, further experiments will be required to elucidate the specific point at which the gene

expression response reaches its maximum threshold, beyond which the genotypic response decreases. Our moderate level of exposure may not have reached the maximum threshold, whereas our high Cu exposure levels may have either have exceeded the maximum, and/or gene expression may have peaked earlier in the course of the experiment (Fig 3.6, Supplemental Table A.1). Notably there were a few genes that exhibited a linear pattern of expression, and it is these genes that require further examination for an accurate comparison of function and transcriptional response. Already noted is the similar pattern of expression of mt2 and atp1a1, yet there are a number of other candidate genes that should be validated for a linear response to increased Cu dose. Chiefly, these are genes that have an assessable functional component of the resultant protein (e.g. enzyme activity) as well, such as pyruvate kinase, hydroxysteroid 17-beta dehydrogenase, or hydroxysteroid 11-beta dehydrogenase 2 (See Supplemental Table A.1). Measurement of both genomic and phenotypic response is essential in identifying a useful gene expression biomarker of environmental contaminants.

GRE and MRE analysis

This study provides evidence that waterborne Cu exposure can elicit a transcriptomic response in zebrafish, yet we also found that the exposure to Cu additionally induced a general stress response, as indicated by increased whole-body cortisol levels (Fig 3.2). Bury et al (1998) previously demonstrated that cortisol provides a protective effect at low doses against Cu induced necrosis in cultured gill filaments, and our data suggests that Cu induced cortisol increase may play a similar role *in vivo*. This suggests that Cu exposure is a chronic stressor, which instigated an analysis of consensus GREs in the promoter region of significantly regulated genes (Supplemental Table A.2). 257 genes were found to contain at least one or more GREs in the promoter region, representing ~45% of significantly regulated genes (Fig 3.7). We also know that Cu can induce a direct metal response through activation of MTF-1, which binds to MREs and initiates a transcriptional response (Heuchel et al. 1994, Selvaraj et al. 2005). This prompted an additional analysis of MREs in the promoter region of significantly regulated genes as well, which showed 99 genes containing at least one consensus MRE motif, representing ~17% (Fig 3.7). Venn analysis indicated an overlap of 85 genes that contained both MREs and GREs. As such, these candidate genes should be excluded from use as Cu-specific genetic endpoints. Ontological analysis indicated a significant proportion of these genes were involved in transcriptional regulation (hoxa3a, hoxc1a) and signalling cascades (Rho family GTPase 3, protein tyrosine phosphates 3), although no strong relationship was found with metal-associated responses, with the exception of hsp60 (Fig 3.7, Supplemental Table A.2 & A.3). Hsp60, or chaperonin, is an essential mitochondrial chaperone and promotes the folding of many proteins imported into the mitochondrial matrix. Furthermore, hsp60 is known to respond to oxidative stress (a result of excessive cellular Cu) and general stress (Cabiscol et al 2002, Martin et al 1992). This implies that although HSPs are commonly seen in metal stress exposure, they are not a good candidate for genetic endpoints of chronic Cu exposure, as they are not specific to Cu alone.

With respect to genes that only contained GREs (~30%), we found categories essentially relating to transcriptional regulation, and loosely relating to ion transport and binding (Fig 3.7). With increased cortisol levels there are associated changes in ion homeostasis, a familiar phenomenon linked to altered environmental water chemistry (i.e freshwater → seawater migration), additionally indicated by the GO molecular category (Fig 3.7; Madsen 1990, Craig et al 2005). We see a large portion of the molecular function categories indicative of ion homeostasis and the active movement of ions across the cell membrane, regulated by genes such as atp1a1, v-type H^+-ATPase, and voltage dependent anion channel 4. Cu is known to compete with other divalent and monovalent ions, such as Ca^{2+}, Fe^{2+}, and Na^+ (Grosell and Wood 2002, Spry and Wiener 1991; Craig et al. 2009), and therefore interrupt ion homeostasis. According to ontological evidence provided here, gene expression as it relates to ion regulation under chronic Cu exposure may be modulated by cortisol, as opposed to direct stimulation from Cu. Conversely, we see a very short list of genes that contain only MREs in the promoter region (Fig 3.7, Supplemental Table A.3). This list of 14 genes comprises a meagre 2.5% of all genes significantly affected by Cu treatment, and suggests that there is a loose association directly between Cu and a gene expression response. However, we did find that mt2 contains 2 MREs, which further identifies this gene as a genetic endpoint, although not specific to Cu, as other metals (i.e. Zn) may invoke increased MTF-1 binding to MREs (Dalton et al 2000, Hogstrand et al 2008; Supplemental Table A.3). Due to the relatively short list of genes found in this analysis, significant over-representation of GO categories is difficult to ascertain, however it appears that genes containing MREs are associated with ion binding (e.g. mt2) and cellular transport, a broad descriptive category that includes genes such as vegfα. Certainly an analysis of both GREs and MREs provides a more accurate description of the potential regulation of gene transcription as it relates to treatment. However, further empirical evidence is required to fully ascertain the role that Cu plays in initiating a transcriptional response.

In summary, we have identified 2 potential genetic markers, atp1a1 (general stress response) and mt2 (metal stress response), of chronic Cu exposure under softwater conditions. Furthermore, we argue that genetic markers need functional components (enzyme activity and/or protein level) since expression level does not always coincide with a functional response (Fig 3.3; Craig et al 2009). Moreover, we have validated in a tropical species that chronic Cu exposure elicits a general stress response, which in turn, can impact transcriptional regulation of genes thought to be mediated by solely by excessive metals. By determining both direct and indirect effects of contaminant exposure, one can filter out specific, direct effects of a particular contaminant, and label responsive genes as genetic endpoints. This study, along with many others, is taking only the first steps towards identifying genetic endpoints of chronic metal exposure in teleosts.

Table 3.1: Forward (F) and reverse (R) primers used for real-time qPCR validation of microarray results.

Gene	Primer	Accession #	Amplicon size (BP)
atp1a1	F: 5'-CTGCCACTTCAATCTTCCCG-3' R: 5'-TCGAACTGGAAGTCCTCAGGA-3'	NM_131686	51
esr1	F: 5'-AGAAACACAGCCGGCCCTA-3' R: 5'-TGGTGAGCAGGGACATCATG-3'	NM_152959	51
hsf2	F: 5'-ACGTCCCCGCATTTCTTACC-3' R: 5'-TATCCGAATCCTCGACGAGG-3'	NM_131867	50
hsp60	F: 5'-CGCTGTGGAGGAAGGAATTG-3' R: 5'-CAGCGCAGTAAAGCACATCC-3'	NM_181330	50
hsp90a	F: 5'-ATCCGCAAAAACCTGGTCAA-3' R: 5'-TGCCAGTTCGGTGAAGAGATC-3'	NM_131328	51
mt2	F: 5'-CCCATCTGGTTGCAGCAAGT-3' R: 5'-GAATTGCCTTTGCAGACGC-3'	NM_194273	50
vegfa	F: 5'-CATCATCCAGGAGTATCCCGA-3' R: 5'-CAGGACGGGATGTACGTGTG-3'	NM_131408	51
zip1	F: 5'-CGTTTTCGAGGGTTTGGCTA-3' R: 5'-AACACCTTGGCATTCGTCGT-3'	NM_001013540	50
ef1-α	F: 5'-GTGCTGTGCTGATTGTTGCT-3' R: 5'-TGTATGCGCTGACTTCCTTG-3'	NM_131263	201

Table 3.2: Concentrations of water ions (μM), copper (μg/L), pH, and total hardness ($CaCO_3$ in mg/L) for all experimental exposures. Values presented as mean ± SEM. Values that do not share the same letter indicates a significant difference ($p<0.05$, n = 21).

Treatment	Ctrl	Cu (8μg/L)	Cu (15μg/L)
Na^+	84.2 ± 4.5	80.1 ± 4.8	80.6 ± 6.4
Mg^{2+}	26.7 ± 1.5	26.8 ± 1.4	26.6 ± 1.8
Ca^{2+}	66.9 ± 4.7	62.8 ± 5.3	61.6 ± 5.6
Cu	1.8 ± 0.2^A	8.0 ± 0.4^B	14.4 ± 0.6^C
pH	7.01 ± 0.6	6.98 ± 0.7	6.96 ± 0.5
$CaCO_3$	9.3 ± 0.3	8.9 ± 0.5	8.6 ± 0.5

Table 3.3: Direction of change in expression of genes used for microarray validation grouped together according to similar patterns of expression. Arrows indicate direction of significant expression >1.5 fold. The normalization gene elongation factor 1 alpha (ef1a) did not change significantly under any treatment regime.

Gene Symbol	Gene Description	GeneBank® ID	Expression Direction Moderate Cu	Expression Direction High Cu
atp1a1	Na^+,K^+-ATPase1α1	AI105927.1	↑	↑
mt2	Metallothionein 2	AW170968.1	↑	↑
esr1	Estrogen receptor 1	BF717829.1	↑	↓
hsf2	Heat shock factor 2	CR926931.1	↑	↓
hsp60	Heat shock protein 60	BI896507.1	↑	↓
hsp90a	Heat shock protein 90	AI397223.1	↓	↑
vegfa	Vascular endothelial growth factor a	AW233384.1	↓	↑
zip1	Zinc transporter 1	BM857883.1	↓	↑

Fig 3.1
Tissue Cu load in the gills, liver and gut of softwater acclimated zebrafish exposed to control (1.8 ± 0.2 μg/L), moderate (8.0 ± 0.4 μg/L), and high (14.4 ± 0.6 μg/L) levels of waterborne Cu. Bars that do not share a like symbol are significantly different within a tissue group ($p<0.05$, $n=7$).

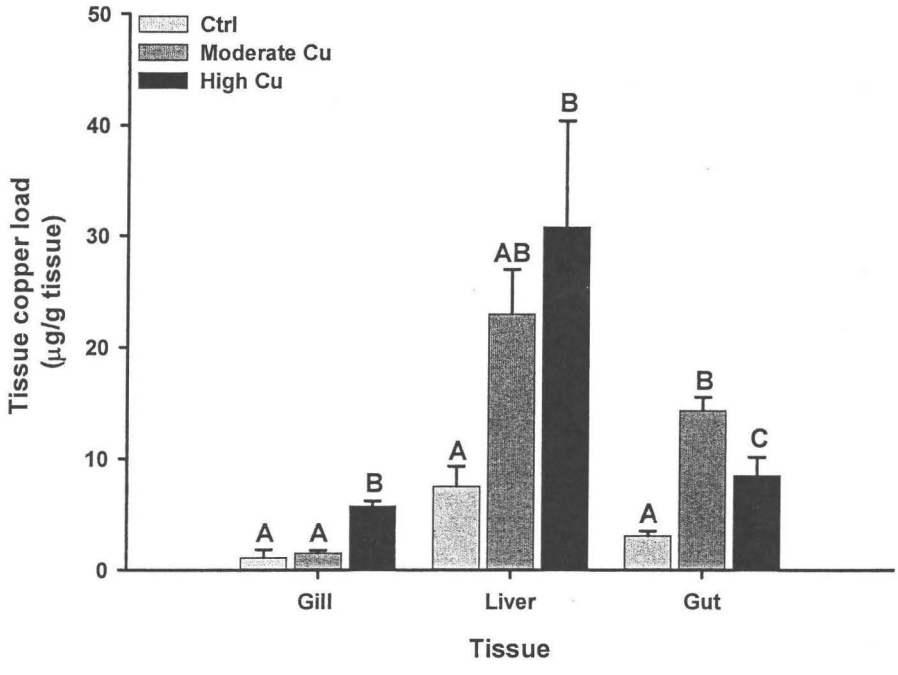

Ctrl
Moderate Cu
High Cu
Tissue copper load (µg/g tissue)
50
40
30
20
10
0
A
A
B
A
AB
B
A
B
C
Gill
Liver
Gut
Tissue

Fig 3.2
Whole-body cortisol levels of softwater acclimated zebrafish exposed to control (1.8 ± 0.2 μg/L), moderate (8.0 ± 0.4 μg/L), and high (14.4 ± 0.6 μg/L) levels of waterborne Cu. Bars that do not share the same letter are significantly different from each other within a specific tissue ($p<0.05$, $n=7$).

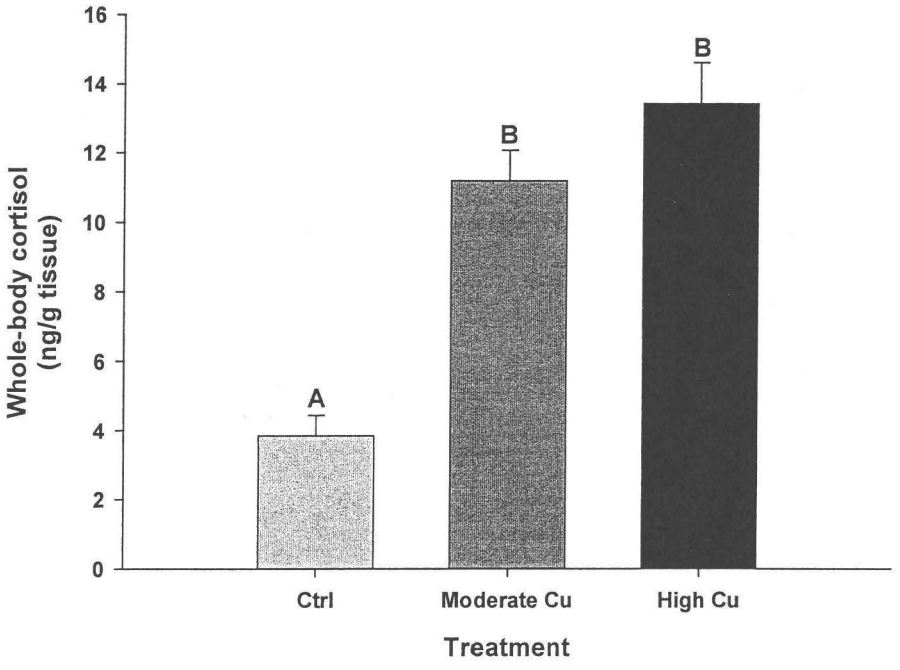

16
14
12
10
8
6
4
2
0
Whole-body cortisol (ng/g tissue)
A
B
B
Ctrl
Moderate Cu
High Cu
Treatment

Fig 3.3
NKA activity (μmol ADP/mg protein/hour; left hand axis) and associated change in gene expression of atp1a1 (normalized to ef1α and relative to the control; right hand axis) in softwater acclimated zebrafish exposed to control (1.8 ± 0.2 μg/L), moderate (8.0 ± 0.4 μg/L), and high (14.4 ± 0.6 μg/L) levels of waterborne Cu. Bars that do not share the same letter are significantly different from each other ($p<0.05$; $n=6$).

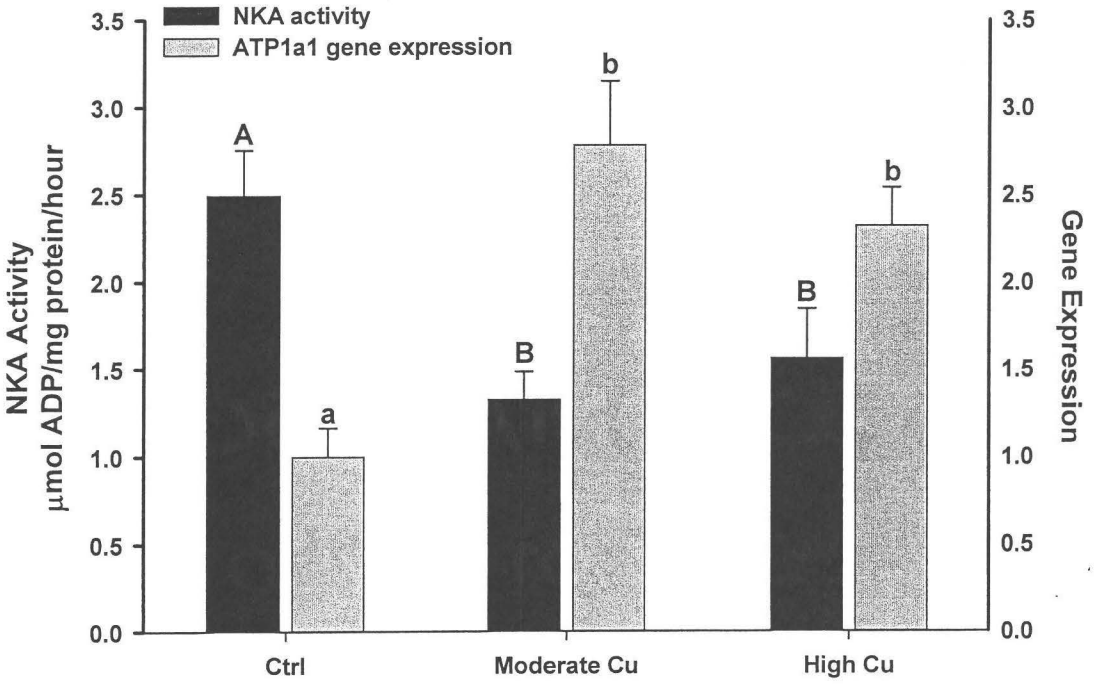

NKA activity
ATP1a1 gene expression
NKA Activity
µmol ADP/mg protein/hour
Gene Expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
a
B
b
B
b
Ctrl
Moderate Cu
High Cu

Fig 3.4
Log_2 transformed regression analysis for the purpose of microarray validation using 8 selected genes (plus the housekeeping gene ef1α) that had a fold change >1.5 from 3 statistically distinct clusters: Na^+K^+ATPase isoform 1a (atp1a1), elongation factor 1 alpha (ef1a), estrogen receptor 1 (esr1), heat shock transcription factor 2 (hsf2), heat shock protein 60 (hsp60), heat shock protein 90a (hsp90a), metallothionein 2 (mt2), vascular endothelial growth factor α (vegfα), and zinc transporter (zip).

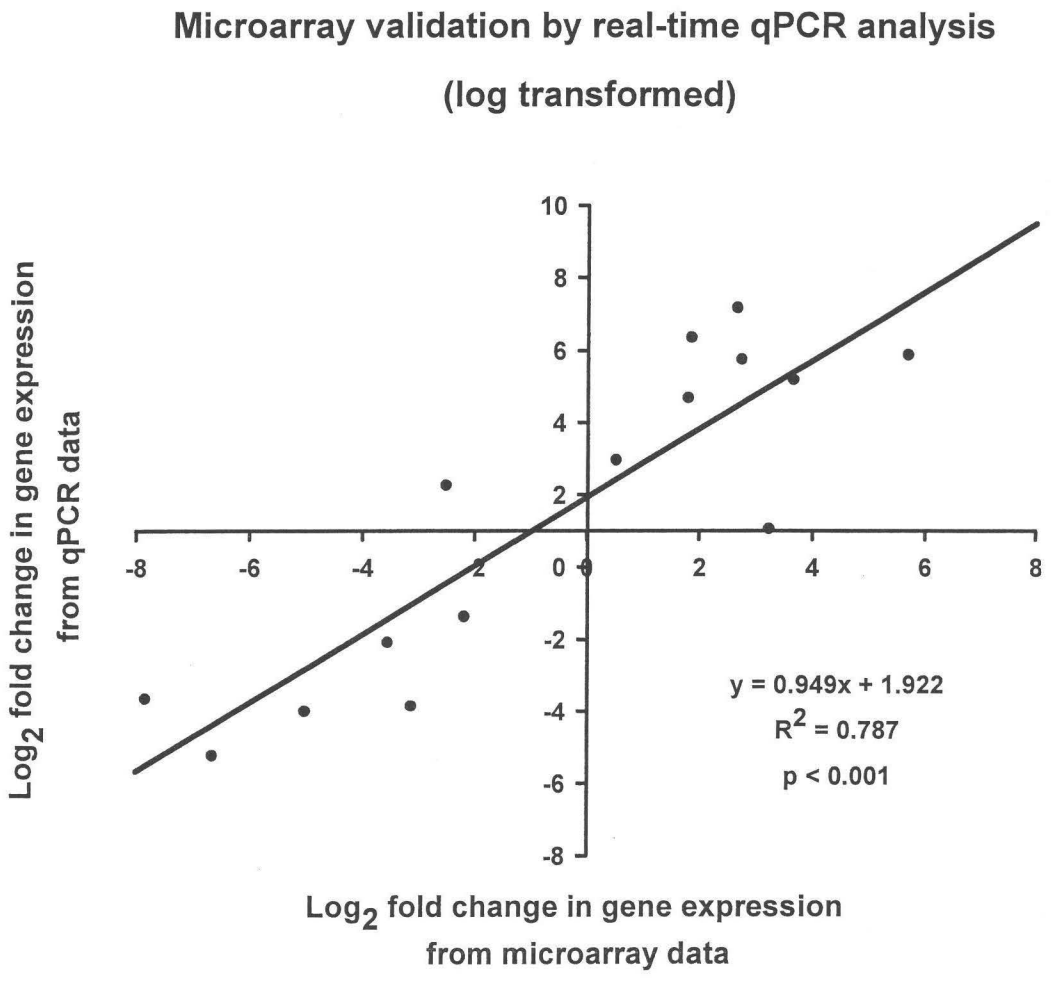
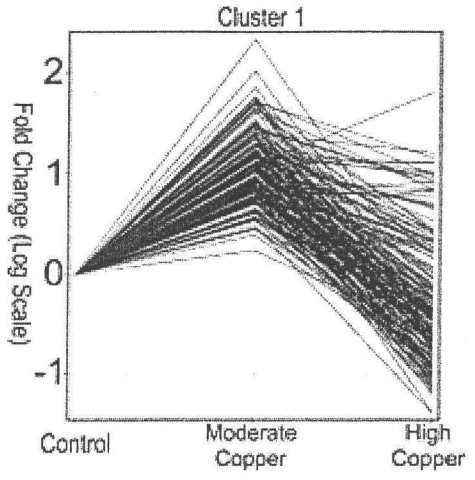
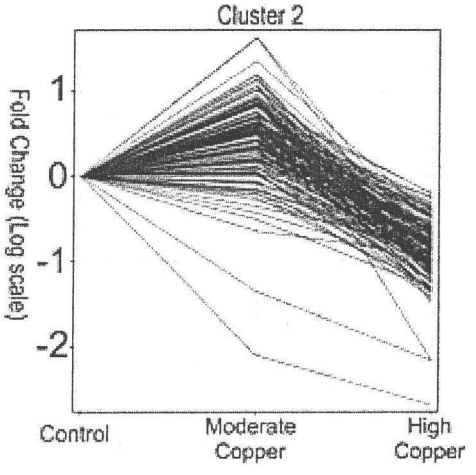

Microarray validation by real-time qPCR analysis
(log transformed)
Log2 fold change in gene expression from qPCR data
Log2 fold change in gene expression from microarray data
y = 0.949x + 1.922
R2 = 0.787
p < 0.001
10
8
6
4
2
0
-2
-4
-6
-8
-8
-6
-4
-2
2
4
6
8

Fig 3.5
Cluster analysis of genes that were statistically up- or down-regulated >1.5 fold versus control genes. Analysis revealed 3 distinct cluster patterns and indicated contradictory responses associated with varied levels of waterborne Cu exposure. Clusters contained 231(A), 201(B), and 141(C) genes, and an associated identification of genes can be found in Supplemental Table 1.

A


Cluster 1
Fold Change (Log Scale)
2
1
0
-1
Control
Moderate Copper
High Copper

B


Cluster 2
Fold Change (Log scale)
1
0
-1
-2
Control
Moderate Copper
High Copper

C

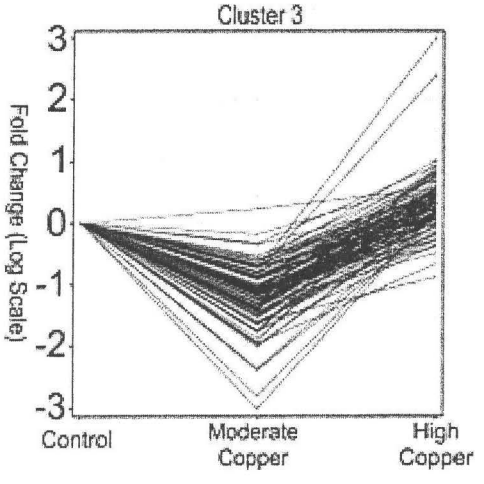
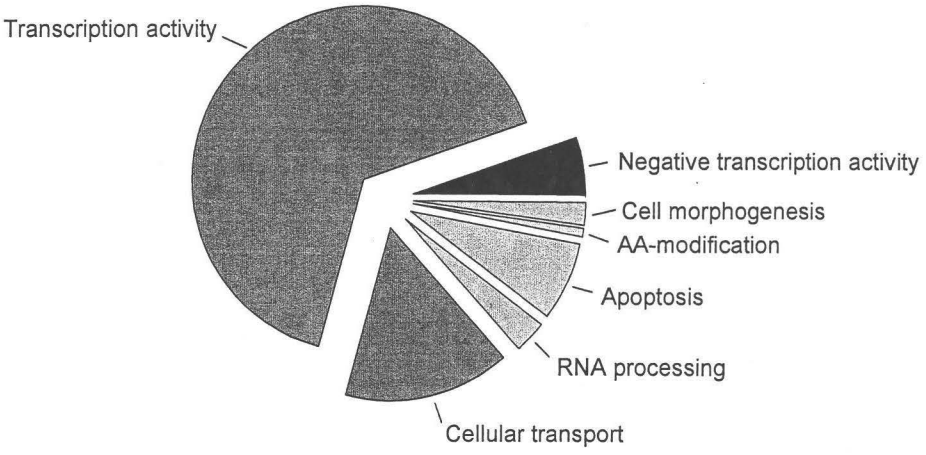

Cluster 3
Fold Change (Log Scale)
3
2
1
0
-1
-2
-3
Control
Moderate Copper
High Copper

Fig 3.6
GO Biological process (A) and molecular function (B) ontological analysis using DAVID. The graph displays terms that are significantly over represented ($p<0.05$) within GO molecular function. Colour identifies the significance level of overrepresented categories: black $p<0.001$; dark grey $p<0.01$, light grey $p<0.05$.

A)


Transcription activity
Negative transcription activity
Cell morphogenesis
AA-modification
Apoptosis
RNA processing
Cellular transport

B)

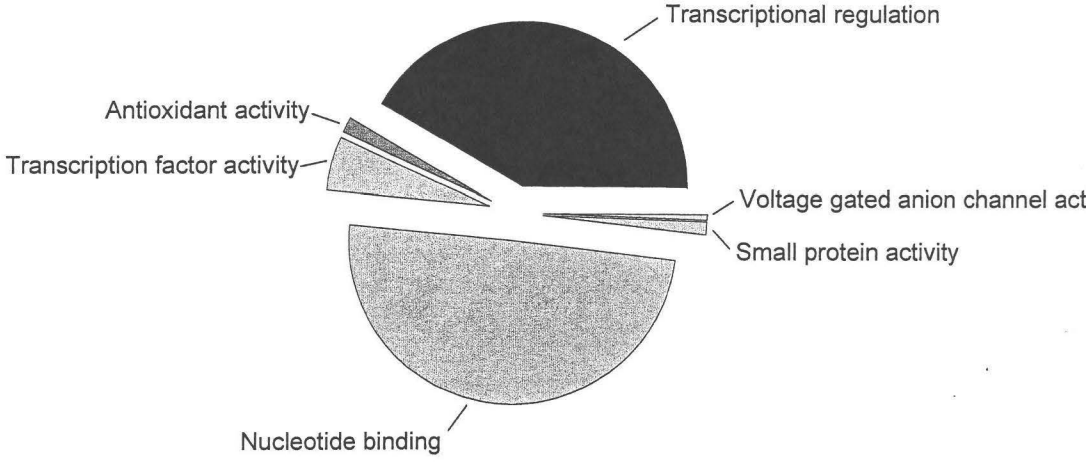
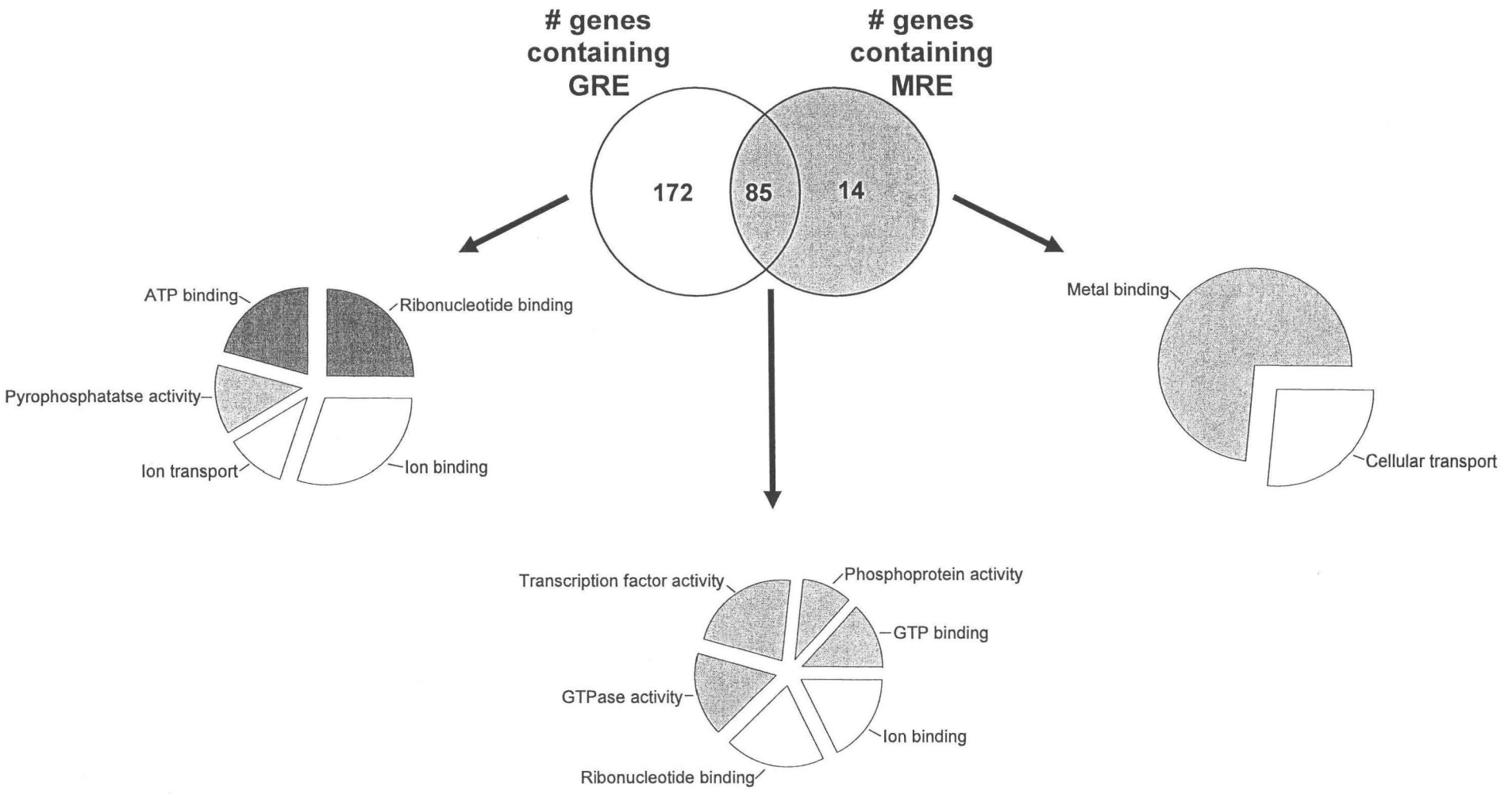

Transcriptional regulation
Antioxidant activity
Transcription factor activity
Voltage gated anion channel act
Small protein activity
Nucleotide binding

Fig 3.7
Venn diagram of assessable genes that contained at least one consensus GRE or MRE, indicating the number of genes that contained the consensus promoter. Further details on specific genes can be found in Supplemental Table 3. Sequences were submitted to DAVID for ontological analysis of the molecular function category. Colour identifies the levels of overrepresented category: dark grey $p<0.01$, light grey $p<0.05$, white $p<0.1$.


genes containing GRE
genes containing MRE
172
85
14
ATP binding
Ribonucleotide binding
Pyrophosphatatse activity
Ion transport
Ion binding
Metal binding
Cellular transport
Transcription factor activity
Phosphoprotein activity
GTP binding
GTPase activity
Ion binding
Ribonucleotide binding

CHAPTER 4

OXIDATIVE STRESS RESPONSE AND GENE EXPRESSION WITH ACUTE COPPER EXPOSURE IN ZEBRAFISH (*DANIO RERIO*)

Abstract

In fish, environmental pollution is one factor that induces oxidative stress and this can disturb the natural antioxidant defence system. Oxidative stress has been well characterized *in vitro*, yet the *in vivo* effects of metal-induced oxidative stress have not been extensively studied. In 2 experiments, we examined the impacts of copper (Cu) on gene expression, oxidative damage, and cell oxidative capacity in liver and gill of zebrafish. In the first experiment, soft-water acclimated zebrafish were exposed to 8 & 15 μg/L Cu for 48 h. This exposure resulted in significant increases in gene expression of cytochrome c oxidase 17 (COX 17) and catalase (CAT), associated with both increased Cu load and protein carbonyl concentrations in the gill and liver after 48 h. In addition, we examined the potential protective effects of increased waterborne Ca^{2+} (3.3mM) and Na^{+} (10mM) on acute Cu toxicity. While both treatments were effective at reducing liver and/or gill Cu loads and attenuating oxidative damage at 48 h, 10 mM Na^{+} was more protective than 3.3 mM Ca^{2+}. There were variable changes in the maximal activities of cytochrome c oxidase (COX) and citrate synthase (CS), indicating possible alterations in cell oxidative capacity. Moreover, Cu affected COX/CS ratios in both gill and liver suggesting that Cu alters normal mitochondrial biogenic processes possibly though metallochaperones like COX 17. Overall, this study provides important steps in determining the transcriptional and physiological endpoints of acute Cu toxicity in a model tropical species.

Introduction

Zebrafish have long been an excellent model for developmental and reproductive biology due to their rapid rate of growth and clear, well-characterized embryonic development. Zebrafish are now becoming a popular model for aquatic toxicological and physiology studies (e.g. Briggs 2002, Craig et al 2007, Neumann & Galvez 2002) due to their publicly available genome and ability to tolerate soft-water (Boisen et al 2003, Craig et al 2007), a key requirement in testing toxic effects of metals without the interfering effects of other cations (Playle et al 1992, 1993). They also answer the need for a tropical species in comparative toxicology. Zebrafish are endemic to the Ganges River which flows through the Indian subcontinent where they are reported to face a wide variety of water chemistries (including moderate soft-water) and a range of Cu concentrations from 6-39 μg/L (Ajmal et al 1985, Krisnaswami et al 1984, Talwar et al 1991).

Copper (Cu) is an essential micronutrient for all organisms and in the case of fish, is acquired by the gills from the surrounding water, as well as from the diet by the digestive tract (Kamunde et al 2002, Kapustka et al 2004). Naturally occurring levels of Cu found in lakes and rivers range from 0.2- 30 μg/L, whereas areas influenced by anthopogenic sources (e.g. mining, industrial discharge, passage though Cu pipes) can exhibit levels ranging from 100 -200,000 μg/L in heavily mined areas (Hem 1989, Robins et al 1997). Elevated aquatic Cu levels cause a range of negative effects on fish including reduced growth (Ricard et al 1998), interference with whole body ionoregulation (Lauren & McDonald 1987, McGeer et al 2000), and endocrine disruption (Teles et al 2005). Many of these responses are in part due Cu's high reactivity with H_2O_2 and potential to undergo redox reactions to form reactive oxygen species (ROS), a process known as the Fenton reaction. The resulting cellular damage can be in the form of membrane lipid peroxidation, DNA damage, and protein carbonyl production (Di Giulio et al 1989). Like other organisms, fish combat elevated levels of ROS with protective ROS-scavenging enzymes such as superoxide dismutase (SOD) and catalase (CAT), which convert the superoxide anions into H_2O_2 and further into H_2O and O_2, respectively. Once these enzymes are overwhelmed by excessive ROS production, irreversible cellular damage and death can occur.

It is well established that Cu toxicity is most aggressive in the absence of any competitive ions, such as in soft-water (Pagenkopf 1983, Playle et al 1992, 1993). Current theory suggests that the mechanisms for acute Cu toxicity in fish occurs mainly at the gills, which involves ionic Cu^{2+} out-competing Na^+ at apical Na^+-channels and ultimately inhibiting the basolateral Na^+K^+ATPase, effectively inhibiting normal Na^+ uptake (Grosell & Wood 2002, Lauren & McDonald 1987, Wood 2001). With increased waterborne Na^+ concentrations, gill Cu uptake is reduced most likely due to this competition at the Na^+ uptake sites (Grosell & Wood 2002, Playle et al 1992, 1993). However, there is evidence that elevated ambient Ca^{2+} concentrations can also effectively inhibit Cu accumulation (Playle et al 1993, Spry & Weiner 1991). Ca^{2+} appears to bind to the apical surface of the gill, thereby reducing membrane permeability and stabilizing tight junctions (Hunn 1985). Indeed the Biotic Ligand Model (BLM; reviewed by Niyogi & Wood 2004, Paquin et al 2002), a relatively new regulatory model that is now being used to determine the allowable levels of Cu in freshwater, takes into account these

protective effects of water chemistry, and is based on a fixed relationship between short-term gill Cu accumulation and ultimate toxicity. Many studies have examined the effects of metal toxicity on zebrafish (e.g. Airaksinen et al 2003, Dave & Xiu 1991, Linbo et al 2006), yet none have examined the combined effects of protective ions and oxidative stress when fish are exposed to elevated copper. However, in preliminary experiments, we have found that 3.3 mM Ca^{2+} and 10 mM Na^{+} have identical protective effects (80% inhibition) against short-term (3h) gill Cu binding in zebrafish (as assessed by the accumulation of radiolabelled Cu in the gill tissue). Yet they had differential protective effects against lethality, where Cu in soft-water + 3.3mM Ca^{2+} is 11-fold more protective and Cu in soft-water + 10mM Na^{+} is 25-fold more protective than Cu in soft-water alone. There is therefore a need to explore the physiological, biochemical and gene expression effects of these exposures to understand the nature of the toxic effects and this differential protection.

Although the gills are the main site of Cu uptake from the ambient medium, very little Cu accumulates within the gills after the initial exposure (Grosell et al 1997). In fact, Cu is exported from the gill cells to the blood, transported to the liver where it is incorporated into ceruloplasmin, transcuprein, and also bound to albumin. It is then exported in these forms for use by other parts of the body. In addition, in the liver, Cu is also sequestered by metal chaperone proteins, such as metallothionein, detoxified and finally excreted via the bile (Grosell et al 1997, Marr et al 1995). With high ambient Cu concentrations, the pathological accumulation of Cu within the liver is inevitable, ultimately increasing ROS production and leading to hepatocyte death (Manzl et al 2003, Manzl et al 2004). Prior to cell death, trout hepatocytes demonstrating elevated Cu levels exhibited cellular respiration rates which were increased by 50% *in vitro*, although there was no impact on energy (ATP) production (Manzl et al 2003). Cu's impact on cellular respiration may be in part due to its role in the formation of cytochome c oxidase (COX), the terminal enzyme of the electron transport chain. In vertebrates, COX is composed of 13-subunits, and a further 30 proteins are required for proper COX assembly (Tzagoloff & Dieckmann 1990). One of these proteins, COX-17, is of interest in particular, since it aids in the assembly of the Cu centre of COX (Carr & Winge 2003, Glerum et al 1996). COX-17 is a metal chaperone that binds to free cytoplasmic-Cu further transporting Cu to the mitochondria intermembrane space where it delivers Cu to Sco1 (another mitochondrial copper chaperone) for COX assembly (Horng et al 2004). Excess Cu may have an impact on this pathway, however to date no studies have examined this as a potential mechanism of Cu toxicity.

The goal of this study was to examine the *in vivo* effects of short-term sub lethal Cu exposure on zebrafish to elucidate the physiological and transcriptional endpoints of acute toxicity. Additionally, we hypothesised that increased Ca^{2+} (3.3 mM) and Na^{+} (10 mM) in the ambient medium would mitigate the deleterious effects of Cu on zebrafish, but in a differential fashion. We examined the mRNA expression and enzyme activity patterns of SOD and CAT to assess the antioxidant defences with Cu exposure. COX-17 mRNA expression and enzymatic activity of COX and citrate synthase (CS) were examined as indices of changes in the oxidative capacity in liver and gills with respect to increased Cu exposure. The results of this study will further our understanding of acute

Cu exposure, especially in terms of gene expression and oxidative stress, and in a tropical species of genomic importance. These data can also be applied to improve regulatory models such as the BLM (Niyogi & Wood 2004) that can predict both acute and chronic toxicity levels of Cu.

Materials & Methods

Animals

Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local pet supply store (PetsMart, Canada) and acclimated to soft-water (Na^+ 115 ± 3.3 μM, Ca^{2+} 51 ± 1.0 μM, Mg^{2+} 26 ± 1.6 μM, Cu^{2+} 1.8 ± 0.5 μg/L; pH 6.8) over a 7 day period in an aerated 40L aquarium as described previously (Craig et al 2007a). After acclimation, zebrafish were housed in multiple 3L self-cleaning AHAB tanks racked in a soft-water recirculating stand-alone AHAB filtration system (Aquatic Habitats, Apopka, Fl). Fish were fed daily with a commercial tropical fish food (Topfin, Phoenix, AZ) and maintained on a 12-h light, 12-h dark photoperiod regime. Zebrafish were fasted for 24h prior to the beginning of experimentation. All procedures used were approved by the McMaster University Animal Research Ethics Board, and conform to the principles of the Canadian Council for Animal Care.

Experiment 1

Zebrafish (n = 120) were removed from their recirculating tanks and placed in static, aerated 3L AHAB tanks (n=10 per tank). Fish were either exposed to control soft-water (n = 40), soft-water plus 8 μg/L Cu (n = 40), or soft-water plus 15 μg/L Cu (n = 40) using a concentrated Cu solution made from $CuSO_4$ dissolved in 0.05% HNO_3, which had no measurable impact on water pH. Zebrafish were quickly euthanized by cephalic concussion (rather than terminal anaesthesia, so as to avoid changes in indices of oxidative stress) after 1, 4, 24, and 48h exposure. Fish were sampled for gill and liver tissues, which were collected and immediately frozen in liquid N_2 for further analysis of protein carbonyls, Cu load, enzyme activity, and gene expression levels. At the time of sampling, a 10ml water sample was taken from each tank, filtered though a 0.45μm filtration disc (Pall Corporation, East Hills, NY), added to a plastic tube containing 100μl HNO_3 and kept at 4°C for analysis of ion content and Cu concentration.

Experiment 2

After acclimation to soft-water, zebrafish (n=40) were then acclimated to soft-water plus 3.3mM $CaCl_2$, or soft-water plus 10mM NaCl in separate 40L aquaria over a period of 14 days before the Cu exposures. Fish were placed in 3L static AHAB tanks containing either soft-water + 3.3mM $CaCl_2$ or soft-water + 10mM NaCl, and exposed to 15 μg/L Cu for 48 h. Control tanks contained soft-water, soft-water + 3.3mM $CaCl_2$ or soft-water + 10mM NaCl, yet had no additional Cu added. Fish were sampled in the same manner as before, and tissues were immediately frozen in liquid N_2 for analysis. Water samples were also taken at each time point. Additionally, liver and gill samples were analysed for Na^+ and Ca^{2+} tissue load.

Water and tissue ion & Cu levels

Gill and liver tissue was first digested in 1ml of 1N HNO_3 for 48 h at 60°C. Gill and liver digests were diluted 10× and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z, Varian, Palo Alto, CA) compared to a 40 μg/L Cu standard (Fisher Scientific, Ottawa, ON). Water samples were measured undiluted. Both tissue and water ion composition were measured by flame atomic absorption spectroscopy (Spectra AA 220FS, Varian, Palo Alto, CA) after 10× dilutions were made with 1% HNO_3 (Na^+) or 0.5% $LaCl_3$/1% HNO_3 (Ca^{2+}), and verified using certified Na^+ & Ca^{2+} standards (1 mg/L diluted in 1% HNO_3 or 0.5% $LaCl_3$/1% HNO_3; Fisher Scientific).

Protein carbonyl content

Protein carbonyls were quantified using a commercial kit (Cayman Chemical Company, Ann Arbor, MI). Briefly, tissue was homogenized in 1 ml of homogenization buffer (50mM phosphate buffer, 1mM EDTA, pH 6.7) and centrifuged at 10,000g for 15min at 4°C. Two separate tubes were then prepared (assay & control). To both tubes, 200 μl of supernatant was added plus 800 μl of 2,4-dinitrophenylhydrazine (DNPH) to the assay tube and 800 μl of 2.5M HCl to the control tube, and both were incubated in the dark for 1 h. After incubation, 1 ml of 20% trichloroacetic acid (TCA) was added and the tubes centrifuged at 10,000g for 15min at 4°C. The supernatant was removed and 1ml of 10% TCA was added and the tubes were again centrifuged. The supernatant was removed and the pellet was washed with 3 sequential washes of 1ml 1:1 ethanol/ethyl acetate mixture followed by centrifugation. After the final wash, the pellet was resuspended in 500 μl guanidine hydrochloride (2.5 M). 220 μl from each tube was placed in a 96-well plate and the absorbance was measured between 360-385 nm. The peak absorbance from the control value was subtracted from the assay value, and this corrected absorbance was used to calculate protein carbonyl content. Protein content of the control sample was then measured in a quartz 96-well plate at 280 nm to yield protein carbonyl content in nmol/mg protein. Samples were compared to a BSA standard curve (0.125-1 mg/ml) dissolved in guanidine hydrochloride (2.5M).

Enzyme activities

Chemicals were purchased from Sigma-Aldrich Chemical Co. (St Louis. MO) and reaction buffers were prepared fresh daily. For all enzyme assays both gill and liver tissues were homogenized in ice-cold 20mM Hepes, 1mM EDTA, 0.1% Triton X-100, pH 7.0. All enzymes were assayed in 96-well format on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA). SOD (CuZn-SOD & Mn-SOD) was measured using a modified assay based on the method of Crapo *et al.*(1978). The assay exploits the ability for SOD to out-compete cytochome c for superoxide generated by xanthine oxidase. One unit of SOD is defined as the amount required to inhibit the cytochome c reduction reaction by 50%. The reaction mixture comprised 50mM K-phosphate buffer, 0.1mM EDTA, 100 μM xanthine, 40 mM cytochome c, and 20 U/ml xanthine oxidase. Various amounts of homogenate were added

to the reaction buffer to determine the amount required to inhibit activity by 50%. Absorbance was measured at 550 nm. COX activity was measured by addition of homogenate to a reaction buffer containing 50mM Tris-HCl pH 8 and 50 μM reduced cytochome c, as described previously (McClelland et al 2006). After mixing, the absorbance was measured at 550 nm and the reaction was followed for 90 s. Homogenate volumes were chosen to ensure cytochome c was at saturating concentrations. For citrate synthase (CS) and catalase (CAT) assays, remaining homogenates were frozen overnight, and then sonicated for 30s (Misonix, Inc, New York). For the CS assay, the reaction buffer contained 0.1mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.3mM acetyl CoA, and 0.5mM oxaloacetate in 20mM Tris-HCl buffer, pH 8.0. Absorbance was measured at 412 nm over 3 minutes, and a control well lacking oxaloacetate was used to correct for background thiolase activity. CAT activity was assayed using a modified method based on Clairborne (1985). The reaction buffer consisted of 20mM K-phosphate, pH 7.0, and 20 mM H_2O_2. The reaction was recorded as the decomposition of H_2O_2 at 240nm over 1 min. Protein concentrations for all samples were determined using BioRad kit reagents according to Bradford (7) using bovine serum albumin as a standard.

Quantification of mRNA by real-time RT-PCR.

Total RNA from the gill and liver tissue (frozen in liquid N_2) was extracted within 24 h of sampling using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by UV spectrophotometry at 260 nm, and RNA purity verified by the 260/280 nm ratios of 1.8 or greater. First strand cDNA was synthesized from 1 μg of total RNA treated with DNase I (Invitrogen, Calsbad, CA) and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase (Invitrogen, Calsbad, CA). mRNA expression was quantified in duplicate on a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 μl SYBR green mix, 1 μL of each forward and reverse primer (5 μM), 5.5 μL RNase/DNase free H_2O, and 5 μL cDNA template. Cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec. This was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock liver cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the house-keeping gene EF1α, which did not change over the course of the experiment. Both water and non-reverse transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer3 (Rozen & Skaletsky 2000) and targets verified by gel electrophoresis and sequencing. Target genes of interest are as follows and primers used can be found in Table 4.1: cytochome c oxidase subunit-17 (COX-17), catalase (CAT), Cu zinc superoxide dismutase (Cu/ZnSOD), and housekeeper elongation factor 1 alpha (EF1α). Genes were normalized to EF1α, and each value was expressed as a percentage of the soft-water control, which allowed for the calculation of SEM.

Statistical analysis

Statistical analysis was performed using Sigma Stat (SPSS Inc, Chicago, MI). All data have been expressed as a mean ± SEM. For experiment 1, a 2-way ANOVA and a Tukey's test was performed ($p<0.05$). For experiment 2, a one-way ANOVA and a Dunnett's test was used to test for significance relative to the soft-water control value, and a Student's unpaired t-test was used to compare within a treatment ($p<0.05$).

Results

Experiment 1

After 48h Cu exposure, we saw no morbidity or mortality in any of the experimental or control tanks. Average measured concentrations of dissolved Cu for each treatment were 2.1 ± 0.2 (control), 8.1 ± 0.4 (moderate), and 15.1 ± 0.5 μg/L (high). Average ion concentrations did not fluctuate from original soft-water acclimation values. Associated with the high Cu exposure, we saw a significant increase in the liver Cu load after 24 and 48h, with a corresponding 3-fold increase in protein carbonyl levels after 48 h (Fig 4.1A,C). However, there was no change in either Cu load or protein carbonyls associated with a moderate Cu exposure (Fig 4.1A,C). Similarly, we saw significant increases in gill Cu load after 24 and 48h with high Cu exposure, and increased gill protein carbonyl content after 48h, in comparison to 1 and 4h exposures (Fig 4.1B,D).

Upon examination of mRNA expression, no changes in SOD expression in the liver were detected despite the elevated Cu load and protein carbonyl levels. However, we did see a significant 2.5-fold increase in liver CAT mRNA expression after 48 h exposure to high Cu levels (Fig 4.2A,B). Additionally, we saw a progressive increase in liver COX-17 mRNA expression with a high Cu exposure, with a significant 8-fold increase in expression after 48 h (Fig 4.2C).

Experiment 2

Since the majority of significant changes occurred after 48 h exposure to the higher Cu level (15 μg/L) we examined the potential protective effect of Ca^{2+} and Na^{+} under this treatment. Zebrafish were exposed to either high Ca^{2+} (nominally 3.3 mM, measured chemistry: 3.4 ± 0.1 mM Ca^{+2}, 71.1 ± 3.7 μM Na^{+}, 23.2 ± 2.2 μM Mg^{2+}, 14.5 ± 0.1 μg/L Cu, pH 6.9 ± 0.2) or high Na^{+} (nominally 10 mM, measured chemistry: 59.6 ± 1.9 μM Ca^{2+}, 10.6 ± 0.4 mM Na^{+}, 27.4 ± 3.5 μM Mg^{2+}, 14.7 ± 0.2 μg/L Cu^{2+}, pH 7.0 ± 0.1). Control tanks were maintained with the same water chemistry, yet had baseline levels of Cu (1.8 ± 0.6 and 1.5 ± 0.9 μg/L Cu for high Ca^{2+} and Na^{+} exposures, respectively). With two exceptions, total Na^{+} and Ca^{2+} concentrations in both the liver and gills did not change as a result of the various exposures (Table 4.2); however liver Ca levels increased in the Cu-exposed fish under high Ca^{2+}, and gill Na^{+} level increased in the Cu-exposed fish under high Na^{+}. In general, these results indicate that the zebrafish had acclimated to the elevated ion levels, and that ionoregulation was not significantly impacted by Cu during these 48 h exposures, as there were no falls in tissue ion levels. (Table 4.2).

When Cu exposure was performed in the presence of high Ca^{2+}, we saw diminished Cu load in the liver at 48 h and the associated increase in protein carbonyl levels was no longer significantly different from the control although it was of similar magnitude (Fig 4.3A,C). When Cu exposure was performed in the presence of high Na^{+}, we saw no accumulation of Cu above control levels in the liver and no change in protein carbonyl levels (Fig 4.3A,C). The high Ca^{2+} + Cu exposure did not significantly reduce the 48 h Cu load in the gills, but again the associated increase in protein carbonyl levels was no longer significant though of similar magnitude. However, the high Na^{+} + Cu exposure virtually eliminated the accumulation of Cu in the gills, and the increase in gill protein carbonyl level was no longer significant (Fig 4.3B, D).

Overall, these results suggest that 10 mM Na^{+} offered greater protection than 3 mM Ca^{2+} against Cu uptake and associated oxidative damage. However, we saw changes in mRNA expression levels that we had not predicted. It appeared that elevated Na^{+} plus Cu increased SOD mRNA expression in the liver by 2.5-fold over its respective high Na^{+} control (Fig 4.4A). Likewise we saw an increase in liver CAT mRNA expression after exposure to 10mM Na^{+} plus 15 μg/L Cu (Fig 4B) which was slightly greater than in control fish exposed to high Cu in softwater alone. However this increase did not occur when Cu exposure was combined with elevated Ca^{2+} levels (Fig 4.4B). The general effects of both high Ca^{2+} and high Na^{+} exposures were to greatly attenuate the increases in liver COX-17 mRNA expression caused by high Cu exposure with respect to their controls (Fig 4.4C). Indeed both high Ca^{2+} and high Na^{+} exposures alone appeared to reduce COX-17 expression levels, although there was no significant difference from the soft-water control (Fig 4.4C). There were no significant changes in gill mRNA expression levels for any of the genes tested over the 48 hr experimental period (Table 4.3).

Despite no changes in liver SOD mRNA expression in response to Cu in either the soft-water or high Ca^{2+} treatments (Fig 4.4A), there was a significant increase in SOD enzymatic activity in the liver of zebrafish exposed to 15 μg/L Cu (Fig 4.5A) in these exposures. In contrast, there was no increase in liver SOD activity (Fig 4.5A), despite an increase in liver SOD mRNA expression in zebrafish exposed to high Na^{+} and Cu (Fig 4.4A). In the gill, SOD activity was 6-fold higher in the soft-water plus Cu exposure, and activities declined to levels not significant different from controls with high Na^{+} or Ca^{2+} plus Cu exposures (Fig 4.6A). Although there were no changes in CAT enzyme activity in the liver (Fig 4.5B), there was a significant inhibition of CAT in the gill associated with Cu exposure both in soft-water and in combination with high Ca^{2+} (Fig 4.6B).

The induction of COX-17 mRNA expression by Cu (Fig 4.2C, 4.4C) warranted the examination of the potential changes in oxidative capacity by examining COX and CS enzymatic activity. In the soft-water plus Cu exposure, we saw significant increases in liver COX and CS activity (Fig 4.5C, D), yet only a significant increase in gill COX activity (Fig 4.6C) with no change in gill CS activity (Fig 4.6D). We found that Cu exposure decreased COX: CS ratios in liver and increased COX:CS in gill (Fig 4.7A,B). Zebrafish exposed to high Ca^{2+} plus Cu, exhibited only increased CS activity in the liver (Fig 4.5D). Those fish exposed to high Na^{+} demonstrated decreased liver COX activity (Fig 4.5C). Interestingly, gill CS activity decreased in the Na^{+} control compared to soft-water values, although CS activity did increase in the Na^{+} plus Cu group, compared to its

respective control (Fig 4.6D). Similar to both the soft-water and high Ca^{2+} treatments, high Na^{+} plus Cu exposure group showed a decreased COX:CS ratio (Fig 4.7A).

Discussion

This is the first study to examine the effects of acute Cu exposure on adult zebrafish with respect to markers of oxidative stress. Exposure to sublethal Cu concentrations (15 μg/L) for 48 h in soft-water elicited significant changes in markers of oxidative damage, enzymes in the oxidative stress response pathways, and altered mitochondrial properties. Acute Cu exposure also induced changes in genes involved in oxidative stress response and in a key metalochaperone. Furthermore, results of this study can be use to extend current predictive toxicity models (e.g. the BLM) to a tropical species. In addition it will contribute the framework for future extension of this model to exposures using chronic, environmentally realistic Cu levels that will be necessary for constructing a chronic BLM and lifetime species protection.

Acute Cu exposure in soft-water

In the absence of any protective ions, there was a significant increase after 48 h in the Cu load and oxidative damage, as indicated by increased protein carbonyls, in both the gill and liver of zebrafish (Fig 4.1). Cu accumulation data are congruent with a majority of freshwater Cu exposure studies where elevated Cu levels have been found to increase Cu load in the gill and liver (De Boeck et al 2004, Hansen et al 2006a,b, Kamunde et al 2002, McGeer et al 2000). There were significant reductions in the Cu load in the liver and/or gills at 48 h as well as in the degree of oxidative damage at this time associated with high ambient Cu (15 μg/L) when Ca^{2+} and Na^{+} were added back to the soft-water. However 3.3 mM Ca^{2+} did not seem to provide the same level of protection as 10 mM Na^{+} in this respect (Fig 4.3). While this conflicts with the observation that these levels of Ca^{2+} and Na^{+} have identical protective effects against short-term (3 h) gill Cu accumulation, it is congruent with the finding that 10 mM Na^{+} has a greater protective effect against lethality (25-fold increase) than does 3.3 mM Ca^{2+} (11-fold increase).

It is commonly agreed that both acute and chonic Cu exposures result in elevated ROS production in both mammals and fish (Britton 2002, Manzl et al 2003, 2004). For this reason, we examined representative genes that encode proteins involved in combating oxidative stress (SOD, CAT) to determine if these might serve as molecular endpoints of acute Cu toxicity. We found significant changes in liver CAT expression (Fig 4.2B), indicative that transcription of this gene is stimulated by oxidative stress with soft-water Cu exposure. Surprisingly, we saw no changes in SOD mRNA expression with acute Cu exposure in soft-water (Fig 4.2A). Contrary to these transcriptional changes, we found a significant increase in liver SOD enzymatic activity but no change in CAT enzymatic activity with Cu in soft-water (Fig 4.5A, B). This suggests that some antioxidant enzymes are not initially controlled by transcriptional means, yet rather by enzyme activation, at least in the case of SOD (Fig 4.2A). Moreover, it is known that superoxide radicals can directly inhibit CAT activity (Kono & Fridovich 1982). Therefore, if the initial Cu effect is an increase in superoxide radicals, then CAT activity might be inhibited, and this in

turn could stimulate transcript expression. In contrast, other studies seem to indicate increased expression of antioxidant enzyme transcripts in the liver occurring 1-2 weeks post-exposure (Hansen et al 2006a, 2006b). We saw no changes in the gene expression of SOD, CAT, or COX-17 gene expression in the gills (Table 4.3). However, in zebrafish gills, SOD activity is increased and CAT activity decreased at 48 h of Cu exposure (Fig 4.6A, B). This pattern, along with protein carbonyl production suggests a rapid rise in oxidative stress and comparably rapid response. Future studies should examine the response to chronic Cu and oxidative stress to elucidate the long-term changes in gene expression and enzyme activities of the ROS scavenging system.

Protective effects of Ca^{2+} and Na^{+}

Since it is well documented in other fish species that certain cations can have a protective effect against both metal uptake and metal toxicity (Baldisserotto et al 2004, Morgan et al 2004, Pagenkopf 1983, Paquin et al 2002), we added Na^{+} and Ca^{2+} back into the soft-water medium. Our results confirm in a tropical species the protective effect of these cations against Cu toxicity. While these were added as chloride salts, there is extensive evidence that protection against Cu toxicity is provided by the cations, rather than by Cl^{-} anions. (Paquin et al 2002).

It has long been know that Cu disrupts Na^{+} homeostasis through competition for apical uptake sites and inhibition of basolateral $Na^{+}K^{+}$ATPase in the gills (Grosell & Wood 2002, Lauren & McDonald 1987, Wood 2001), which explains the protective effects of elevated Na^{+}. However, the protective effects of Ca^{2+} to Cu toxicity are poorly understood, since disruption in Ca^{2+} uptake is mainly attributed to other metals which are direct calcium analogues such as cadmium and lead (Capkova et al 2002, Rogers &, Wood 2004, Verbost et al 1987,1989, Wong & Wong 2000, Wood 2001). Certainly this study indicates that Ca^{2+}, and therefore water hardness, does lessen acute Cu toxicity, but the mechanisms by which this occurs are currently unclear. Potentially unreduced Cu^{2+} may travel across the gill apical surface via epithelial calcium channels (ECaC) in the same fashion as Cd^{2+} (Verbost et al 1987, 1989). With soft-water acclimation alone, ECaC mRNA expression and protein levels increase 4 and 3-fold, respectively, and this change in transporter abundance may accentuate Cu sensitivity in zebrafish (Craig et al 2007). However, the partial protection in the current study by elevated Ca^{2+} may have effectively increased competition with Cu at Ca^{2+} uptake sites, yet still allowed Cu to travel though other pathways such as apical Na^{+} channels. However, in zebrafish, a primary uptake pathway for Cu appears to be the apical Na^{+} channels, similar to the situation in trout (Grosell & Wood 2002), since we saw a significant reduction in both Cu load and oxidative damage in both the gill and liver when 10mM Na^{+} was included in the high Cu exposure (Fig 4.3).

With the Ca^{2+} plus Cu treatment, we saw no change in SOD or CAT gene expression in the liver, indicating that increased Ca^{2+} limited the impact of Cu on expression of these genes (Fig 4.4A, B). However, in gill, the apparent repression of CAT activity was still present in this exposure, possibly indicative of ROS-induced inhibition (Fig 4.6B), although there were no transcriptional changes (Table 4.3). A surprising result was the large increase in both SOD and CAT transcription levels in the liver of fish

exposed to high Na^+ plus Cu (Fig 4.4A, B) despite the fact that Cu load and oxidative damage is reduced (Fig 4.3). Furthermore, there was no association between increased transcription and enzyme activity, highlighted by the lack of change in SOD and CAT activity in both tissues (Fig 4.5A, B; Fig 4.6A, B, respectively). One possible explanation is that high Na^+ levels by themselves resulted in increased oxidative stress, which was exacerbated by the addition of Cu, although elevated tissue Na^+ levels were only apparent in the gill, not the liver (Table 4.2). High NaCl has been shown to induce oxidative stress in the renal medullary cell cultures and isolated perfused rat livers (Zhang et al 2004, Saha et al 1992, respectively). Moreover, there was a significant repression of gill CAT activity in the 10mM Na^+ control group compared to the soft-water control (t-test, $p=0.048$). Again, this may be oxidative stress related, although to a lesser extent than in soft-water, since we did not see any significant increase in protein carbonyls in gills (Fig 4.3D). In total, Ca^{2+} and Na^+ did provide protection against Cu toxicity, with Na^+ effectively reducing Cu load by 50%, and protein carbonyl levels 60% over reductions due to Ca^{2+} in the liver. Likewise, Na^+ provided 40% greater protection than Ca^{2+} when gill Cu load was examined. By reducing the Cu load, both liver and gill tissues are protected from the acute effects of high Cu exposure.

Cu toxicity and mitochondrial targets

One of the main targets of metal toxicity is the mitochondrion, and there is a close relationship between metal-induced oxidative stress and proper mitochondrial function, as seen in mammals and fish (Krumschnabel et al 2005, Pourahmad & O'Brien 2000, Risso-de Faverney et al 2004). Many studies have examined the *in vitro* impact of metals on mitochondrial respiration and energetics in fish (Manzl et at 2003, 2004), but to our knowledge, this is the first the look at the *in vivo* effects of Cu exposure on markers of mitochondrial density (CS) and oxidative capacity of the inner mitochondrial membrane (COX). After 48h exposure to Cu, we saw a significant increase in the mRNA expression of COX-17 (Fig 4.2C), which is a key metalochaperone essential in the proper assembly of COX (12, 20). Upon addition of Ca^{2+} and Na^+, the increased COX-17 expression persisted but at a reduced level, despite the apparent protective effects of Ca^{2+} and Na^+ (Fig 4.4C). Whether this is due to the interactions between Cu levels and COX-17 - Cu handling and stimulation of transcription are unclear and further investigation into the changes in protein abundance, and amount of COX-17 bound Cu with varying cytosolic Cu is warranted. However, we did see an increase in maximal COX activity in both the gill and liver of zebrafish exposed to soft-water plus Cu (Fig 4.5C, 4.6C). COX is a key enzyme in the electron transport chain, located in inner mitochondrial membrane. An increase in Vmax for COX suggests an increase in COX protein and both elevated oxygen consumption potential but also an increase in oxidative ATP production capacity. Manzl *et al* (2003) found that in isolated trout hepatocytes, Cu can increase cellular O_2 consumption, yet there is no change in ATP production compared to basal values, indicating mitochondria were in a partially uncoupled state. It has been shown in mammals that uncoupling respiration from ATP production leads to a reduction in ROS production in the mitochondria (Arsenijevic et al 2000, Duval et al 2002) since there is a relationship between membrane potential and ROS production (Krumschnabel et al

2005). Potentially, Cu exposed fish may use uncoupled respiration as an adaptive response to Cu-induced ROS production, since increased proton leak lowers the membrane potential and leads to a significant reduction of ROS production (Marcienk 2006). Answering these questions represents a fruitful area for future research.

We also measured CS activity as a marker of mitochondrial volume density (Reichmann et al 1985), which significantly increased in liver with the soft-water plus Cu exposure (Fig 4.5). Increases in the COX:CS ratio can be used as an indicator of relative oxidative capacity or a decrease in this ratio to can be used to indicate general mitochondrial dysfunction (Capkova et al 2002). We saw a significant increase in COX:CS in the gill, yet a substantial decrease in this ratio within liver with acute Cu toxicity. These changes may in part be due to the gill morphological changes associated with soft-water acclimation which might be further stimulated with Cu exposure. For instance, in soft-water exposed rainbow trout, there is a distinct increase in the number of mitochondrial rich chloride cells, impacting the blood-to-water diffusion distance, thereby impacting gas transfer (Greco et al 1996). This may explain why the control Ca^{2+} and Na^{+} treatments lowered liver COX:CS ratios compared to the soft-water control. As for the impact of Cu on the COX:CS ratio, we saw a significant drop in liver compared to the soft-water control (Fig 4.7A). This may by indicative of mitochondrial dysfunction as the increased Cu may inhibit proper COX assembly, although this is only apparent in the soft-water plus Cu exposure group. An alternate explanation is the impact that ROS can have on the lipid membrane of mitochondria. ROS are known to increase lipid peroxides (Bagnyukova et al 2006, Radi & Matkovics 1988) thereby altering the lipid membrane of the mitochondria. Changes in lipid membrane composition have been shown to alter the activity of membrane bound COX in carp (Wong & Wong 2000). This may explain the increases found in COX activity in both the gill and liver of Cu exposed zebrafish (Fig 4.5C, 4.6C). In the gills, there was no change in the relative oxidative capacity between the Ca^{2+} and Na^{+} treatments, again demonstrating potential protective effects of water hardness and ions on Cu toxicity.

Perspectives

We show here that zebrafish are a powerful model for the study of oxidative stress and metal toxicology *in vivo*. A relatively low, environmentally relevant level of Cu in soft-water (15 μg/L) has immediate impacts on oxidative damage and antioxidant protection pathways in the zebrafish. Furthermore, by increasing either the water Ca^{2+} or Na^{+} concentration, we can protect against the acute toxic effects of elevated waterborne Cu, although 3.3 mM Ca^{2+} is not as effective as 10 mM Na^{+}. Interestingly, with the exception of COX-17, we are able to abolish the transcription expression effects of Cu by 3.3 mM Ca^{2+}, and it appears that 10 mM Na^{+} allows for a transcriptional response to occur. Furthermore, 10 mM Na^{+} exposure with Cu decreased the oxidative damage and overall Cu load in both the gills and liver. With increased Cu exposure, there is an increase in the relative oxidative capacity at the gills, but indications of mitochondrial dysfunction in the liver, indicating these as possible physiological endpoints of acute toxicity. Even more apparent is the relationship between increasing ambient Cu concentration and induction of COX-17 mRNA expression (R^2= 0.851; p=0.009), which

provides a key molecular determinant of acute toxicity. Combined, the rapid induction of these physiological and transcriptional endpoints with acute exposures suggests that they should be applied further to chronic Cu studies. Moreover, as a representative tropical species zebrafish will provide essential information for the development of water quality guidelines.

Table 4.1: Forward (F) and reverse (R) primers used for real-time PCR.

Gene	Accession #	Primer	Amplicon
COX-17	NM001004652	F - gcagcgcagaaaaagccact	200
		R - acgcaagcagtcacacacat	
CAT	AF170069	F - agggcaactgggatcttaca	499
		R - tttatgggaccagaccttgg	
Cu/ZnSOD	Y12236	F - ggccaaccgatagtgttaga	157
		R - ccagcgttgccagtttttag	
EF1α	NM131263	F - gtgctgtgctgattgttgct	201
		R - tgtatgcgctgacttccttg	

Table 4.2: Liver and gill tissue concentrations of sodium and calcium for soft-water acclimated control zebrafish and after exposure to 3.3mM Ca^{2+}, and 10mM Na^{+} for a 48h period with and without 15 μg/L Cu. Values presented as mean ± SEM. * indicates significant difference from respective control ($p<0.05$, n=6 for all treatments).

	Treatment	Liver		Gill	
		Control	15 μg/L Cu	Control	15 μg/L Cu
Na load					
	Soft-water	38.9 ± 6.5	40.4 ± 8.5	45.6 ± 3.6	48.6 ± 2.7
	3.3mM Ca	54.1 ± 1.6	62.6 ± 18.5	54.1 ± 1.6	58.4 ± 6.3
	10mM Na	43.9 ± 3.5	38.0 ± 9.8	55.5 ± 1.3	60.7 ± 2.8*
Ca Load					
	Soft-water	11.6 ± 3.7	15.5 ± 4.5	126.8 ± 10.3	122.3 ± 13.5
	3.3mM Ca	8.1 ± 0.8	21.9 ± 6.7*	125.1 ± 16.1	136.4 ± 11.8
	10mM Na	12.5 ± 4.8	8.3 ± 3.7	125.3 ± 18.5	123.2 ± 12.7

Table 4.3: mRNA expression of superoxide dismutase (SOD), catalase, and cytochrome oxidase subunit-17 (COX-17) in the gills of soft-water acclimated zebrafish exposed to 15 μg/l Cu for 48h in either soft-water, soft-water + 3.3mM Ca^{2+}, or soft-water + 10mM Na^{+}. Gene expression values were normalized to EF1α, expressed as a ratio of the control (control = 1), and are presented as mean ± SEM. There were no significant difference when compared to control values (n=6 for all treatments, $p>0.05$).

Treatment		Gill	
		Control	15 μg/L Cu
SOD			
	Soft-water	1.00 ± 0.37	1.55 ± 0.48
	3.3mM Ca	1.00 ± 0.56	0.67 ± 0.23
	10mM Na	1.00 ± 0.23	0.81 ± 0.24
CAT			
	Soft-water	1.00 ± 0.25	0.67 ± 0.18
	3.3mM Ca	1.00 ± 0.41	1.28 ± 0.35
	10mM Na	1.00 ± 0.37	1.15 ± 0.48
COX-17			
	Soft-water	1.00 ± 0.11	1.11 ± 0.08
	3.3mM Ca	1.00 ± 0.18	0.96 ± 0.12
	10mM Na	1.00 ± 0.16	0.95 ± 0.11

Fig 4.1

Liver and gill Cu load (μg/g tissue; **A** & **B** respectively) and protein carbonyl levels (nmol/mg protein; **C** & **D**, respectively) from soft-water acclimated zebrafish exposed to 8 μg/L or 15 μg/L Cu for 1, 4, 24, or 48 h. Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).

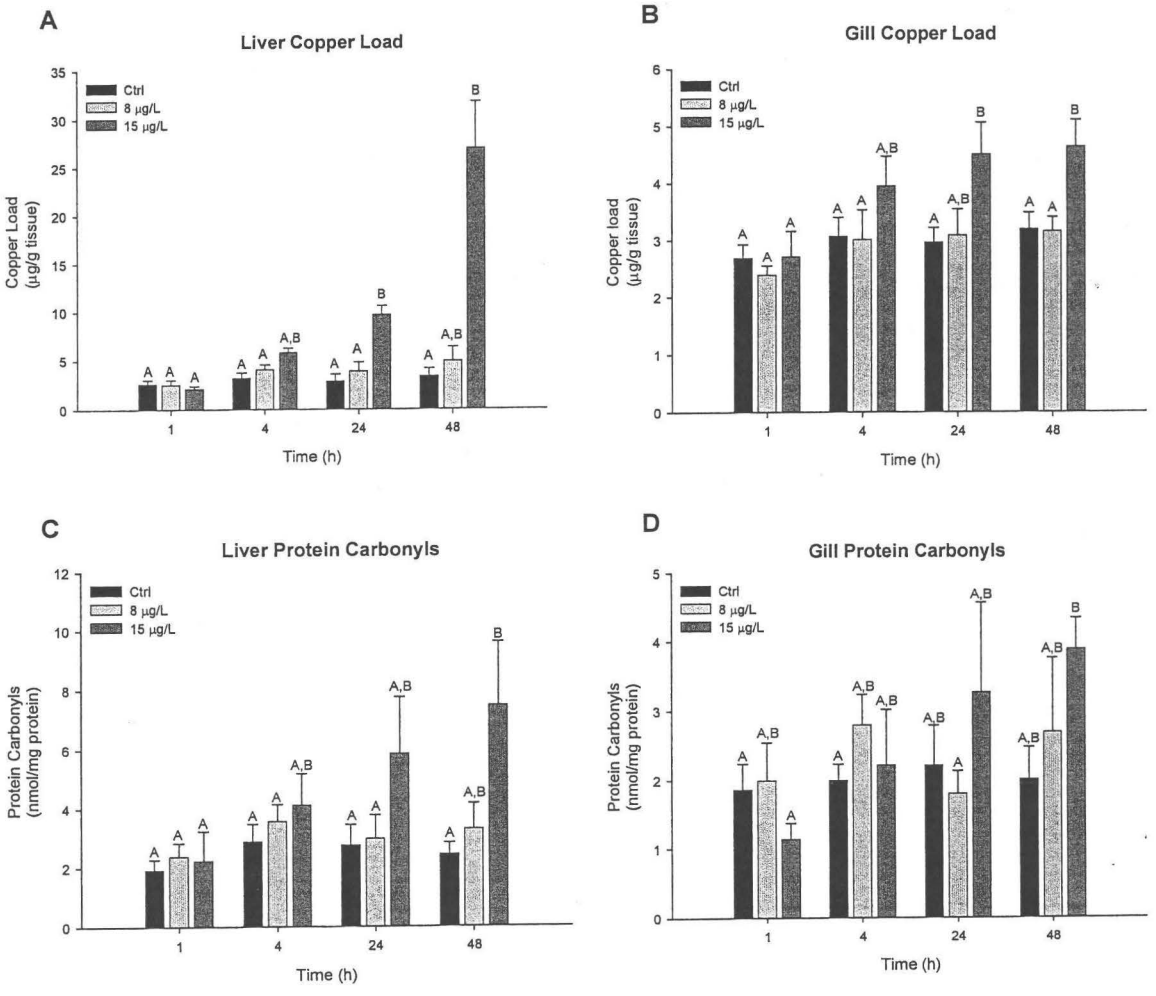
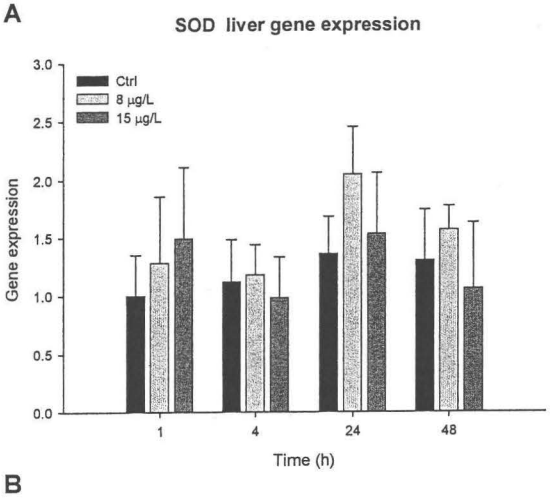
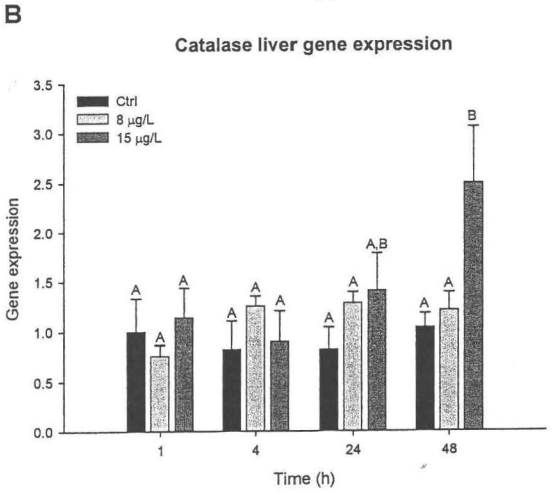

A
Liver Copper Load
Ctrl
8 µg/L
15 µg/L
Copper Load (µg/g tissue)
Time (h)
B
Gill Copper Load
Copper load (µg/g tissue)
C
Liver Protein Carbonyls
Protein Carbonyls (nmol/mg protein)
D
Gill Protein Carbonyls

Fig 4.2

mRNA expression of (**A**) superoxide dismutase (SOD), (**B**) catalase, and (**C**) cytochrome oxidase subunit-17 (COX-17) from the liver of soft-water acclimated zebrafish exposed to 8 μg/L or 15 μg/L Cu for 1, 4, 24, or 48 h. Gene expression values were normalized to EF1α, expressed as a ratio of the control (control = 1), and are presented as mean ± SEM. Treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).


A
SOD liver gene expression
Ctrl
8 µg/L
15 µg/L
Gene expression
Time (h)
1
4
24
48


B
Catalase liver gene expression
Ctrl
8 µg/L
15 µg/L
Gene expression
Time (h)
A
A,B
B

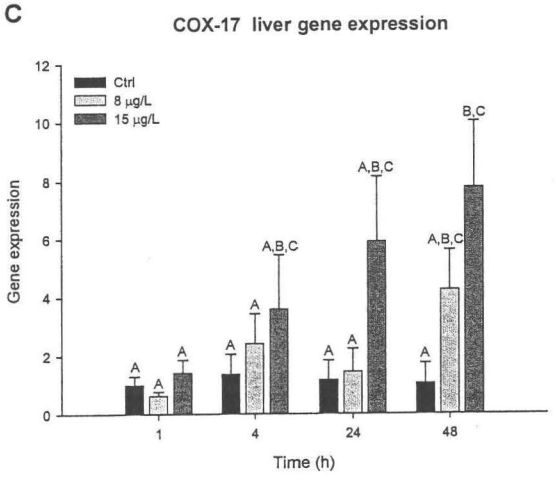

C
COX-17 liver gene expression
Ctrl
8 µg/L
15 µg/L
Gene expression
Time (h)
A
A,B,C
B,C

Fig 4.3

Liver and gill Cu load (μg/g tissue; **A** & **B** respectively) and protein carbonyl levels (nmol/mg protein; **C** & **D**, respectively) from soft-water acclimated zebrafish exposed to 15 μg/l Cu for 48h in either soft-water, soft-water + 3.3mM Ca^{2+}, or soft-water + 10mM Na^{+}. * indicates significance from the soft-water control, and § indicates significance within a treatment. Values are presented as means ± SEM (n=6 for all treatments, $p<0.05$).

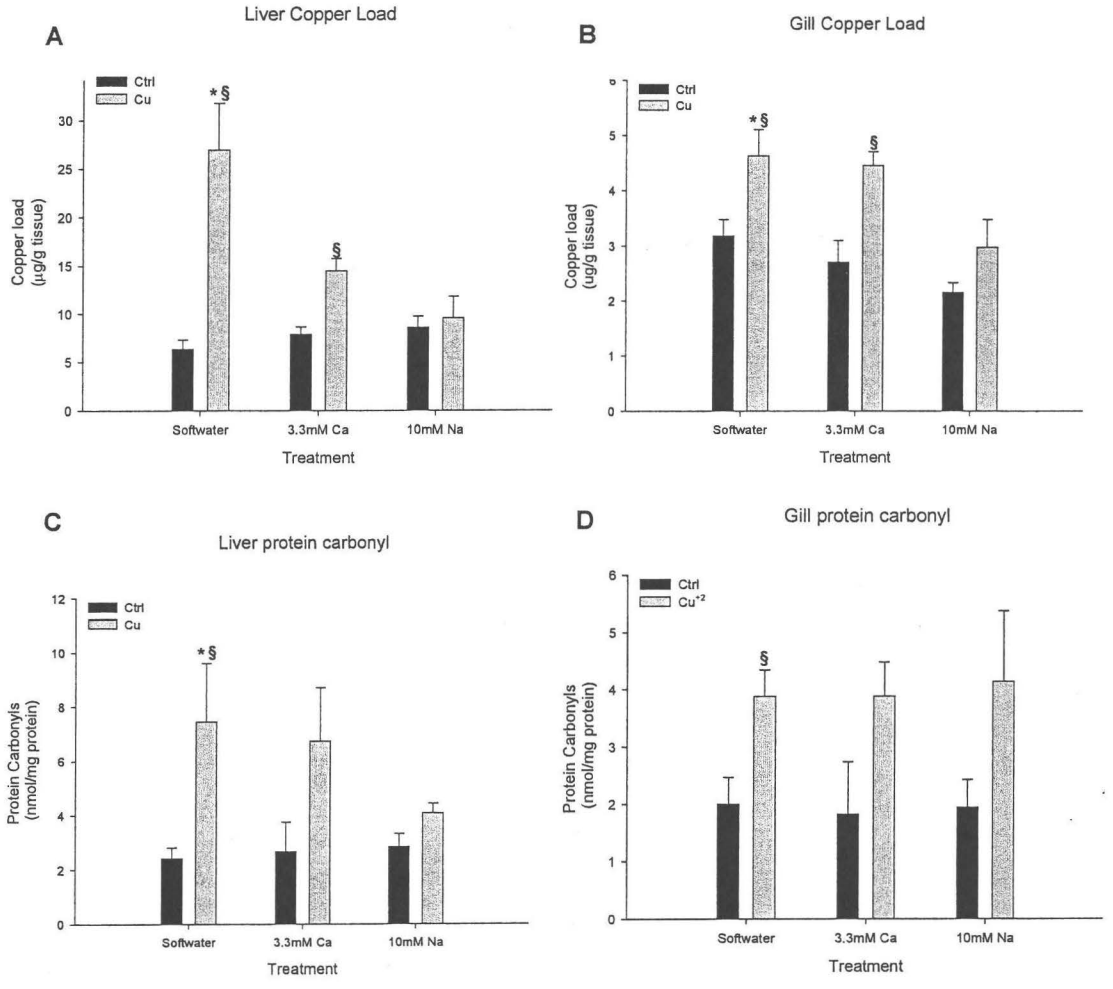
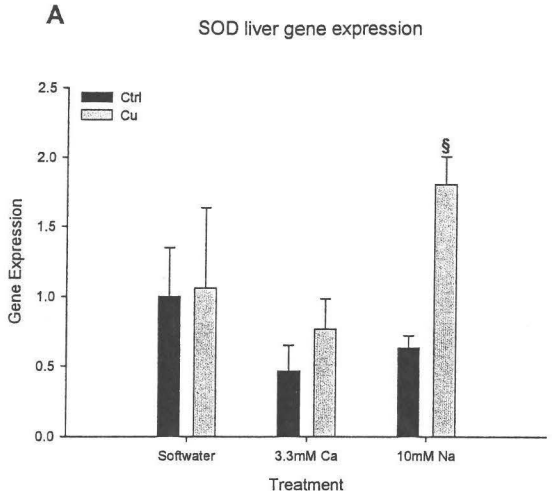
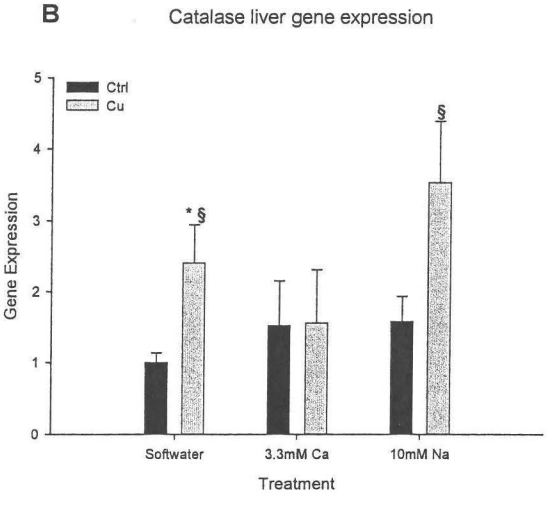

A
Liver Copper Load
Ctrl
Cu
*§
§
Copper load (µg/g tissue)
0
5
10
15
20
25
30
Softwater
3.3mM Ca
10mM Na
Treatment
B
Gill Copper Load
Ctrl
Cu
*§
§
Copper load (ug/g tissue)
0
1
2
3
4
5
Softwater
3.3mM Ca
10mM Na
Treatment
C
Liver protein carbonyl
Ctrl
Cu
*§
Protein Carbonyls (nmol/mg protein)
0
2
4
6
8
10
12
Softwater
3.3mM Ca
10mM Na
Treatment
D
Gill protein carbonyl
Ctrl
Cu+2
§
Protein Carbonyls (nmol/mg protein)
0
1
2
3
4
5
6
Softwater
3.3mM Ca
10mM Na
Treatment

Fig 4.4

mRNA expression of (**A**) superoxide dismutase (SOD), (**B**) catalase, and (**C**) cytochome oxidase subunit-17 (COX-17) from the liver of soft-water acclimated zebrafish exposed to 15 μg/l Cu for 48h in either soft-water, soft-water + 3.3mM Ca^{2+}, or soft-water + 10mM Na^{+}. Gene expression values were normalized to EF1α, expressed as a ratio of the control (control = 1), and are presented as mean ± SEM. * indicate significance from the soft-water control, and § indicates significance within a treatment. Values are presented as means ± SEM (n=6 for all treatments, $p<0.05$).


A
SOD liver gene expression
Ctrl
Cu
Gene Expression
2.5
2.0
1.5
1.0
0.5
0.0
§
Softwater
3.3mM Ca
10mM Na
Treatment


B
Catalase liver gene expression
Ctrl
Cu
Gene Expression
5
4
3
2
1
0
* §
§
Softwater
3.3mM Ca
10mM Na
Treatment

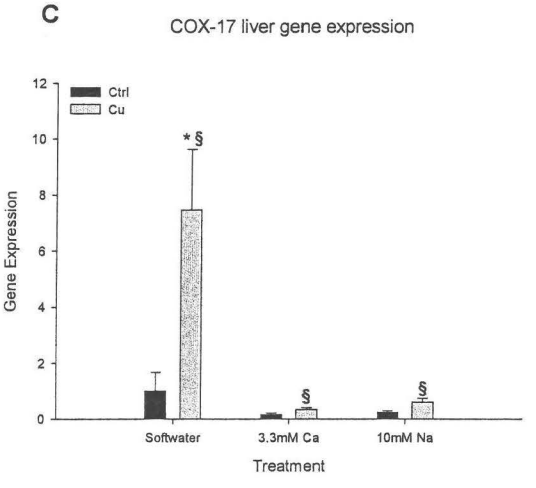

C
COX-17 liver gene expression
Ctrl
Cu
Gene Expression
12
10
8
6
4
2
0
* §
§
§
Softwater
3.3mM Ca
10mM Na
Treatment

Fig 4.5

Liver enzyme activity (U/mg protein) for (**A**) superoxide dismutase (SOD), (**B**) catalase, (**C**) cytochrome C oxidase (COX), and (**D**) citrate synthase (CS) from the liver of soft-water acclimated zebrafish exposed to 15 μg/l Cu for 48h in either soft-water, soft-water + 3.3mM Ca^{2+}, or soft-water + 10mM Na^{+}. * indicate significance from the soft-water control, and § indicates significance within a treatment. Values are presented as means ± SEM (n=6 for all treatments, $p<0.05$).

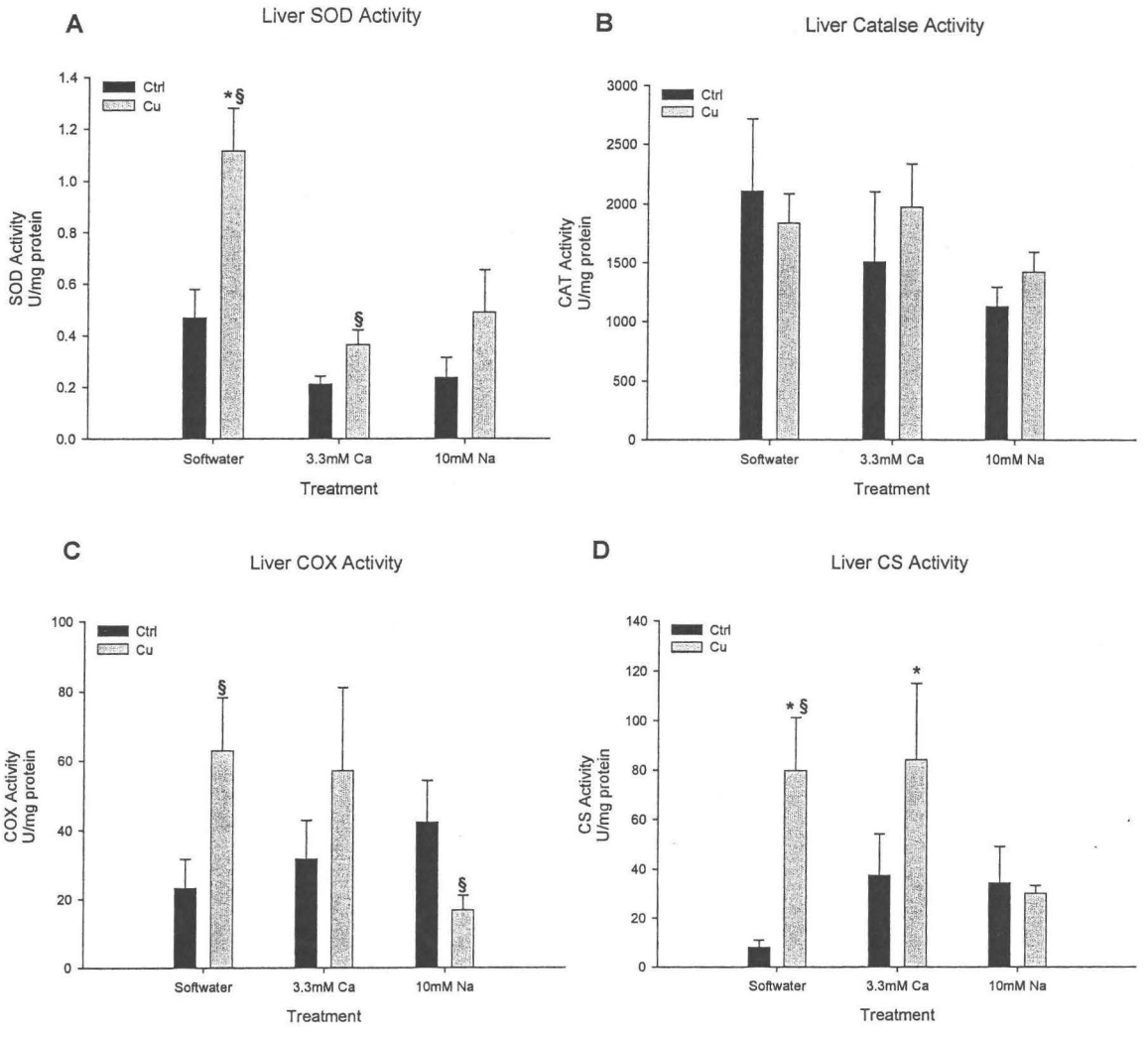

A
Liver SOD Activity
SOD Activity U/mg protein
Ctrl
Cu
*§
§
Softwater
3.3mM Ca
10mM Na
Treatment
B
Liver Catalse Activity
CAT Activity U/mg protein
Ctrl
Cu
Softwater
3.3mM Ca
10mM Na
Treatment
C
Liver COX Activity
COX Activity U/mg protein
Ctrl
Cu
§
§
Softwater
3.3mM Ca
10mM Na
Treatment
D
Liver CS Activity
CS Activity U/mg protein
Ctrl
Cu
*§
*
Softwater
3.3mM Ca
10mM Na
Treatment

Fig 4.6

Gill enzyme activity (U/mg protein) for (**A**) superoxide dismutase (SOD), (**B**) catalase, (**C**) cytochrome C oxidase (COX), and (D) citrate synthase (CS) from the liver of soft-water acclimated zebrafish exposed to 15 μg/l Cu for 48h in either soft-water, soft-water + 3.3mM Ca^{2+}, or soft-water + 10mM Na^{+}. * indicates significance from the soft-water control, and § indicates significance within a treatment. Values are presented as means ± SEM (n=6 for all treatments, $p<0.05$).

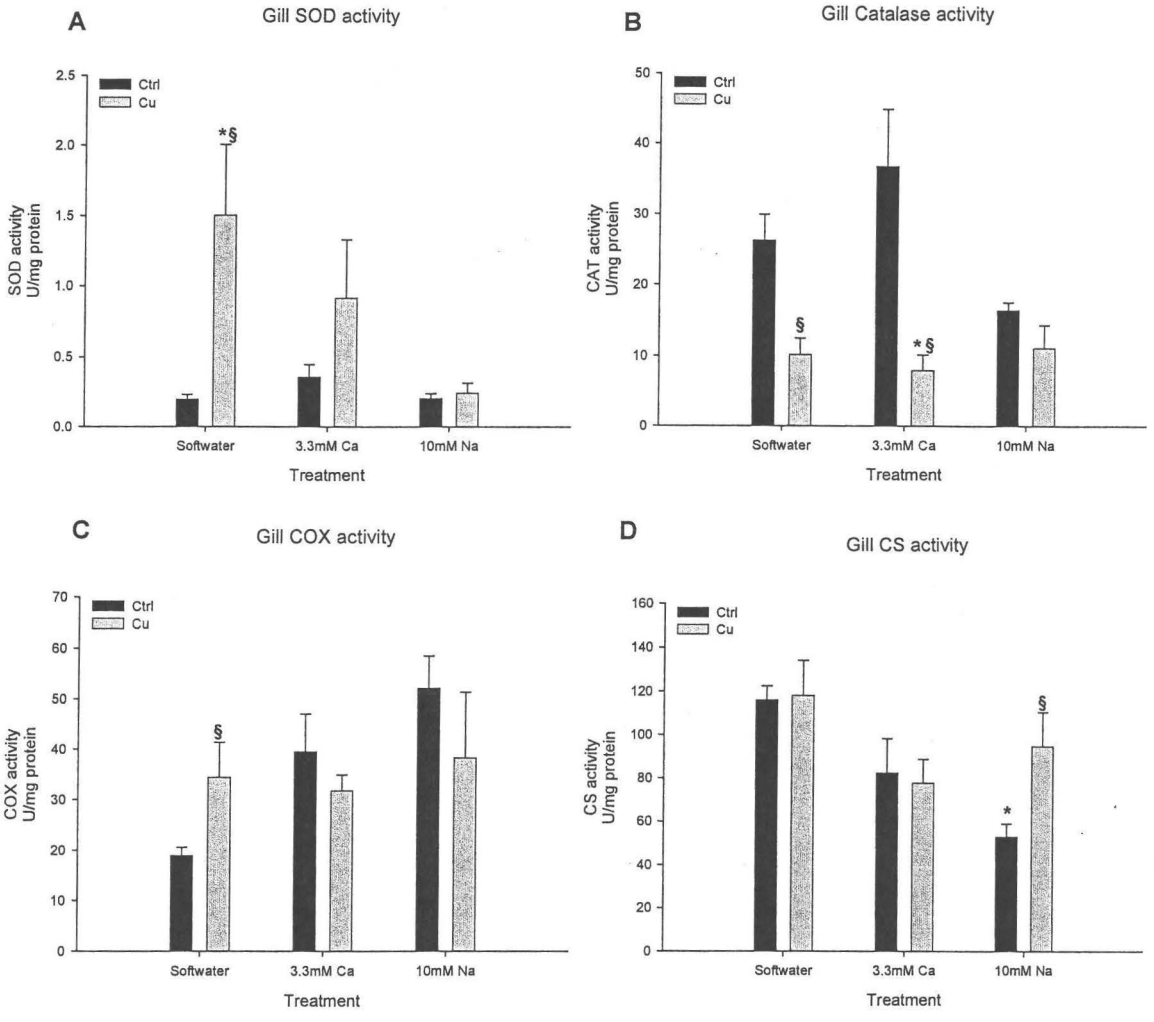
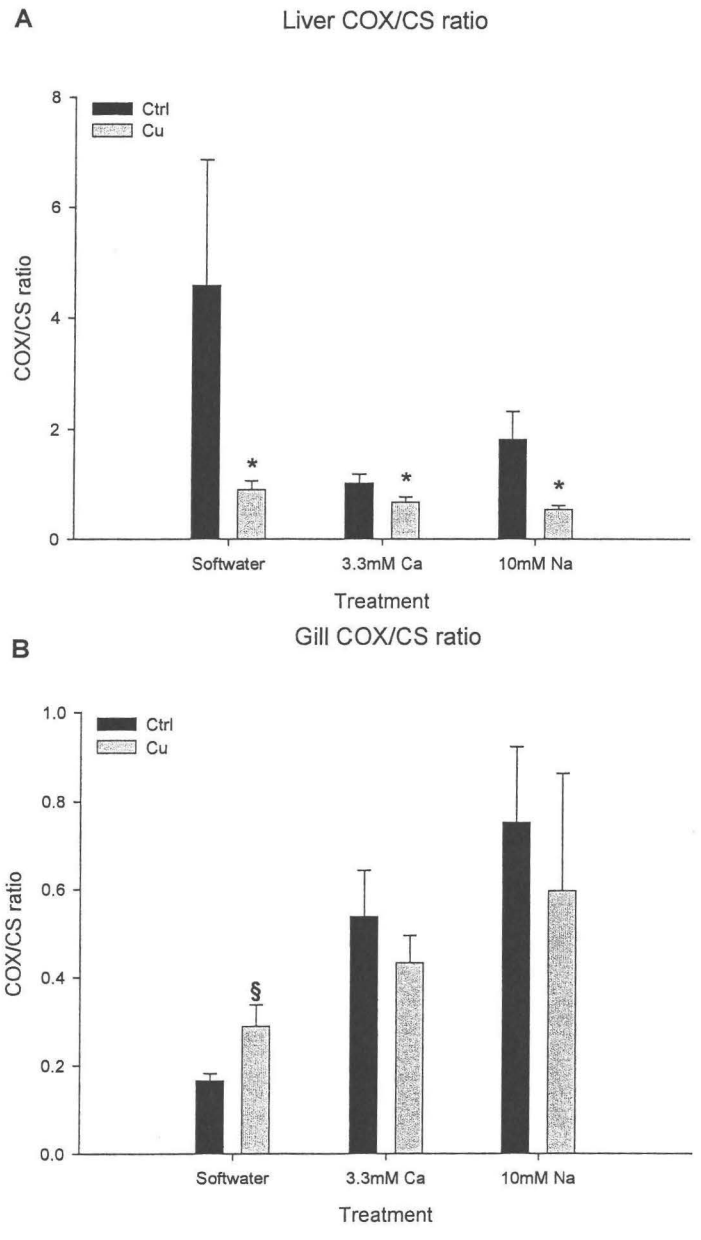

A
Gill SOD activity
SOD activity U/mg protein
Ctrl
Cu
*§
Softwater
3.3mM Ca
10mM Na
Treatment
B
Gill Catalase activity
CAT activity U/mg protein
§
*§
C
Gill COX activity
COX activity U/mg protein
D
Gill CS activity
CS activity U/mg protein
*

Fig 4.7
(**A**) Liver and (**B**) gill cytochrome C oxidase to citrate synthase (COX/CS) ratios for soft-water acclimated zebrafish exposed to 15 μg/l Cu for 48h in either soft-water, soft-water + 3.3mM Ca^{2+}, or soft-water + 10mM Na^{+}. * indicates significance from the soft-water control, and § indicates significance within a treatment. Values are presented as means ± SEM (n=6 for all treatments, $p<0.05$).


A
Liver COX/CS ratio
Ctrl
Cu
COX/CS ratio
8
6
4
2
0
*
*
*
Softwater
3.3mM Ca
10mM Na
Treatment
B
Gill COX/CS ratio
Ctrl
Cu
COX/CS ratio
1.0
0.8
0.6
0.4
0.2
0.0
§
Softwater
3.3mM Ca
10mM Na
Treatment

CHAPTER 5

WATER CHEMISTRY ALTERS GENE EXPRESSION AND PHYSIOLOGICAL ENDPOINTS OF CHRONIC WATERBORNE COPPER EXPOSURE IN ZEBRAFISH, *DANIO RERIO*

Abstract

This is the first study to implement a genomic approach to ascertain both transcriptional and functional endpoints of chronic Cu toxicity in fish associated with experimentally manipulated water chemistries. Over 21d, soft-water acclimated zebrafish were exposed to: soft-water (Ctrl), 12μg/L Cu (Cu), 3.3mM Na^+ (Na), 3.3 mM Na^+ + 12μg/L Cu (NaCu), 3.3mM Ca^{2+} (Ca), or3.3mM Ca^{2+} + 12μg/L Cu (CaCu). Although effective at reducing Cu load in all tissues, Na^+ in the presence of Cu did not decrease the degree of oxidative damage, particularly in the gill and gut. In contrast, we found that CaCu treatment enhanced the degree of Cu load in both the liver and gut, although oxidative damage appeared to be minimized. Transcriptional analysis of candidate genes of interest indicated a majority of downregulated transcripts associated with the Cu only treatment, and CaCu treatment rehabilitated only some of the genes to control transcript levels. Conversely, the NaCu treatment had a strong, opposing affect when compared to that of Cu alone. On a more global scale, using zebrafish Affymetrix GeneChips®, we found significantly clustered patterns of expression, where quantitative changes in expression induced by Cu appeared to be opposite to the majority of the other treatments. Moreover, we have compiled data on the preventative or enhancing effects of Na^+ and Ca^{2+} both alone and in the presence of Cu, which may in future facilitate the incorporation of gene expression endpoints into a Biotic Ligand Modelpredicting chronic Cu toxicity in this tropical species.

Craig, P.M.., Wood, C.M., and McClelland, G.B. To be Submitted to Environmental Science & Technology July/2009.

Introduction

Water chemistry is the major factor influencing the toxic effects of contaminant metals in the natural environment. Water hardness, principally Ca^{2+} and Mg^{2+} concentrations, provides protection to an organism since these ions mitigate toxicity by competing with metal for uptake sites (Zitko & Carson 1976). Furthermore, factors such as water pH and dissolved organic carbon (DOC) can modify metal speciation and in turn modulate toxicity (Hutchinson & Collins 1978, Pagenkopf 1983). These and other factors, such as Na^{+} concentrations, determine the physiochemical properties of water and shape the toxicological effects of metal contaminants in the natural environment. The Biotic Ligand Model (BLM; Paquin et al 2002, Niyogi & Wood 2004), is a predictive model which takes into account the effects of protective ions and other aspects of water chemistry. This model is based on a fixed relationship between short-term gill metal accumulation and ultimate toxicity to an organism. However, the BLM model is based on acute endpoints (metal accumulation and mortality), and does not take into account non-phenotypic changes related to increased metal contamination, such as changes in gene expression that can have potent effects on a chronic time scale. With advanced techniques in genome-wide assessment (microarrays) now commercially available for many aquatic species (e.g., . channel catfish, medaka, zebrafish, rainbow trout), incorporating gene expression endpoints into models of chronic toxicity for use in environmental protection standards is possible. The primary objective of this study on zebrafish was to use a microarray approach to examine the global gene expression response to chronic waterborne copper (Cu) exposure, at environmentally realistic levels, under various water chemistries

Zebrafish have been studied extensively in the past, as a model for developmental biology studies and to a lesser extend for examining vertebrate physiology. Yet more recently, zebrafish have gained popularity as a model for aquatic toxicology, particularly due to their softwater tolerance, a key facet in assessing metal toxicity in aquatic species in the virtual absence of competing cations and complexing anions (Craig et al 2007a, 2009). Cu is an essential micronutrient taken up primarily by the digestive tract, and in part, via the gills, through both specific (e.g., copper transporter 1; Mackenzie et al 2004) and non-specific mechanisms (e.g., Na^{+} dependent transport; see review Handy et al 2002, Burke & Handy 2005). Further evidence suggests that Cu uptake can be modulated by elevated ambient Ca^{2+}, which has been found to inhibit Cu accumulation. Instead of affecting a particular membrane transport process, this effect is probably associated with Ca^{2+} reducing membrane permeability by stabilizing tight junctions (Hunn 1985, Playle et al 1992). A previous experiment examined the impact that increased concentrations of competitive ions (Ca^{2+} and Na^{+}) had on acute waterborne copper toxicity, and found that Na^{+} had a greater protective effect over Ca^{2+} in reducing gill and liver Cu load (Craig et al 2007b). Certainly this provides evidence that elevated waterborne Na^{+} and Ca^{2+} concentrations are protective against Cu on an acute scale. However, to our knowledge no studies have examined the protective effects of equal ion concentrations on a chronic time frame in zebrafish.

Despite Cu being an essential micronutrient, excessive Cu has a broad range of detrimental effects including increased oxidative damage, disrupted ion-regulation and

growth, and Cu can play a role in endocrine disruption (Dethloff et al 1999, Zahner et al 2006, Craig et al 2007b, 2009, Mohanty et al 2009). Certainly, these responses may only occur under relatively high levels of Cu exposure, yet chronic and environmentally meaningful exposure levels may not necessarily elicit such a pronounced response. Measuring gene expression provides a useful tool to identify a transcriptomic response at lower metal levels, and possibly before a more dramatic response occurs. Essentially, there are two approaches in determining the gene expression endpoints of chronic exposure. This study used both approaches. The first is a direct approach where candidate genes of particular interest are selected because *a priori*, they are expected to be affected by metals. We have selected several candidate genes not only to validate genes that changed on our array experiment, but also genes of interest that relate to Cu exposure. The second is the application of microarray techniques that take more of a shotgun approach, where thousands of genes can be assessed at a given time point.

Of particular interest to understanding Cu toxicity are genes related to Cu transport, specifically the apical copper transporter (crt-1) and the basolateral Cu-ATPase (atp7a). Although both are well characterized in mammalian systems (Lee et al 2002, Sharp 2002) and identified as essential Cu transporters during embryogenesis (Mackenzie et al 2004, Mendelsohn et al 2006, Madsen 2008), there are relatively few studies (Craig et al 2009) that have examined the expression levels of these transporters in adult zebrafish. We have previously shown that softwater acclimation alone can alter the mRNA expression of ctr-1, so it was of interest to look at transcript levels of this transporter with increased waterborne cations (Ca^{2+} and Na^{+}, Craig et al 2007a). With respect to waterborne Cu exposure (8 μg/L), we have seen that increased Cu induces a transcriptional response in both ctr-1 and atp7a, with an increase in expression but decreased functional activity and protein levels of ctr-1 (Craig et al 2009). As previously mentioned, Ca^{2+} can additionally play a protective role, thus it is essential to examine the apical epithelial calcium channel transcript levels to assess the competitive interactions between Cu and Ca^{2+}.

Cu plays a role in the stimulation of the hypothalamic-pituitary-interrenal axis in fish (Solaiman et al 2001, McGeer et al 2000). Moreover, we have shown that 21d Cu exposure increases whole body cortisol levels. There is an additional transcriptomic response, depending on the dose of Cu, related to estrogen receptors, validated both via microarray experimentation and qPCR analysis (Craig et al 2009). Cu has recently been shown to interact with estrogen receptors, along with Zn and Cd, indicating a potential role in endocrine disruption (Denier et al 2009), and therefore warranting us to investigate changes in estrogen receptor gene expression (esr-1) in this study.

This study employs commercially available Affimetrix GeneChip® Zebrafish Genome Array to ascertain the impact of waterborne Cu on the transcriptomic profile in zebrafish and help identify gene expression endpoints of chronic Cu exposure. Furthermore, by altering concentrations of Ca^{2+} and Na^{+}, we can gain further insight into the protective effects of these cations on both a functional (enzymatic/ oxidative damage) and transcriptional level. The BLM incorporates the competition of free metal ions (e.g. Cu^{2+}) with other naturally occurring cations (e.g. Na^{+} & Ca^{2+}) for binding with the biotic ligand, the site of toxic action for the organism, and our manipulation of water chemistry

related to chronic Cu exposure allows for future incorporation of gene expression endpoints into a BLM to predict chronic Cu toxicity in this tropical species.

Methods & Materials

Animals

Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local pet fish distributor (DAP International, Canada) and acclimated to soft-water (Na^+ 83.9 ± 1.1 μM, Ca^{2+} 63.4 ± 1.4 μM, Mg^{2+} 26.0 ± 0.3 μM, Cu^{2+} 2.9 ± 0.1 μg/L, hardness 8.9 ± 0.1 mg/L $CaCO_3$; pH 7.0) over a 7 day period in an aerated 40L aquarium as described previously (Craig et al 2007). After acclimation, zebrafish were housed in multiple 3L self-cleaning AHAB tanks racked in a soft-water re-circulating stand-alone AHAB filtration system (Aquatic Habitats, Apopka, Fl). Fish were fed daily with a commercial tropical fish food (Topfin, Phoenix, AZ, Ion content: 8.3 ± 0.2 mg Cu · kg^{-1} food, 338.6 ± 5.4 mg Na^+ · kg^{-1} food, 762.1 ± 24.7 mg Ca^{2+} · kg^{-1} food, 105.8 ± 2.0 mg Mg^{2+} · kg^{-1} food) and maintained on a 12-h light, 12-h dark photoperiod regime. Zebrafish were fasted for 24h prior to the beginning of experimentation. All procedures used were approved by the McMaster University Animal Research Ethics Board, and conform to the principles of the Canadian Council for Animal Care.

Experimental Protocol

Zebrafish (n=420; 70/treatment) were weighed and placed in 8L aerated tanks, served with flow-through soft-water at 25ml/min. A combination of Mariotte bottles were used to dose tanks with Cu (concentrated Cu solution made from $CuSO_4$ dissolved in 0.05% HNO_3), Na (concentrated NaCl solution), and Ca (concentrated $CaCl_2$ solution) to 6 treatment regimes of either control (Ctrl), Cu only (Cu), high Ca only (Ca), Ca + Cu (CaCu), high Na only (Na), and Na + Cu (NaCu; See Table 1 for ion concentrations). Nitric acid had no measurable impact on water pH. Fish were fed 2% body weight of commercial tropical fish food, once per day. Tanks were monitored daily for mortality and cleaned of any food or waste that had accumulated. Water samples (10 ml) were taken from each tank, filtered though a 0.45μm filtration disc (Pall Corporation, East Hills, NY), added to a plastic tube containing 100μl HNO_3 and kept at 4°C for analysis of ion content and Cu concentration. Throughout the experiment, there were no mortalities in any of the tanks. At the end of the exposure period, fish were quickly euthanized by overdose of buffered aesthetic (MS-222, Sigma) and sampled for gill, liver, and gut, which were immediately frozen in liquid N_2 for further analysis of Cu burden, gene expression, and enzyme activity.

Water and tissue ion & Cu levels

All tissues were first digested in 1ml of 1N HNO_3 for 48 h at 60°C. Tissue digests were diluted 10× and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z, Varian, Palo Alto, CA) compared to a 40 μg/L Cu standard (Fisher Scientific, Ottawa, ON). Water samples were measured undiluted. Both tissue and water ion composition were measured by flame atomic absorption spectroscopy (Spectra AA 220FS, Varian, Palo Alto, CA) after 10× dilutions

were made with 1% HNO_3 (Na^+) or 0.5% $LaCl_3$/1% HNO_3 (Mg^{2+}, Ca^{2+}), and verified using certified Na^+, Mg^{2+}, and Ca^{2+} standards (1 mg/L diluted in 1% HNO_3 or 0.5% $LaCl_3$/1% HNO_3; Fisher Scientific).

Protein carbonyl & enzyme activities

Protein carbonyls in gill, liver and gut tissues were quantified using a commercially purchased kit (Cayman Chemical Company, Ann Arbor, MI), following the protocol as described by Craig et al (2007b). Chemicals were purchased from Sigma-Aldrich Chemical Co. (St Louis. MO) and reaction buffers were prepared fresh daily. For enzyme assays, gill, liver and gut tissues were homogenized in ice-cold 50 mM phosphate buffer, containing 1mM EDTA, pH 6.7. Catalase (CAT) activity was assayed at 28°C using a modified method based on Clairborne (1985). The reaction buffer consisted of 20mM K-phosphate, pH 7.0, and 20 mM H_2O_2. The reaction was recorded as the decomposition of H_2O_2 at 240nm over 1 min. Protein concentrations for all samples were determined using BioRad kit reagents following the Bradford (1976) protocol using bovine serum albumin as a standard. Tissue Na^+/K^+-ATPase activity (NKA) was determined using the microassay method of McCormick (1993). All samples were homogenized in SEI buffer (150mM sucrose, 10mM EDTA, 50 mM imidazole, pH 7.3) containing 0.3% Na deoxycholic acid. Both NKA activity and Bradford protein assays (Bio-Rad, Hercules, CA) were run in 96-well format on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA).

RNA purification and microarray analysis

Total RNA was extracted from pooled liver tissue (8 fish; n=4) of zebrafish exposed to each treatment using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by UV spectrophotometry at 260 nm, and RNA purity verified by the 260/280 nm ratios of 1.8 or greater on a Nanodrop spectrophotometer (ThermoScientific, Wilmington DE). The integrity of RNA was assessed by capillary electrophoresis on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto CA,) with all samples demonstrating sharp 18S and 28S ribosomal RNA bands. 5 μg of total RNA was used to prepare biotin labelled complimentary RNA (cRNA) which was hybridized onto Affymetrix Zebrafish Genome arrays. All protocols were done according to the Affymetrix Technical Manual (Affymetrix Santa Clara, CA). Raw fluorescence data (cel files) were converted to normalized expression indices using the RMA algorithm in Gene Spring v.10.0.1 (Agilent Technologies, Palo Alto CA, http//www.agilent.com). Differential expression analysis was performed using one-way ANOVA with a Tukey post hoc test, and genes which demonstrated 2.0 fold or higher change in expression between any 2 treatments were selected for further analysis. Principle component analysis (PCA) was performed to identify the inherent physiological variability both within and between treatments. K-means clustering algorithm was applied to genes with a p-value of 0.05 or less in the ANOVA analysis to gain understanding of the major changes in gene expression following experimental treatment. The list of significantly expressed genes (fold change in expression >2) was submitted to the

Database for Annotated, Visual, and Integrative Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp; Denis et al 2003, Huang et al 2009) for functional annotation clustering using the default settings, minus cellular component ontology. Functional annotation was ranked based on significant overrepresentation ($p>0.05$) and an elevated enrichment score, indicating a significant likelihood that genes fall within the listed annotation cluster. Complete results of this microarray experiment were submitted to the public archive ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) in accordance with Microarray Gene Expression Data Society (MGED) recommendations.

Quantification of mRNA by real-time RT-PCR.

First strand cDNA was synthesized from total RNA of gill, liver and gut tissues (extracted as described above) using 1 μg treated with DNase I (Invitrogen, Calsbad, CA) and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase (Invitrogen). mRNA expression was quantified in duplicate on a Stratagene MX3000P (La Jolla, CA) real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 μl SYBR green mix, 1 μL of each forward and reverse primer (5 μM), 5.5 μL RNase/DNase free H_2O, and 5 μL cDNA template. Cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec. This was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock liver cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the house-keeping gene ef1α, which did not change over the course of the experiment. Gene expression data were calculated using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen 2001). Both water and non-reverse transcribed RNA were assayed on each plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA) and targets verified by gel electrophoresis. Target genes of interest are as follows and primers used can be found in Table 5.2: Menkes transporter (atp7a), copper transporter 1 (ctr-1), estrogen receptor 1 (esr-1), epithelial calcium channel (ecac), and the housekeeping gene, elongation factor 1 α (ef1α).

Statistical Analysis

Statistical analysis separate from microarray analysis was performed using Sigma Stat (SPSS Inc, Chicago, MI). In particular, a one-way ANOVA and a post-hoc Tukey's test were used to test for pairwise significance for all data, with the exception of gene expression when related to control, where a Dunnett's test was used. Additionally, regression analysis was used for qPCR validation, ($p<0.05$). All data have been expressed as a mean ± SEM.

Results

Copper tissue burden

Using Mariotte bottles, we were able to maintain constant water chemistry in all tanks for the duration of the 21 d for each given exposure (Table 5.1). Cu levels were constant and elevated in all of the Cu exposure groups (11.96 ± 0.6 μg/L) compared to control tanks. Waterborne Cu alone increased gill Cu burden 11-fold over Ctrl values, although CaCu and NaCu treatments exhibited no significant increase in gill Cu levels (Fig 5.1A). Furthermore, we saw significantly increased Cu load in both the gut and liver tissues under Cu and CaCu conditions (Fig 5.1B,C). Interestingly, the CaCu fish had significantly more Cu in the gut than the Cu only animals, although there was no difference between these treatments in the liver (Fig 5.1B,C). Examining the effects of varying Na^+ and Ca^{2+} on tissue Na^+, Ca^{2+} and Mg^{2+} levels, we found no significant differences in gill tissue amongst all treatment groups (data not shown), although we did find a significant elevation in Ca^{2+} levels under CaCu and NaCu treatments in the gut and liver (Figure 5.2).

Oxidative damage and enzyme activity

As an index of oxidative damage, we found elevated protein carbonyl levels under Cu only conditions in both gill and liver tissue (Fig 5.3A,C). Additional protein damage was apparent in the CaCu and NaCu treatments in gut, but protein carbonyls were only elevated in the gills of NaCu treated fish (Fig 5.3A,B). In contrast, treatment with Na and Ca alone did not affect protein carbonyl levels. As an assessment of the response to oxidative stress, catalase activity was measured and was unchanged by any treatment in both the gill and gut, although there seemed to be a statistically non-significant 30-50% decrease in activity across the majority of treatments (Fig 5.4). Gill NKA activity was significantly reduced under Cu only treatment and this reduction was reversed in the presence of Na (Fig 5.5A). We saw a 1.6 fold increase in gill NKA activity under Ca only conditions, yet this increase was muted in the CaCu treatment group (Fig 5.1A). In the gut, Cu only had a strong positive influence over NKA activity, as no other treatments displayed a similar response (Fig 5.5B). In the liver, NKA activity was reduced in all treatment groups when compared to Controls (Fig 5.5C).

Microarrays were validated by real-time qPCR using 2 genes of interest, ctr-1 and esr-1, which revealed a significant, positive correlation (R^2=0.776; $p<0.001$) between the Log_2 transformed expression found on the arrays compared that of the qPCR measurements. Correlation between and within treatments was assessed using PCA, which indicated a high degree of inherent physiological variability, visible by the spatial distance between dots (Fig 5.6).

Microarray analysis

Significantly regulated genes were submitted for k-means clustering algorithm which revealed 4 distinct patterns of expression, indicating that Cu alone has a strong opposing effect to other treatments (Fig 5.7). A complete list of genes significantly regulated greater than 2-fold can be found in Supplemental Table A.4. Cluster 1 (275 genes, 67 genes >2-fold) indicated that Cu had a strong positive effect on gene

expression, whereas all other treatments, with minor exceptions, distinctly down-regulated gene expression (Fig 5.7A). DAVID functional clustering analysis indicated the majority of clustered genes were involved in active membrane transport (e.g. slc6a19) , lipid metabolic processes (e.g. 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1), and responses to chemical stimuli (e.g. glutathione peroxidase 1b, Cytochrome P450, member 51; Fig 5.7A). Cluster 2 (441 genes, 35 genes >2-fold) demonstrated that Cu significantly decreased gene expression, whereas all other treatments appeared to increase gene expression, however the magnitude of expressional change was not large (Fig 5.7B). DAVID functional clustering revealed that the majority of effects were related primarily to transcriptional and translational changes in gene expression as indicated by the high enrichment scores associated with those categories (e.g. numerous ribosomal proteins, MAPK1, estrogen receptor 1). Furthermore, this cluster contained several transport mechanisms associated with ion and amino acid transport (e.g. slc20a1b, slc31a1, slc43a1). Similarly, cluster 3 (288 genes, 125 genes > 2-fold) exhibited a functional clustering related to transcription and translation as well, although the pattern of expression related to treatment was distinctly different. In this cluster, Cu had a significant influence in down-regulating gene expression, despite increased waterborne Na and Ca levels (Fig 5.7C). Additionally, the Na and Ca only treatment increased gene expression when compared to the Ctrl treatment (Fig 5.7C). Beyond transcriptional regulation, functional clustering revealed regulation of growth and development (e.g. insulin-like growth factor binding protein) and responses to endogenous and stress stimuli (e.g. cytochrome P450 family 24, HSP90b). Cluster 4 (260 genes, 75 genes > 2-fold) had a unique expression pattern in that Ca, Cu, and Na only treatments appeared to moderately increase gene expression, although when in combination (CaCu & NaCu), there was a general pattern of decreased expression. DAVID function clustering indicated that this group of genes are related to solute transport and cellular communication (e.g. slc26a1, HIF1a, Ras family proteins), and to a lesser extent lipid modification and vitamin binding (e.g. lysophospholipase 3, glutamic-oxaloacetic transaminase 2).

Further analysis of microarray data was directed towards establishing gene expression endpoints for possible future incorporation into a chronic BLM for the zebrafish. This analysis evaluated all significant changes in gene expression (1265 genes, $p<0.05$) to compare the effects of all experimental treatments. Analysis revealed the shared and unique significant changes induced by Cu that were prevented or enhanced by the addition of Na^+ and Ca^{2+} (Supplemental Table A.5). In total (i.e. unique and shared genes), we found 3.5% of genes were modified in the presence of elevated waterborne Na^+ and Ca^{2+} (Supplemental Table A.5). Likewise, we examined the shared and unique significant changes induced by Na^+ and Ca^{2+} alone that were prevented or enhanced by NaCu and CaCu exposure, and found that 4.5% of genes were modified with NaCu and CaCu treatments (Supplemental Table A.6). However, it appears that Na^+ treatment alone had a significant effect on gene expression, as Na^+ treatment contained a number of significantly regulated genes (2.9%; Supplemental Table A.6A).

QPCR validation of target genes

Relative expressional levels (compared to control values) were assessed using qPCR not only for microarray validation, but also to identify changes in gene expression in other target tissues. Four genes (ctr-1, esr-1, ecac, and atp7a) were assessed for expressional changes in the gill, liver, and gut (Table 5.3). Although there were no changes in the gill, we found a significant reduction in the expression of ctr-1 in both the gut and liver under Cu only conditions. Additionally, there was a significant reduction in ctr-1 expression under CaCu and Na treatments in the gut. With the NaCu treatment, we found a 2.5-fold increase in ctr-1 expression in the liver (Table 5.3). Examining esr-1, Cu and Ca treatments invoked a significant decrease in expression across all tissues examined, although the CaCu treatment only resulted in a decrease in esr-1 expression in the gill (Table 5.3). Likewise, the Na only treatment resulted in a decreased expression in the liver and gut, although in the presence of increased waterborne Cu, there was a dramatic 9-fold increase in esr-1 expression in the liver, and a 2-fold increase in the gut (Table 5.3). We examined ecac to look at how calcium homeostasis may be disrupted due to Cu exposure. There were no significant changes in the liver and gut, although in the gill, we found a significant up-regulation under the Cu and NaCu treatment. Surprisingly, there were no changes in ecac expression associated with Ca treatment, and decrease in expression with CaCu ($p=0.06$). Finally, we assessed atp7a, and found that Cu alone increased expression in the liver, and decreased expression in the gills (Table 5.3). Furthermore there was a decrease in atp7a expression in the gills and gut with CaCu exposure (Table 5.3).

Discussion

Overview

Toxicogenomics is an emerging discipline which allows scientists to assess the genomic effects of environmental contaminants on a whole organism scale as they pertain to classical toxicological experiments. This is the first study in zebrafish to implement a genomic approach associated with varying water chemistries to ascertain endpoints, both transcriptional and functional components, of chronic waterborne Cu toxicity. Classically, both Ca^{2+} and Na^{+} are protective against acute Cu toxicity. In contrast, we have found that at the transcript level, these cations may not be as protective during chronic Cu exposure. Although effective at reducing Cu load in the all tissues, increased Na^{+} in the presence of Cu did not decrease the degree of oxidative damage, particularly in the gill and gut (Fig 5.3B). Likewise, we found that CaCu treatment enhanced the degree of Cu load in both the liver and gut, although oxidative damage appeared to be minimized (Fig 5.1B,C; Fig 5.3). Transcriptional analysis of our candidate genes of interest indicated the majority of changes associated with the Cu only treatment appeared as a down regulation, and CaCu treatment returned only some of the transcripts to control levels (Table 5.3). Conversely, NaCu treatment exerted a strong, opposing effect when compared to that of Cu alone (Table 5.3). It remains to be determined whether the changes involved are beneficial or not. On a more global scale, we found significantly clustered patterns of expression, where Cu appeared to have an opposing effect to the majority of other treatments based on the magnitude of expression (Fig 5.7). A key feature of this study was the

identification of physiological variability inherent in the population of animals under study. As such, using a heterogeneous population of zebrafish reflects a more realistic depiction of a natural response, as opposed to experiments performed on genetically identical populations (Fig 5.6). Moreover, we have compiled data on the preventative or enhancing effects of Na^+ and Ca^{2+}, both alone and in the presence of Cu, to help interpret endpoints of gene expression for possible future incorporation into a chronic BLM for this tropical model species of genomic importance.

Copper accumulation

In the absence of any competitive ion, there was a significant increase in Cu load in gill, gut, and liver tissues of zebrafish exposed to waterborne Cu (Fig 5.1A). Although it appeared that elevated waterborne Na^+ and Ca^{2+} both provided some protection by reducing Cu accumulation in the gills, Ca^{2+} appeared to enhance Cu load in both the liver and gut (Fig 5.1). Primary absorption of Cu is thought to occur in the gut (Watanabe et al 1997, Carpene et al 1999), but Cu is known to be taken up from the external medium via the gills, and it is well established that Na^+ and Ca^{2+} compete with Cu for uptake and binding sites at the level of the gill (Lauren & McDonald 1987; Playle et al 1993; Clearwater et al 2002; Grosell & Wood 2002; Kamunde et al 2002). However, less is known about protection in the liver by waterborne cations. It is well-documented that in fish, the primary site of accumulation of excess Cu is the liver, which is then excreted into the gut associated with the bile (Craig et al 2007b, 2009, Grosell et al 1998, 2001). In studies on both mammals (Prince et al 1984) and teleosts (Adbel-Tawwab 2007), treatment with a high Ca^{2+} diet in conjunction with a high Cu diet provides a degree of protection from accumulation of Cu within the liver and also reduces the amount of Cu related liver damage. Interestingly, although we see an increase in both liver Cu and Ca^{2+} load associated with the CaCu treatment (Fig 2B, 1C), we do not see a parallel increase in protein carbonyls (Fig 3C). This result suggests that Ca^{2+} provides a moderate degree of protection in the liver, but does not out-compete Cu uptake at the level of the gills, and implies that Cu and Ca^{2+} do not share similar uptake pathways.

Shared uptake pathways in the gills between Na^+ and Cu are well established (Lauren & McDonald 1987; Grosell & Wood 2002), and further supported in this study by the reduced Cu load with NaCu treatment in the liver (Fig 5.1C). However, although it appears that Cu uptake is inhibited by high external Na^+ levels; we saw significant levels of protein carbonyls in both the gut and gill of NaCu treated zebrafish (Fig 5.3AB). Previous experiments have shown high NaCl levels can induce oxidative stress in both rat renal medulary cell cultures (Zhang et al 2004) and isolated perfused rat livers (Saha et al 1992), and therefore, it may be the combination of high external Na and Cu that leads to increased oxidative damage, as there was no difference between the Na treatment versus control and the NaCu treatment with respect to protein carbonyl levels (Fig 5.3). However, on an acute timeframe, Craig et al (2007b) found no increase in oxidative damage in the gills or liver under combined conditions of increased Na^+ and Cu, and likewise, no increased carbonyls in the gill or liver associated with Na^+ exposure alone, an indication that prolonged NaCu exposure may be more detrimental in this instance.

A fascinating aspect of our data is the large increase in Cu load associated with the CaCu treatment. It is known that fish in marine environments drink copious amounts of seawater to avoid dehydration, and a resulting effect is the increase in pH and HCO_3^- concentration due to the apical Cl^-/HCO_3^- exchange in the intestine (Wilson et al 1996, Grosell et al 2001, Wilson et al 2009). The high levels of HCO_3^- and pH cause Ca^{2+} and Mg^{2+} to precipitate as insoluble carbonates which are then excreted (Wilson et al 2002). This precipitation of osmolytes from the intestinal lumen lowers osmotic pressure in the chyme, thereby facilitating fluid uptake, and it appears Ca^{2+}, rather than Mg^{2+} plays the more important role. Although the level of Ca^{2+} to which the fish were exposed is ⅓ of seawater values, Ca^{2+} appears to have exerted a strong effect in initiating an osmoregulatory response, as there were significant increases in NKA activity associated with both Ca only and CaCu treatments in all tissues (Fig 5.5). Additionally, there were significant increases in Ca^{2+} levels in the gut of fish exposed to both CaCu and NaCu (Fig 5.2A). Therefore, it is possible that Ca^{2+} triggers an osmoregulatory response that may aid in detoxification and precipitation of Cu in a carbonate form. Although speculative, this definitely warrants further investigation, as this may be a key aspect of protection against divalent metal contaminants in marine environments.

Enzyme activities

It has been established that Cu competes with Na^+ for entry pathways at the apical side of gill ionocytes, and ultimately inhibits basolateral NKA activity. Indeed, decreased activity of NKA was observed in the gills and liver of Cu treated zebrafish (Fig 5.5; Lauren & McDonald 1987; Grosell & Wood 2002). Not surprising was a rescue of decreased NKA activity linked to increased ambient Na^+ particularly in gills (Fig 5.5A). Increased NKA activity is routinely associated with freshwater/seawater migration in euryhaline teleosts (Lasserre 1971; Stuenkel & Hillyard 1980; Doneen 1981; Stagg & Shuttleworth 1982; Woo & Chung 1995). As previously mentioned, Ca^{2+} has an effect by increasing NKA activity in the gill and gut in both Ca only and CaCu treated zebrafish (Fig 5.5A). Ca^{2+} has been shown to have variable effects on NKA activity, depending on the species and tissue used. For example, high Ca^{2+} has been shown to stimulate gill NKA activity in *Cyprinodon variegatus* but to decrease its activity in *Mysidopsis bahia,* two euryhaline species commonly used for toxicological studies (Price et al 1990). In the present study, there was a parallel decrease across all treatments in liver NKA and catalase activities (Fig 5.5C, 5.6C). Studies have shown that increased reactive oxygen species (ROS) can have a negative impact on both catalase (Kono & Fridovich 1982) and NKA (McKenna et al 2006), and indications of increased ROS (elevated protein carbonyls) can be seen with most treatments (Fig 5.3C).

Gene expression and microarrays

We have taken two approaches to assess the transcript profile associated with altered water chemistry and increased waterborne Cu. One approach used was the candidate gene approach which allowed us to validate microarray data and to extend gene expression measures to tissues not used in the microarray experiments. Secondly, the microarray experiment revealed a high degree of inherent physiological variability (Fig

5.6), which is not surprising when using commercially purchased, heterogeneous zebrafish with unknown population origins, and validates our study as a more natural depiction of gene response to environmental disruption at the population level. Our results from liver tissue of zebrafish revealed 4 distinct clustered patterns of expression, and predominantly indicated that Cu alone had an opposing effect when compared to the other treatment groups. Chiefly, Cu alone had a distinct impact on the transcriptional and translational machineries as determined by functional annotation, a finding further supported in a previous study examining various levels of Cu exposure in softwater conditions (Fig 5.7; Craig et al Submitted).

Cluster 1 indicates that Cu had an up-regulatory effect on liver transcripts, and the other treatments resulted in a decrease in transcript expression, as compared to the control (Fig 5.7A). Here we see overrepresentation in 3 separate categories, yet predominantly, a greater number of genes are involved in active transmembrane transport. This is not surprising as Cu is known to interrupt ion homeostasis (Lauren & McDonald 1987; Krumschnabel et al 1999, Grosell & Wood 2002), and the compensatory response may include up-regulation of transport genes, although this may not necessarily be reflected at the protein and functional levels. Several solute carriers controlling amino acid and neurotransmitters are up regulated in Cu treatment, as well as a vacuolar type H^+-ATPase, which has been shown to be essential in maintaining apical ion regulative processes in fish (Schredelseker & Pelster 2004). Also not surprising was a stimulation of exogenous chemical response genes, such as glutathione peroxidase, a known antioxidant enzyme scavenger related to increased ROS initiated by excessive Cu (Evans & Halliwell 2001; Hansen et al 2007). Interestingly, we did see a response involved in lipid metabolic processes, as Cu induced ROS results in lipid peroxides (Radi & Matkovics 1988). This response may be important in combating lipid damage, because preliminary data from our lab indicates a shift from polyunsaturated lipids to saturated lipids in the liver of zebrafish chronically exposed to Cu, suggesting a preventative measure in reducing the number of double bonds available for ROS attack. Further analysis revealed that 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1, a key rate limiting enzyme in the production of cholesterol (Vock et al 2008), is the only significantly regulated gene common to the three treatments that involve Cu (Cu, Ca,Cu, NaCu). Changes in cholesterol may be essential in maintaining proper fluidity in membranes high in unsaturated fatty, although Huster *et al* (2007) have found a distinct decrease in cholesterol biosynthesis in the liver of mice exposed to high Cu. This provides novel evidence that Cu may play a significant role in lipid membrane processes, and merits further investigation in teleosts.

The second cluster revealed a unique pattern associated with Cu, Ca^{2+}, and Na^+ having a strong negative impact on gene expression, whereas CaCu and NaCu in combination minimized this effect, potentially indicative of a protective transcriptional response (Fig 5.7B). Functional ontological analysis revealed a significant impact on initiation of transcription, translation, and protein modification and transport (Fig 5.7B). This could indicate reduced energetics associated with Cu, where non-essential processes, such as performance (McGeer et al 2000), growth (Mohanty et al 2009), and reproduction (Levesque et al 2003) are reduced for higher, essential functions. Further revealed are 2 key genes of interest associated with Cu exposure, esr-1 and ctr-1 (Supplemental Table

A.3B). Quantitative PCR analysis was clear in validating our microarray experiment, as indicated by the significant regression analysis (R^2=0.776; p<0.001), and additionally demonstrated that there was similar pattern of expression related to other tissues (Table 5.3). Cluster 3 had similar functional characteristics as cluster 2, although the pattern of expression was altered. All treatments involving Cu had negative impacts on transcription, with an attenuated effect in the NaCu and CaCu treatments, as there were not as many genes significantly expressed >2 fold in these treatment groups (Fig 5.7C). Again we see factors involved in transcriptional and translational responses, which could be related to diversion of energy towards essential processes; however the functional clustering had a weak affiliation as indicated by both p-value and enrichment score. Here we saw responses in association to stress and endogenous stimuli, such as cyp24a1, which besides a role in vitamin D regulation also plays a role in calcium homeostasis (Sakaki et al 2005). Under the influence of all Cu treatments, this gene was down-regulated, indicating probable Ca^{2+} homeostasis disruption due to excessive Cu. Cluster 4 had a weak pattern of expression, and contained the lowest significance related to functional, ontological annotation (Fig 5.7D). Predominant increases in transcripts were associated with Cu, Ca, and Na treatments, whereas CaCu and NaCu treatments induced a negative response (Fig 5.7D). A feature of this cluster is changes in HIF1α expression, which is known to be induced by increased ROS in zebrafish (van der Meer et al 2005). Overall, upon examination of microarray results with multiple treatments, the patterns of expression were more important than specific genes involved, and although we attempted to highlight potential factors involved in transcript regulation, it is evident that global transcriptional regulation is a complex response. What makes interpretation even more difficult is the fact that gene endpoints are not necessarily applicable as common endpoints, such as metallothioneins, which are not responsive in this particular experiment.

A more viable approach to gene endpoint assessment is a directed gene analysis in which we can examine a handful of known responsive genes under our exposure regime. Our approach examined mechanisms involved in Cu uptake/transport (ctr-1, atp7a, ecac) and endocrine disruption (esr-1). Copper uptake and transport is primarily regulated by ctr-1 and atp7a, and by altering levels of external Cu and ion concentrations, there are associated changes in transcript levels (Mackenzie et al 2004, Craig et al 2007a, Craig et al 2009). Within the liver, we found a significant increase in ctr-1 and a decrease in atp7a under the Cu only treatment, which validated our microarray experiment, although it should be noted that atp7a is not found on the Affymetrix GeneChip® (Table 5.3). Changes in Cu transporters may signify an attempt to reduce Cu load within the liver, by an increased expulsion of Cu from the liver, in an effort to reduce Cu related damage. Craig et al (2009) found a similar pattern of atp7a expression in the liver of zebrafish chronically exposed to 8μg/L Cu and suggested this as a compensatory response to promote cellular assimilation and translocation of Cu. However, in the gills of zebrafish, we found no such evidence of changes in transcription with either ctr-1 or atp7a associated with the Cu only treatment, although atp7a did decrease in the CaCu exposure (Table 5.3). The importance of linking expressional changes to a functional aspect is highlighted by Craig et al (2009), where they found that both protein and uptake of

radioactive ^{64}Cu were, in effect, opposite to gene expression. Nevertheless, upon closer examination, CaCu treatment does significantly reduce ctr-1 expression compared to Cu only exposure (Table 5.1), which may explain the reduced Cu load in the gills between these two treatments (Fig 5.1A). Although it is recognized that Ca^{2+} may play an indirect role in Cu uptake at the level of the gill (Spry & Weiner 1991, Playle et al 1993), further inquiry into Ca^{2+} direct competitive effects associated with Cu are yet to be determined. The gut seems to demonstrate a similar response with a down-regulation of transcript expression for both ctr-1 and atp7a, and this again may be a preventative response to allow further absorption of Cu from the gut, as there is a substantial increase in Cu load in the gut associated with both Cu and CaCu treatments (Fig 5.1B).

The microarray analysis revealed that Ca^{2+}, both alone and in combination with Cu played a significant role in altering gene expression, when compared to treatment with Na^{+} and prompted investigation into mechanisms of Ca^{2+} uptake (Fig 5.7, Supplemental Table A.4). Tissue Ca^{2+} uptake occurs through ecac in the apical membrane, and transported basolaterally through Ca^{2+}-ATPase or Na^{+}/Ca^{2+} exchangers (Flik et al 1984, Verbost 1994). Upon analysis of the ecac expression profile we found no response in any treatment in the liver or gut. However, there was a considerable increase in ecac transcript expression associated with Cu and NaCu treatment in the gill (Table 5.3). Previously, Craig et al (2007a) showed that acclimation to a Ca^{2+} deficient environment induces an increase in the protein and gene expression profile of gill ecac, an important factor to consider in this experiment as ambient Ca^{2+} concentrations were low in the Cu, and NaCu exposure groups. However, low ambient Ca^{2+} levels were associated with the Ctrl and Na exposure groups (Table 5.1) and there was no significant change in gill ecac expression (Table 5.3). This represent a potential competition between Cu and Ca^{2+}, as only the Cu related treatments associated with low ambient Ca^{2+} increased gill ecac expression (Table 5.3), although, this has yet to be functionally determined.

Our final approach was to examine the potential endocrine disruptive capacity of Cu exposure in zebrafish. Chronic exposure to Cu may have prolonged effect on reproductive capacity, which in turn can have an impact at the population level. In mammalian studies, it has long be known that Cu, in a dose dependent manner, reduces both estrogen and progesterone concentrations, and at one point was used as a means of contraception through the use of intra-uterine devices (Castro et al 1986, Castro 1988, Hill 1997). Furthermore, estrogen receptors are known metal-binding proteins, and contain zinc binding sites which promote binding to estrogen response elements (ERE, Danielsen 1991). Divalent metals have been shown to interact with this site and prevent binding to EREs (Predki & Sarkar 1992). Furthermore, in mammalian cell lines, exposure to copper, and other divalent metals decreased the concentration of estrogen receptor proteins and mRNA by 40-60% (Martin et al 2003). Likewise, there was a substantial decrease in transcript expression under Cu or Ca only conditions in all tissue (Table 5.3). Furthermore, esr-1 expression decreased in gill tissue with CaCu exposure, and liver and gut demonstrated a decrease associated with Na treatment (Table 5.3). Potentially, Ca^{2+} alone may interact with metal binding sites on the estrogen receptor in a similar fashion to other divalent metals, although this does not explain how CaCu treatment had no effect on esr-1 expression in the liver (Table 5.3). Conversely, a large increase in esr-1

expression associated with NaCu treatment, yet the mechanistic reason is unclear (Table 5.3). Taken as a whole, evidence suggests that Cu plays a significant role in estrogen receptor disruption, which in turn can reduce the sensitivity to estradiol, which can impact reproductive capacity in fish (Katsu et al 2007), although further investigation in this area are warranted.

BLM application & perspectives

Analysis directed towards establishing genes for use in a chronic tropical BLM primarily revealed that Na^+ alone initiates a large transcriptional response, and when associated with Cu, the response is principally prevented (Fig 5.7, Supplemental Table A.6). Furthermore, this novel approach identifies specific genes that are 'rescued' or returned to control expression levels by the addition of Na^+ and Ca^{2+}, although it is unknown if these are protective effects to a Cu challenge (Supplemental Tables A5 & A6). Effectively we have provided a short list of candidate genes that are under the influence of combined treatments associated with Cu, Na^+, and Ca^{2+}. Moreover, we show that Na^+ and Ca^{2+} alone play a significant role in changing gene expression, and interpretation of metal related transcriptional expression in experiments associated with altered water chemistry requires careful interpretation, as relatively benign ions may induce significant expressional patterns.

This study recognizes the importance of examining multiple water chemistries to elucidate the transcription and physiological effects of Cu in zebrafish. Traditional approaches to studying Cu impacts are mostly associated with measurements related of mortality, and environmental standards are based on these predictions of toxicity. However, chronic, low doses, although may not have direct and readily visible effects, can show distinct transcriptional patterns associated with decreased growth, impaired ion homeostasis, and endocrine disruption. Although the protective effects of ions, such as Ca^{2+} and Na^+ and functionally well documented (Lauren & McDonald 1987; Playle et al 1993; Clearwater et al 2002; Grosell & Wood 2002; Kamunde et al 2002), the protection that cations provide at the level of the genome is poorly understood. By using microarrays, we effectively demonstrated that, although there is some degree of protection given in conjunction with Na^+ and Ca^{2+}, transcriptionally, there is a wealth of data that needs to be reviewed to fully assess the complete protective effect of these ions.

Table 5.1: Water chemistry parameters of each exposure group, showing essential competitive ions, pH, and water hardness averaged over the 21 day exposure period. Values with a * indicate a significant difference from all other treatments ($p<0.05$).

	Treatment					
Parameter	**Ctrl**	**Cu**	**Ca**	**CaCu**	**Na**	**NaCu**
Na^+ (μM)	84.3 ± 1.7	85.9 ± 2.3	86.1 ± 1.2	81.5 ± 2.2	3137.3 ± 141.9*	3012.7 ± 93.3*
Mg^{2+} (μM)	26.8 ± 0.7	25.9 ± 0.6	26.0 ± 0.5	26.3 ± 0.5	26.0 ± 0.7	25.3 ± 0.6
Ca^{2+} (μM)	60.0 ± 3.1	61.6 ± 3.6	3307.8 ± 136.5*	3357.2 ± 88.4*	65.7 ± 1.5	66.5 ± 2.2
Cu^{2+} (μg/L)	3.2 ± 0.2	12.5 ± 0.7*	3.0 ± 0.3	11.4 ± 0.8*	2.7 ± 0.3	12.0 ± 0.4*
pH	7.04 ± 0.02	7.02 ± 0.02	7.03 ± 0.02	7.03 ± 0.02	7.06 ± 0.04	7.06 ± 0.04
$CaCO_3$ (mg Ca^{2+}/L)	8.7 ± 0.3	8.8 ± 0.5	334 ± 30.6*	339 ± 40.2*	9.1 ± 0.5	9.1 ± 0.7

Table 5.2: Forward (F) and reverse (R) primers used for real-time qPCR validation of microarray results.

Gene	Primer	Accession #	Amplicon size (bp)
atp7a	F: 5'-GGCTCGACTTCTCGCAGCT-3' R: 5'-ATTCCGCATTTTCACTGCCT-3'	NM_001042720	50
ctr-1	F: 5'-GAATCAGGTGAACGTGCGCT-3' R: 5'-CCATCAGATCCTGGTACGGG-3'	AY077715	51
ecac	F: 5'-ACTTGGTCAACCGCAGAAAG-3' R: 5'-CAGATTCCACTTGAGCGTGA-3'	AY383562	197
ef1α	F: 5'-GTGCTGTGCTGATTGTTGCT-3' R: 5'-TGTATGCGCTGACTTCCTTG-3'	NM_131263	201
esr1	F: 5'-AGAAACACAGCCGGCCCTA-3' R: 5'-TGGTGAGCAGGGACATCATG-3'	NM_152959	51

Table 5.3: Quantitative expression levels assessed by qPCR of ctr-1, esr1, ecac, and atp7a from the liver, gill and gut of acclimated zebrafish exposed to control (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu). Fold change values that do not share a like symbol are significantly different from each other ($p<0.05$). Directional arrows indicate a significant fold change compared to that of the control (Dunnet's Test, $p<0.05$; fold change >2).

Gene	Tissue		Treatment Ctrl	Cu	Ca	CaCu	Na	NaCu
ctr-1	Liver	Fold Change	1.01 ± 0.10^{a}	0.35 ± 0.08^{ab}	1.09 ± 0.03^{a}	0.51 ± 0.06^{a}	0.92 ± 0.22^{a}	2.46 ± 0.16^{c}
		Direction	—	↓	—	—	—	↑
	Gill	Fold Change	1.12 ± 0.34	1.07 ± 0.14	1.29 ± 0.11	0.57 ± 0.07	1.29 ± 0.13	1.98 ± 0.52
		Direction	—	—	—	—	—	—
	Gut	Fold Change	1.08 ± 0.24^{a}	0.27 ± 0.02^{b}	0.65 ± 0.05^{ab}	0.28 ± 0.01^{b}	0.46 ± 0.01^{b}	0.71 ± 0.03^{ab}
		Direction	—	↓	—	↓	↓	—
esr-1	Liver	Fold Change	1.02 ± 0.13^{a}	0.02 ± 0.01^{b}	0.09 ± 0.01^{ab}	1.58 ± 0.19^{abc}	0.22 ± 0.09^{ab}	9.03 ± 0.44^{d}
		Direction	—	↓	↓	—	↓	↑
	Gill	Fold Change	1.14 ± 0.39^{a}	0.21 ± 0.01^{b}	0.26 ± 0.03^{b}	0.15 ± 0.02^{b}	0.43 ± 0.02^{ab}	0.59 ± 0.06^{ab}
		Direction	—	↓	↓	↓	—	—
	Gut	Fold Change	1.17 ± 0.40^{a}	0.02 ± 0.01^{b}	0.20 ± 0.10^{b}	0.39 ± 0.08^{ab}	0.18 ± 0.02^{b}	2.21 ± 0.26^{c}
		Direction	—	↓	↓	—	↓	↑
ecac	Liver	Fold Change	1.16 ± 0.37	1.18 ± 0.88	0.31 ± 0.09	0.57 ± 0.17	1.40 ± 0.67	0.86 ± 0.39
		Direction	—	—	—	—	—	—
	Gill	Fold Change	1.11 ± 0.33^{a}	5.26 ± 0.41^{ab}	1.88 ± 0.26^{a}	0.24 ± 0.04^{a}	3.66 ± 0.68^{a}	13.86 ± 4.99^{b}
		Direction	—	↑	—	—	—	↑
	Gut	Fold Change	1.05 ± 0.19^{ab}	1.86 ± 0.47^{ab}	0.315 ± 0.11^{ab}	0.32 ± 0.06^{a}	2.04 ± 0.69^{ab}	0.45 ± 0.07^{a}
		Direction	—	—	—	—	—	—
atp7a	Liver	Fold Change	1.29 ± 0.58^{a}	4.88 ± 1.14^{b}	2.38 ± 0.48^{ab}	0.42 ± 0.03^{a}	1.79 ± 0.24^{a}	1.00 ± 0.14^{a}
		Direction	—	↑	—	—	—	—
	Gill	Fold Change	1.06 ± 0.20^{a}	0.65 ± 0.04^{ab}	0.91 ± 0.04^{ab}	0.35 ± 0.04^{b}	0.84 ± 0.06^{ab}	0.69 ± 0.24^{ab}
		Direction	—	—	—	↓	—	—
	Gut	Fold Change	1.07 ± 0.22^{a}	0.37 ± 0.04^{b}	1.09 ± 0.13^{a}	0.41 ± 0.01b	1.19 ± 0.16^{a}	0.85 ± 0.02^{a}
		Direction	—	↓	—	↓	—	—

Fig 5.1
Copper (Cu) load (μg/g tissue) in gill (A), gut (B), and liver (C) tissue from zebrafish exposed to ctrl (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu). Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=10 for all treatments, $p<0.05$).

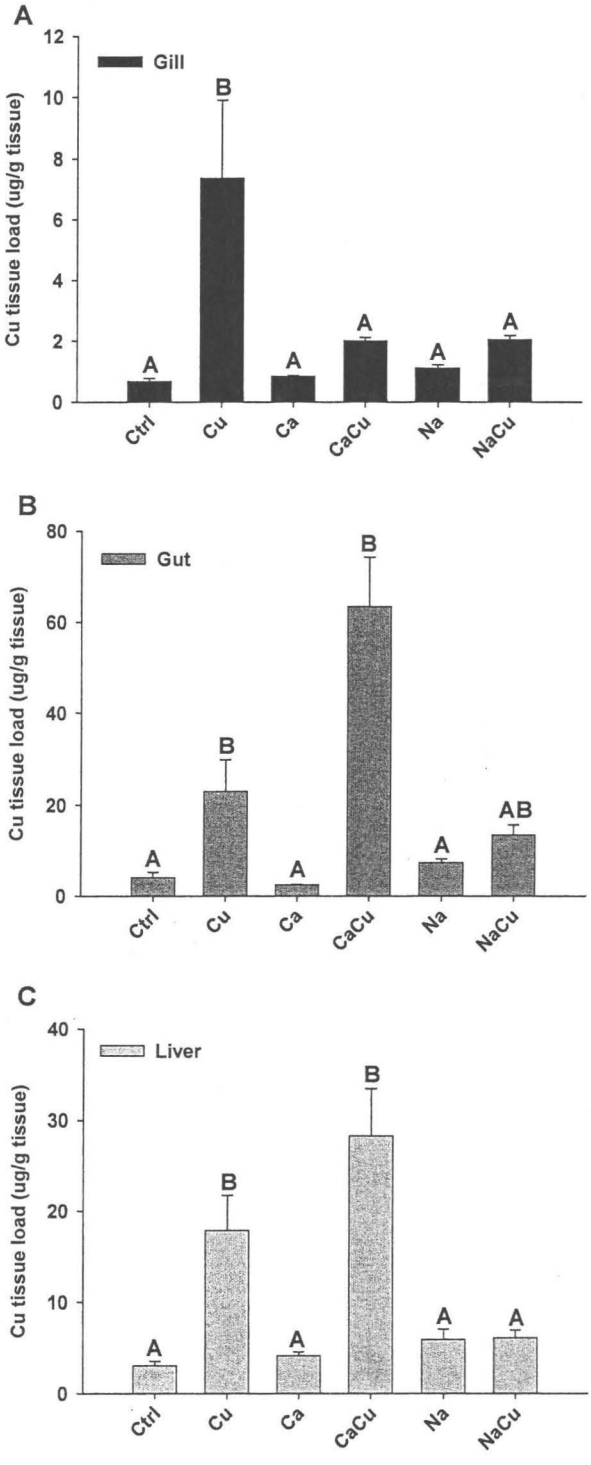

A
Gill
Cu tissue load (ug/g tissue)
12
10
8
6
4
2
0
B
A
A
A
A
A
Ctrl
Cu
Ca
CaCu
Na
NaCu
B
Gut
Cu tissue load (ug/g tissue)
80
60
40
20
0
B
B
A
A
A
AB
Ctrl
Cu
Ca
CaCu
Na
NaCu
C
Liver
Cu tissue load (ug/g tissue)
40
30
20
10
0
B
B
A
A
A
A
Ctrl
Cu
Ca
CaCu
Na
NaCu

Fig 5.2
Calcium (Ca) tissue load (mmol/kg tissue) in the gut and liver of zebrafish exposed to ctrl (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu). Gill tissue did not contain any significant differences (data not shown). Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=10 for all treatments, $p<0.05$).

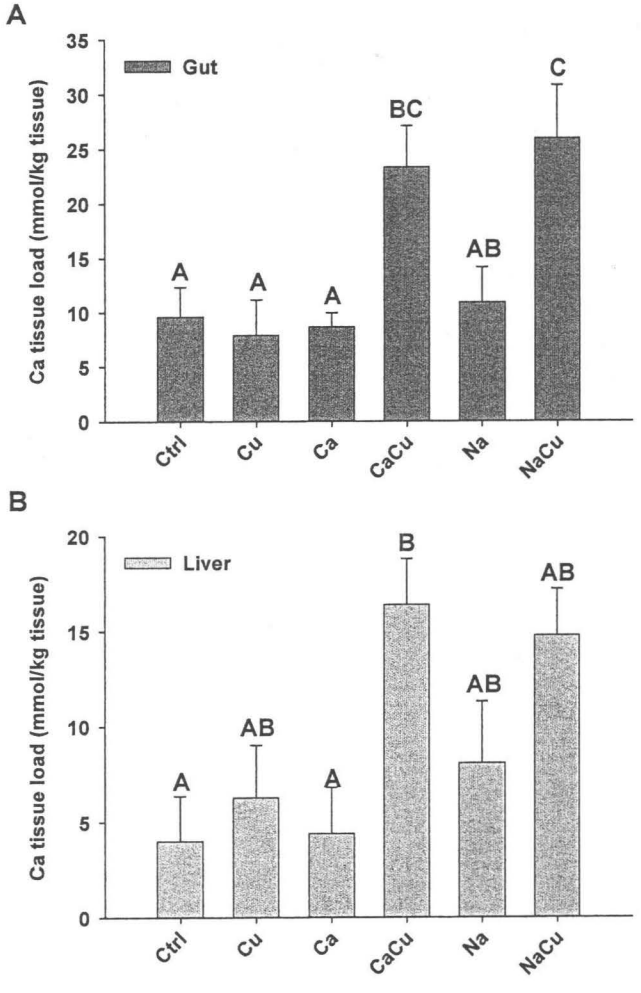

A
Gut
Ca tissue load (mmol/kg tissue)
35
30
25
20
15
10
5
0
A
A
A
BC
AB
C
Ctrl
Cu
Ca
CaCu
Na
NaCu
B
Liver
Ca tissue load (mmol/kg tissue)
20
15
10
5
0
A
AB
A
B
AB
AB
Ctrl
Cu
Ca
CaCu
Na
NaCu

Fig 5.3
Protein carbonyl levels (nmol/mg protein) in gill (A), gut (B), and liver (C) tissue from zebrafish exposed to ctrl (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu). Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=10 for all treatments, $p<0.05$).

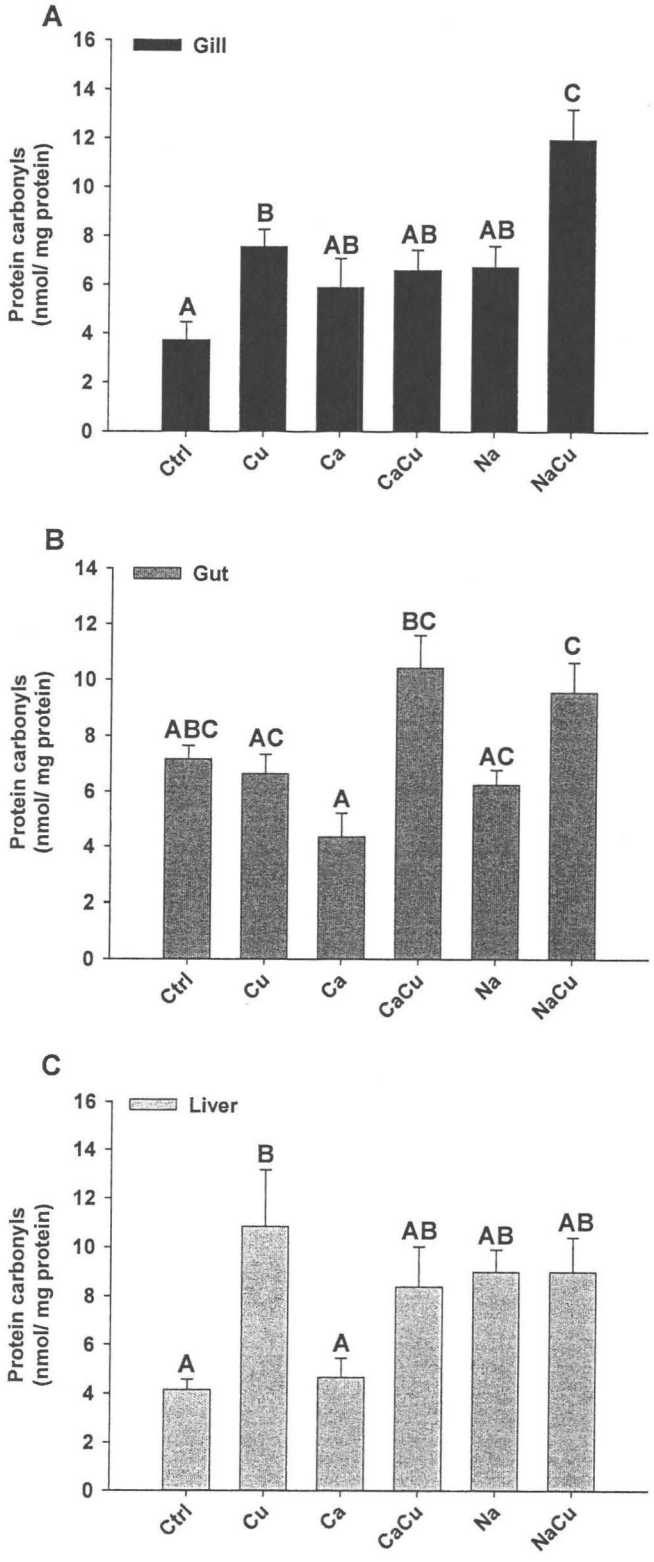

A
Gill
Protein carbonyls (nmol/ mg protein)
16
14
12
10
8
6
4
2
0
A
B
AB
AB
AB
C
Ctrl
Cu
Ca
CaCu
Na
NaCu
B
Gut
Protein carbonyls (nmol/ mg protein)
14
12
10
8
6
4
2
0
ABC
AC
A
BC
AC
C
Ctrl
Cu
Ca
CaCu
Na
NaCu
C
Liver
Protein carbonyls (nmol/ mg protein)
16
14
12
10
8
6
4
2
0
A
B
A
AB
AB
AB
Ctrl
Cu
Ca
CaCu
Na
NaCu

Fig 5.4
Catatalse activity (CAT; U/mg protein) in gill (A), gut (B), and liver (C) tissue from zebrafish exposed to ctrl (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu). Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=10 for all treatments, $p<0.05$).

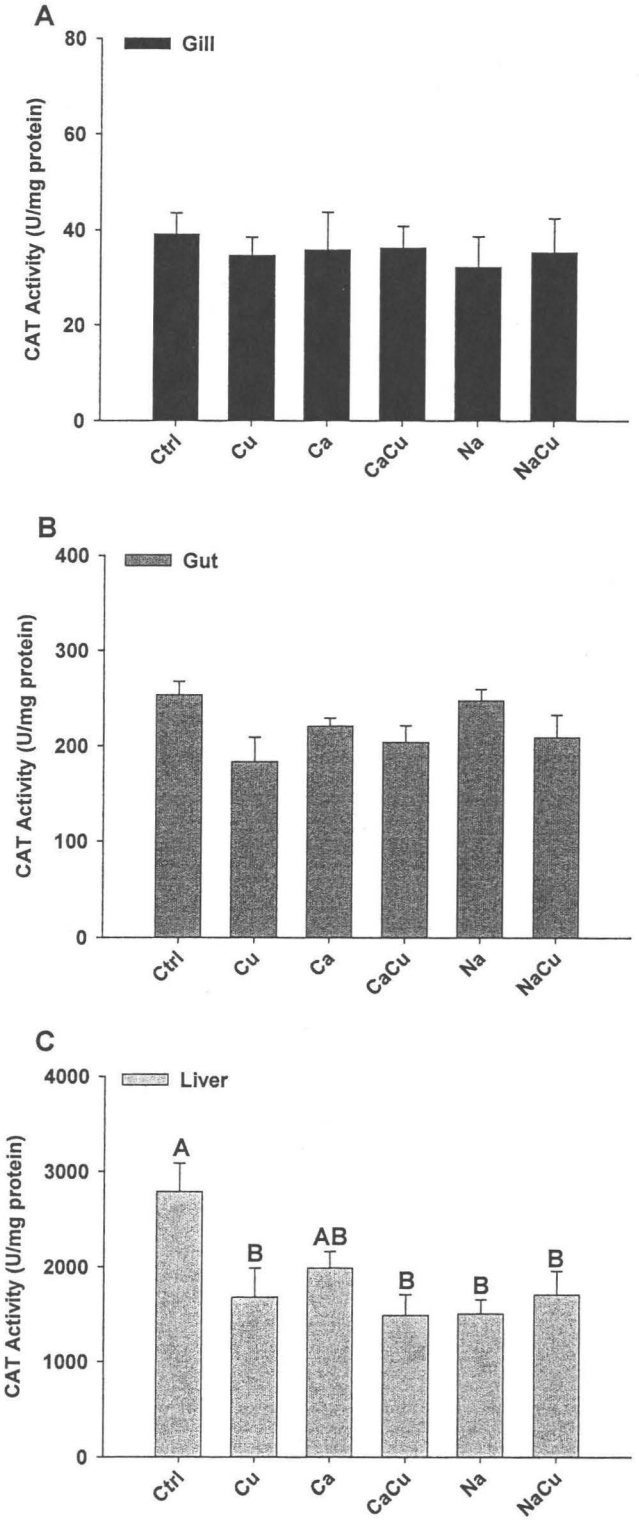

A
Gill
CAT Activity (U/mg protein)
0
20
40
60
80
Ctrl
Cu
Ca
CaCu
Na
NaCu
B
Gut
CAT Activity (U/mg protein)
0
100
200
300
400
Ctrl
Cu
Ca
CaCu
Na
NaCu
C
Liver
CAT Activity (U/mg protein)
0
1000
2000
3000
4000
A
B
AB
B
B
B
Ctrl
Cu
Ca
CaCu
Na
NaCu

Fig 5.5
Na^+K^+ATPase activity (NKA; mmol ADP mg protein^{-1} hr^{-1}) in gill (A), gut (B), and liver (C) tissue from zebrafish exposed to ctrl (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu). Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=10 for all treatments, $p<0.05$).

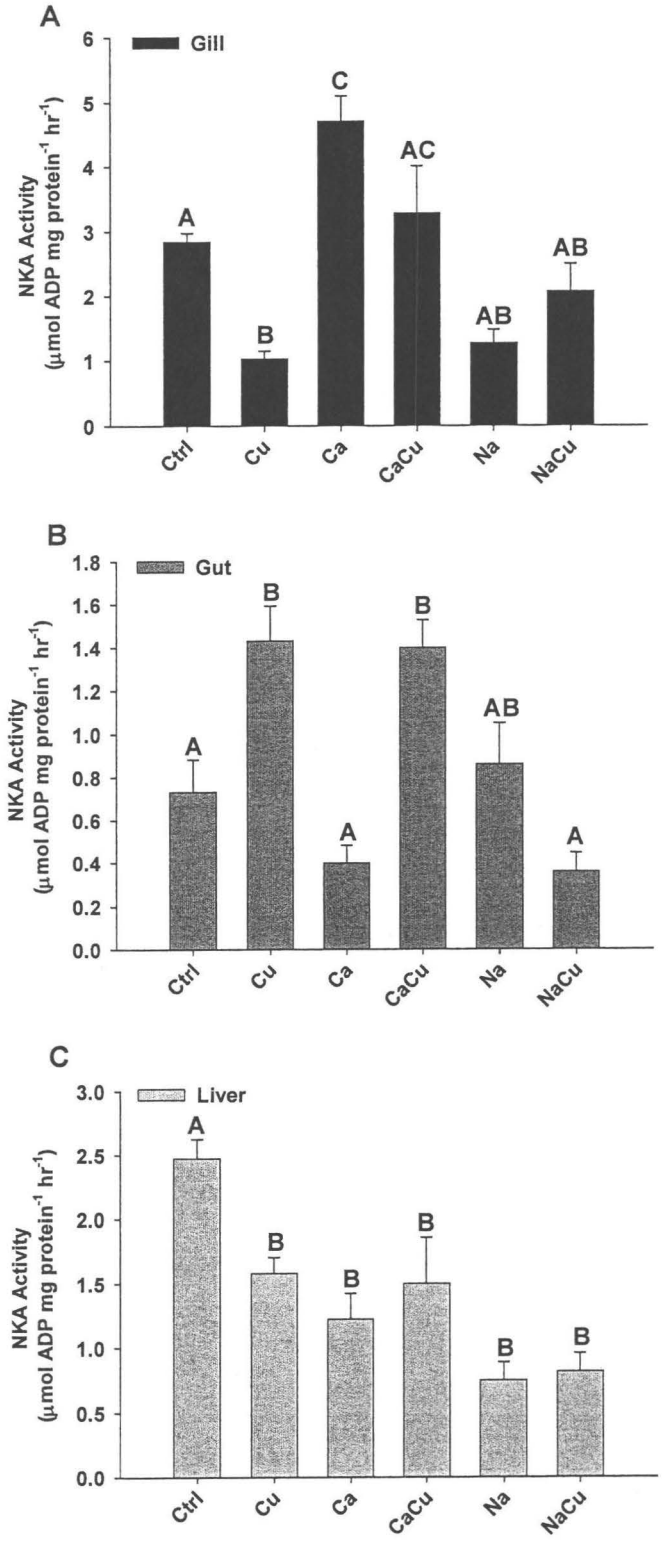
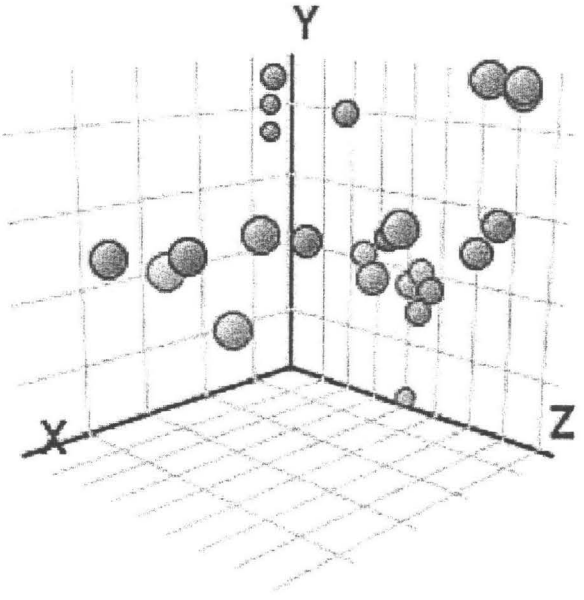

A
Gill
NKA Activity
(µmol ADP mg protein-1 hr-1)
6
5
4
3
2
1
0
A
B
C
AC
AB
AB
Ctrl
Cu
Ca
CaCu
Na
NaCu
B
Gut
NKA Activity
(µmol ADP mg protein-1 hr-1)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
B
A
B
AB
A
Ctrl
Cu
Ca
CaCu
Na
NaCu
C
Liver
NKA Activity
(µmol ADP mg protein-1 hr-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
B
B
B
B
B
Ctrl
Cu
Ca
CaCu
Na
NaCu

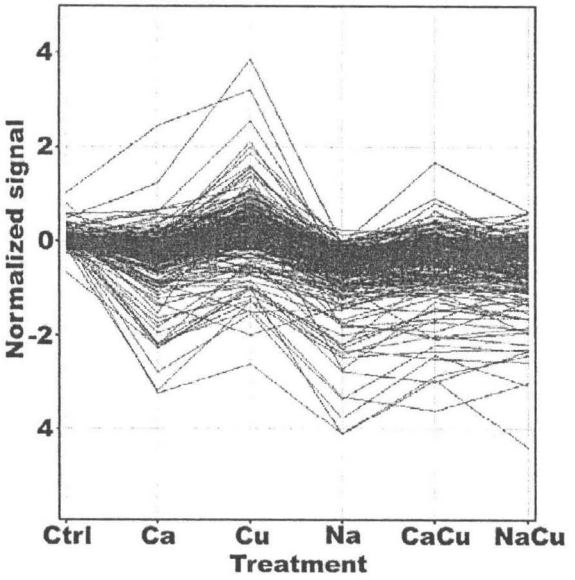
Fig 5.6
Visual description of principle component analysis of microarray results from the liver tissue of zebrafish exposed to ctrl (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu) indicating inherent physiological variability both within and between treatments, as represented by the spatial distance between coloured dots; the greater the distance apart, the higher degree of variability.

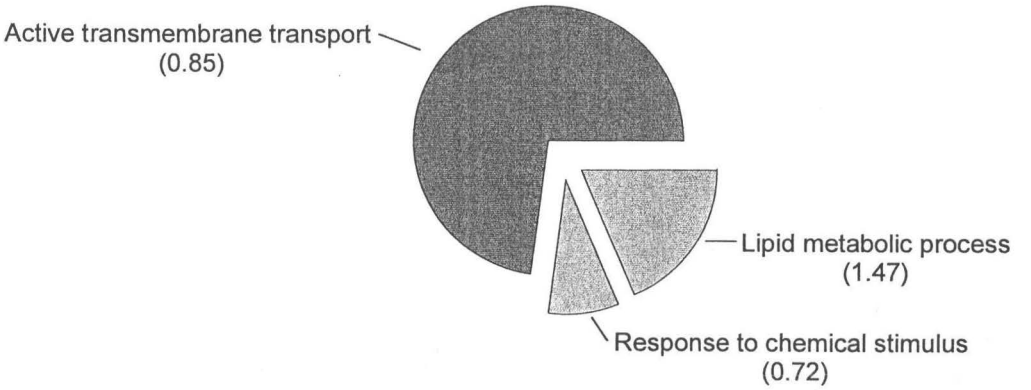


Legend:
Blue - Control
Brown - Cu
Red - Ca
Grey - CaCu
Green - Na

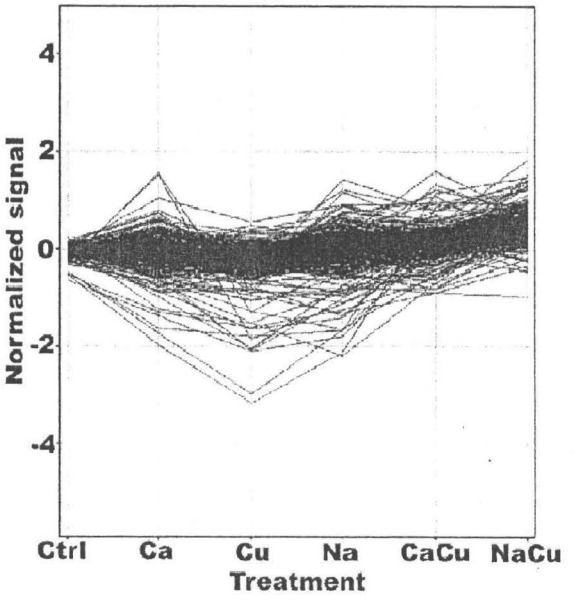
Fig 5.7
Pictorial cluster analysis of microarray results from liver tissue of zebrafish exposed to ctrl (softwater), Cu (12μg/L), Ca (3.3mM), CaCu (3.3mM Ca + 12μg/L Cu), Na (3.3mM), and NaCu (3.3mM Na + 12μg/L Cu) and DAVID functional annotation clustering results ($p<0.05$). Four distinct cluster patterns were identified and normalized signal is represented in Log_2 format, and signal intensities greater than 1 were used for functional annotation. Numerical values in brackets after annotation are the enrichment scores, where the higher the score, the greater likelihood that selected genes from a given cluster fall within the annotation indicated.

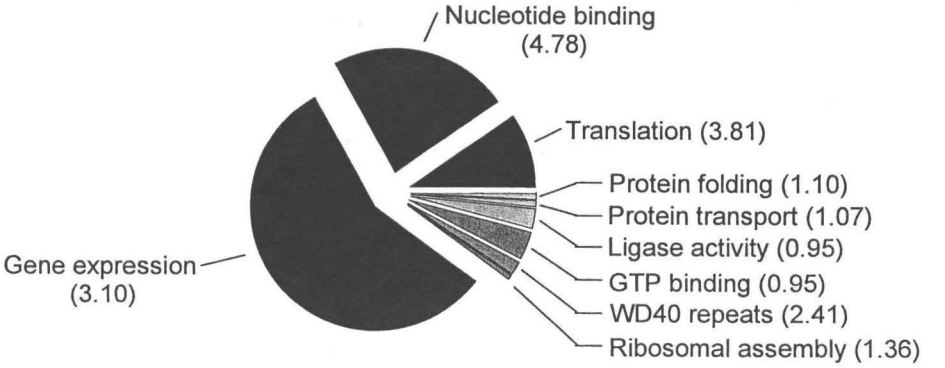
(A) Cluster 1



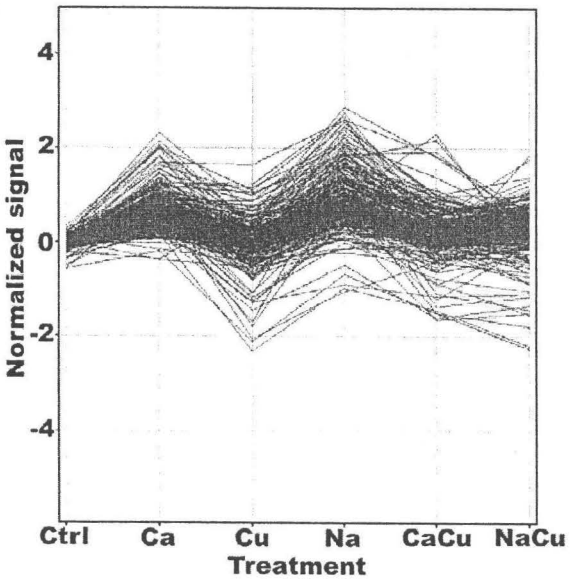


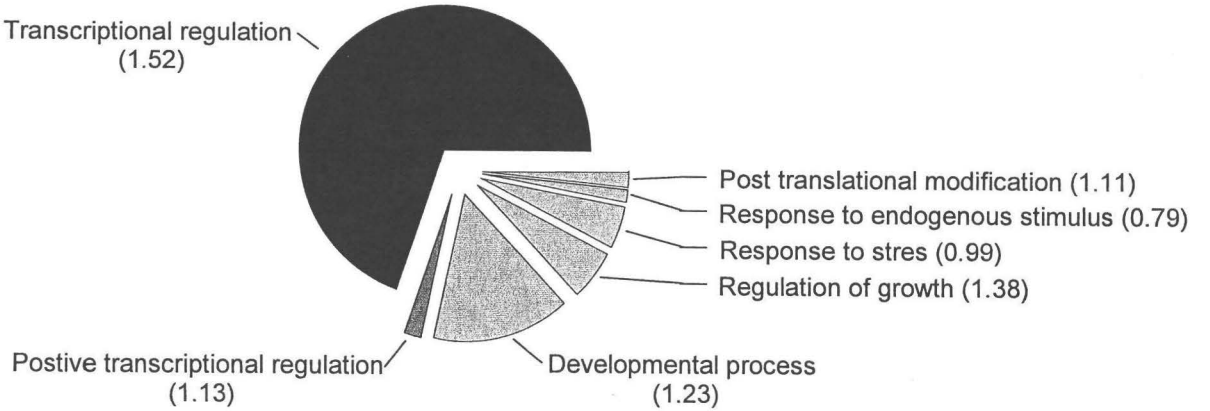
(B) Cluster 2



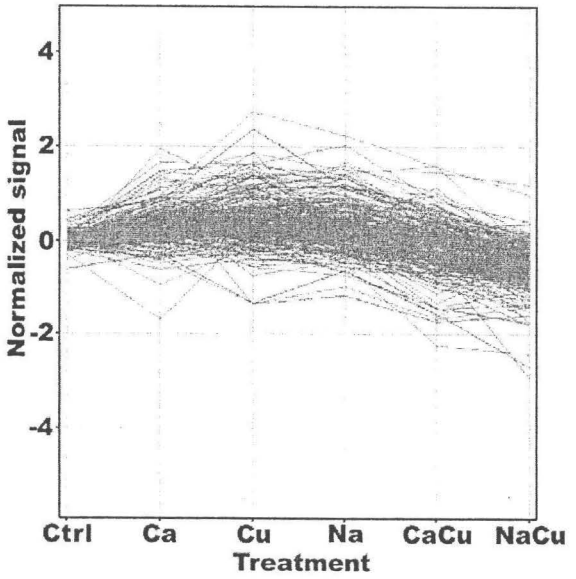


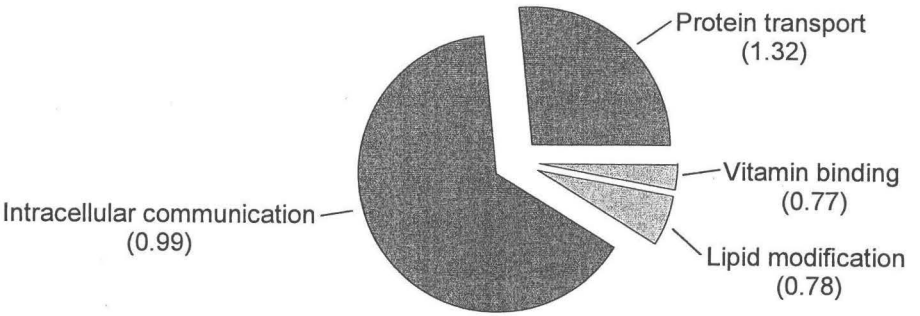
(C) Cluster 3





(D) Cluster 4





CHAPTER 6

DIETARY IRON ALTERS WATERBORNE-COPPER INDUCED GENE EXPRESSION IN SOFTWATER ACCLIMATED ZEBRAFISH (*DANIO RERIO*)

Abstract

Metals like iron (Fe) and copper (Cu) function as integral components in many biological reactions and in excess, these essential metals are toxic, and organisms must control metal acquisition and excretion. We examined the effects of chronic waterborne Cu exposure and the interactive effects of elevated dietary Fe on gene expression and tissue metal accumulation in zebrafish. Softwater acclimated zebrafish exposed to 8μg/L Cu, with and without supplementation of a diet high in Fe (560 vs 140 mg Fe/kg food) for 21d demonstrated a significant reduction in liver and gut Cu load relative to waterborne Cu exposure alone. Gene expression levels for divalent metal transport (DMT)-1, copper transporter (CTR)-1 and the basolateral metal transporter ATP7A in the gills and gut increased when compared to controls, but the various combinations of Cu and high Fe diet revealed altered levels of expression. Further examination of the basolateral Fe transporter, ferroportin, showed responses to waterborne Cu exposure in the gut, and a significant increase with Fe treatment alone in the liver. Additionally, we examined metallothionein 1 & 2 (MT1 & MT2), which indicated that MT2 is more responsive to Cu load. To explore the relationship between transcription and protein function, we examined both CTR-1 protein levels and gill apical uptake of radiolabelled Cu^{64}, which demonstrated decreased Cu uptake and protein abundance in the elevated Cu treatments. This study shows that high dietary Fe can significantly alter the genetic expression pattern of Cu transporters at the level of the gill, liver, and gastrointestinal tract.

Introduction

Trace elements such as copper (Cu), and iron (Fe) are essential micronutrients for all organisms due to their high redox potential, and importance as cofactors for a variety of metabolic proteins, such as cytochrome C oxidase and haemoglobin (Mertz 1981). However, due to increased anthropogenic activities, exogenous concentrations of these trace elements are tending to increase in natural ecosystems, which can be harmful, if not fatal, to aquatic organisms. Due to their persistence in the aquatic environment, it is important to examine the chronic biological impacts of these metals, especially in tropical water systems, where focus has been lacking. Zebrafish, a tropical species, have become an excellent model to study the physiological and genetic impact of increased metal contamination due to their publicly available genome and ability to tolerate softwater, which is key in examining metal impacts without interference of other cations (Craig et al 2007a, 2007b, Neuman & Galvez 2002, Playle at al 1992, 1993). Furthermore, zebrafish are an endemic species to the Indian subcontinent, and may be found in waters that can contain Fe and Cu at levels 100 and 15 times greater, respectively, than those dictated by Indian environmental protection rules (Fe: 3mg/L vs 326mg/L; Cu: 3mg/L vs 48mg/L; Rawat et al 2003) or United States EPA regulations (USEPA 2007) . It is essential to understand the impact metals can have on tropical species in such situations to assess the potential risk of morbidity and mortality which would lead to a population decline.

The primary uptake pathway of trace metals in fish is the diet, although considerable evidence suggests that Cu and Fe can also be taken up by the gills (Clearwater et al 2002, Grosell & Wood 2002, Kamunde et al 2002). Several transport mechanisms associated with Cu and Fe uptake are found in the gill, liver and gastrointestinal tract of zebrafish. Although principally characterized as a Fe transporter, divalent metal transporter-1 (DMT-1) appears to function as a carrier for most divalent metal ions across the apical surface of the cell (Gunshin et al 1997). Its expression has been detected in both the gills and gastrointestinal tract of zebrafish (Cooper et al 2006, Knöpfel et al 2000). Ferroportin, a known Fe exporter, transports Fe from the cell into circulation and has been characterized in both mammalian and fish models (Abboud & Haille 2000, Donovan et al 2000). Interestingly, in macrophages, ferroportin gene expression has been shown to increase in a dose- dependent manner upon increased Cu exposure, which stimulates the release of Fe (Chung et al 2004). Other transporters involved are specifically related to Cu: the apical copper transporter-1 (CTR-1) and the basolateral Cu-ATPase (ATP7A or Menkes gene). CTR-1 is ubiquitously expressed in all tissues in both mammals and other vertebrates, although the largest concentration of CTR-1 in mammals is found in the small intestine, where 90% of total body Cu is absorbed (Sharp 2002). In zebrafish, the CTR-1 gene was first cloned and characterized in studies focusing on embryonic development and the importance of Cu for growth (Mackenzie et al 2004). It was found that altering ambient levels of Cu inversely affected CTR-1 transcripts, indicating CTR-1 expression changes are crucial for normal zebrafish development (Mackenzie et al 2004). However, the CTR-1 gene has not been examined under elevated waterborne Cu in adult zebrafish. Craig et al (2007) found that zebrafish had fluctuations in transcript levels of CTR-1 in the gill during softwater acclimation, indicating that waterborne Cu is required for normal physiological homeostasis. ATP7A

('Menkes' protein) is an essential Cu transport protein involved in both the packaging and transport of cellular Cu into the plasma. Essentially, ATP7A can transfer Cu to the Golgi apparatus to be incorporated into copper-dependent enzymes such as lysyl oxidase or allow for direct secretion of Cu into the circulation (see review by La Fontaine & Mercer 2007). Characterization of this transporter is well established in mammals, due to its importance in human copper deficiency diseases such as Menkes disease (Lee et al 2002). To date, no characterization of ATP7A expression has been performed in adult zebrafish exposed to elevated waterborne Cu. Changes in expression of this protein may be important for the regulation of basolateral Cu transport with changing environmental Cu exposure.

As metals accumulate within cells above ambient levels, several mechanisms are invoked to prevent cellular damage, particularity metal chaperone proteins such as metallothioneins (MT). MTs are metal-binding proteins of low molecular mass that play essential biological roles in metal homeostasis, cytoprotection and detoxification (Kagi & Schaffer 1988). Furthermore, they are increasingly used as biomarkers of metal pollution in the environment (Dallinger et al 2004, Knapen et al 2007, Olsvik et al 2001). Two different isoforms of MTs have been cloned and characterized in zebrafish, MT1 and MT2 and are found in the majority of tissues (Chen et al 2007, Gonzalez et al 2006). These isoforms are particularly sensitive to cadmium (Cd) and Cu, but they are known to bind to various other metals (Kagi & Schaffer 1988). However, the relative responsiveness of the two isoforms has not been studied previously in zebrafish. It is of particular interest to examine which isoform is most sensitive to Cu, as this may aid in determining biomarkers of Cu toxicity.

There is a wealth of data on single metal exposures in fish (Bury & Wood 1999. Bury & Grosell 2003. Chowdhury et al 2008) including zebrafish (Craig et al 2007). However, it is more relevant, both environmentally and physiologically, to examine exposure to metal mixtures. We chose to examine the interactive effects of Fe and Cu since in a pilot study, we found that chronic exposure to 15μg/L waterborne Cu caused a surprising increase in expression of DMT-1 in the gills, which was contrary to our predictions of zebrafish maintaining homeostatic control over Cu. There was also a significant reduction in the Fe load in the liver indicating a potential competition between Cu and Fe, though interpretation was confounded by some mortality (unpublished observations). Many studies have shown that the best characterized link between Cu and Fe is the Cu-derived protein ceruloplasmin, which has been shown to act as a serum ferrioxidase, and is required for Fe mobilization and metabolism in Fe storage tissues (see review Sharp 2004). There is a link between decreased total body Cu and decreased ceruloplasmin levels, which in turn leads to an impairment of iron metabolism resulting in anaemia (Miyajima et al 1987, Osaki & Johnson 1962). Furthermore, under conditions of excess Cu in mammals, haemolytic anaemia occurs due to Cu interruption of glycolysis in erythrocytes, which denatures the haemoglobin (Fairbanks 1967). However, this may not occur in fish since their erythrocytes have a greater aerobic capacity (Eddy et al 1977). If excess Cu in zebrafish leads to altered Fe homeostasis, then supplementing the fishes' diet with Fe should alleviate any Cu-induced anemia.

In the present study, we employed a level of chronic waterborne Cu exposure (8 μg/L) that is environmentally relevant for softwater environments (USEPA 2007), examining its effects on gill, liver, and gut expression of CTR-1, DMT-1, ATP7A, ferroportin, MT1 and MT2. To further characterize the expressional pattern of Cu uptake, we examined how a Fe supplemented diet can alter the gene expression of these transcripts at the gills and the liver. Several studies on fish have demonstrated how a metal- or ion-amended diet can affect uptake of non-essential ions from both the water and food. For example, dietary Ca or Fe can affect branchial cadmium uptake (Baldisserotto et al 2004, Copper et al 2006 in trout and zebrafish, respectively). The goal of this study was to identify the transcription profile of Cu transporters in the gill, liver and gut, of softwater acclimated zebrafish chronically (21d) exposed to moderate levels of waterborne Cu and to determine how dietary Fe can affect both the transcription profile and gill apical uptake of an essential micronutrient. Additionally, as a functional test, short-term uptake of radiolabelled Cu (^{64}Cu) was employed to complement this data to clarify the relationship between transcript levels, protein levels, and functional Cu transport activity.

Materials & Methods

Fish Care

Adult zebrafish of mixed sex (*Danio rerio*) were purchased from a local pet fish distributor (DAP International, Canada) and acclimated to soft-water over a 7 day period in an aerated 40L aquarium as described previously (Craig et al 2007; Table 6.2). After acclimation, zebrafish were housed in multiple 3L self-cleaning AHAB tanks racked in a soft-water recirculating stand-alone AHAB filtration system (Aquatic Habitats, Apopka, Fl). Fish were fed twice daily with a commercial tropical fish food (Topfin, Phoenix, AZ) and maintained on a 12-h light, 12-h dark photoperiod regime until experiments began. All procedures used were approved by the McMaster University Animal Research Ethics Board, and conform to the principles of the Canadian Council for Animal Care.

Exposure to waterborne Cu and Fe diet

Zebrafish (n=224; 40-48 per treatment) were weighed and placed in 8L aerated, flow through soft-water tanks set to 25ml/min. There were four experimental treatments: 0 μg Cu /L(Ctrl), 0 μg Cu/L (Ctrl+Fe diet), 8 μg Cu/L, or 8 μg Cu/L + Fe diet. For the two elevated Cu treatments, Mariotte bottles were used to dose tanks with a concentrated Cu solution made from $CuSO_4 \cdot 5H_2O$ dissolved in 0.05% HNO_3, nitric acid, which had no measurable impact on water pH. Fish were fed 2% body weight once per day of commercial tropical fish food or a diet supplemented with Fe. The high iron diet was formulated by the addition of 240mg $FeSO_4 \cdot 7H_2O$ to 40g of powdered tropical food. Food and Fe was mixed with the addition of a small amount of water, spread on a baking sheet and dried at 35°C for 1h, and then collected and re-powdered. A sample of the normal diet and elevated Fe diet was taken and dissolved in HNO_3 and measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z, Varian, Palo Alto, CA) to verify Fe concentrations. The normal diet contained ~ 140 mg Fe/kg food, whereas the high Fe diet contained ~560 mg Fe/kg food. Tanks were monitored daily for mortality and cleaned of

any food or waste that had accumulated. Daily, a 10ml water sample was taken from each tank, filtered though a 0.45μm filtration disc (Pall Corporation, East Hills, NY), added to a plastic tube containing 100μl HNO_3 and kept at 4°C for analysis of Cu and Fe concentrations. At the end of the exposure period, fish were quickly euthanized by cephalic concussion and sampled for gill, gut, and liver tissue which were immediately frozen in liquid N_2 for further analysis of Cu and Fe burdens and gene expression. Additional gill samples were taken for western blotting.

Water and tissue ion levels

All tissues (and food) were first digested in 1ml of 1N HNO_3 for 48 h at 60°C. Digests were diluted 10× and dissolved Cu levels were measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian, Palo Alto, CA) and compared to a 40 μg/L Cu standard (Fisher Scientific, Ottawa, ON). Water Cu levels were read without dilution. Water Fe levels were measured by graphite furnace and compared to a diluted 1mg/L Fe standard (Fisher Scientific). Both tissue digests (Na^+,Mg^{2+}, Fe^{2+} and Ca^{2+}) and water ion composition (Na^+,Mg^{2+}, and Ca^{2+}), were measured by flame atomic absorption spectroscopy (Spectra AA 22 0FS, Varian, Palo Alto, CA) after 10× dilutions were made with 1% HNO_3 (Na^+) or 0.5% $LaCl_3$/1% HNO_3 (Mg^{2+}, Ca^{2+}, Fe^{2+}), and verified using certified Na^+,Mg^{2+}, Fe^{2+} and Ca^{2+} standards (1 mg/L diluted in 1% HNO_3 or 0.5% $LaCl_3$/1% HNO_3; Fisher Scientific).

Quantification of mRNA by real-time RT-PCR.

Total RNA from the gill, gut and liver tissues were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by UV spectrophotometry at 260 nm, and RNA purity verified by the 260/280 nm ratio (Fisher Scientific, Nanodrop ND-1000, Wilmington, Delaware). First strand cDNA was synthesized from 1 μg of total RNA treated with DNase I (Invitrogen, Calsbad, CA) and reverse transcribed to cDNA using SuperScript II RNase H- reverse transcriptase (Invitrogen, Calsbad, CA). mRNA expression was quantified in duplicate on a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 μl SYBR green mix, 1 μL of each forward and reverse primer (5 μM), 5.5 μL RNase/DNase free H_2O, and 5 μL of 5× diluted cDNA template. Cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 95°C for 15 sec, 60°C for 45sec, 72°C for 30 sec. This was followed by a melting curve analysis to verify the specificity of the PCR products within and between tissues. To account for differences in amplification efficiency between different cDNAs, standard curves were constructed for each target gene using serial dilutions of stock gill, gut and liver cDNA. To account for differences in cDNA production and loading differences, all samples were normalized to the expression level of the house-keeping gene EF1α, which did not change over the course of the experimental treatments. Gene expression data were calculated using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen 2001). Both DNase- and RNase-free water and non-reverse transcribed RNA were assayed on each

plate to ensure there was no contamination present in reagents or primers used. Primers were designed using Primer3 (Rozen & Skaletsky 2000). Target genes of interest are as follows: Menkes transporter (ATP7a), copper transporter-1 (CTR-1), divalent metal transporter-1 (DMT-1), elongation factor-1 alpha (EF1α), ferroportin, metallothionein 1 (MT1), and metallothionein 2 (MT2). Primers and accession numbers can be found in Table 1.

Apical gill uptake of ^{64}Cu

Following final sampling on day 21 (i.e. at the end of the exposure period), an acute (20min) Cu gill uptake study was performed on fish remaining from each of the experimental treatments (n=8 per treatment). ^{64}Cu was prepared from dried $Cu(NO_3)_2$ (300μg) and irradiated at the McMaster nuclear reactor to achieve a radioactivity level of 0.6mCi (half-life = 12.9h). After irradiation, the $Cu(NO_3)_2$ was dissolved in 0.1mM HNO_3 (400μl), 0.01mM $NaHCO_3$ (400μl) and Nanopure water (1.7ml). The resuspended ^{64}Cu stock was added to 1.5L tanks containing aerated softwater 30 min prior to the addition of fish. Water samples (10ml) were taken in duplicate at the beginning and end of the exposure for the measurement of dissolved and radioactive Cu. After exposure, fish were removed, rinsed in a concentrated solution of Cu to remove any loosely bound radioisotope by displacement, and were terminally anesthetised with an overdose of MS-222 (1g/L). Previous tests have validated this procedure (Grosell & Wood 2002). The gills were excised from the body, and both gills and carcass were blotted and weighed prior to gamma counting. The gamma radioactivities of ^{64}Cu of the gills (representative of apical uptake; Grosell & Wood 2002) and carcass were measured on a Minaxi- γ Auto gamma 5530 counter (Canberra Packard, Mississauga, ON) using energy windows of 433-2000 KeV for ^{64}Cu. ^{64}Cu was corrected for decay to a common reference time due to a short half-life (12.9h).

Western blot of gill CTR-1

Whole gill arches were homogenized in buffer (100 mM imidazole, 5mM EDTA, 200 mM sucrose, and 0.1% deoxycholate, pH 7.6), and centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was collected and diluted to 30 μg of protein in 4× loading buffer (48 mM Tris-HCl pH 6.8, 4% glycerol, 3.2% SDS, 600 mM β-mercaptoethanol, 1.6% bromophenol blue). Samples were denatured in boiling water for 5 minutes, and loaded onto a 7.5% SDS-polyacrylamide gel. Proteins were separated by electrophoresis for 1h at 150V. Samples were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Mississauga, ON), and blots were incubated overnight at 4°C in 7.5% skim-milk + PBST (10mM phosphate buffer, 0.09% NaCl, 0.05% Tween-20, pH 7.5, PBST). Blots were washed 3× for 5min in PBST and incubated at room temperature with the primary antibody diluted in 5% skim-milk + PBST. The primary antibody (1:200) used was a human CTR-1 antibody (ab30907; Abcam, Cambridge, MA) which was designed on a highly conserved region of the hCTR protein. The % amino acid homology of the conserved region between the hCTR-1 (accession # NM_001855) and the zebrafish CTR-1 (accession # NM_205717) was 90%. Tubulin was used due to availability and good reactivity with zebrafish tissues and did not change with our

treatments (13). Membranes were washed 3 × 5min in PBST, and incubated for 1 h at room temperature with an HRP-conjugated anti-rabbit IgG (CTR-1, 1:25,000; PerkinElmer, Boston MA) or anti-mouse IgG (tubulin, 1:50,000). After 3× 5min washes with PBST, proteins were visualized with a Western Lightning chemiluminescence kit, following the manufacturer's protocol (PerkinElmer). Blots and band density analysis were completed on a ChemiImager (AlphaInnotech Corporation, San Leandro, CA), which used pixel density to quantify band intensity. Bands were normalized to tubulin, and expressed as a ratio of the control.

Statistical Analysis

Statistical analysis was performed using Sigma Stat (SPSS Inc, Chicago, MI). In particular, a one-way ANOVA and a Student-Newman Keuls post-hoc test was used to test for significance for all pairwise treatments ($p<0.05$). All data have been expressed as mean ± SEM.

Results

Water ion composition & fish weights

Water ion analysis verified that all experiments were conducted in a soft-water environment (hardness as $CaCO_3$ equivalents = 6.9± 0.3 mg/L; Table 6.2). This reduced the protective effects which would be exerted by ions normally present in hard water against metal toxicity. Statistical analysis revealed that a significant difference exists only when comparing Cu concentrations to that of controls (Table 6.2). The elevated Fe diet in experiment 2 did not cause any change in waterborne Fe levels (Table 6.2). Zebrafish were weighed prior to and after exposure to all treatments, with the control and control + Fe diet increasing in weight after 21d, and all other treatments decreasing in weight, by up to 17% for fish exposed to 8 μg /L Cu alone (Table 6.3). There were no mortalities in any of the treatments.

Exposure to waterborne Cu and Fe diet

Significant increases in Cu load were found in all tissues examined in zebrafish exposed to 8 μg/L Cu (Fig 6.1). Interestingly, there was a significant accumulation of Cu in the gills of zebrafish fed the high Fe diet only, which was further exacerbated in fish exposed to 8μg/L Cu + high Fe diet (Fig 6.1A). There was a tendency towards a decrease in Fe tissue burden in both the gut and gill of fish exposed to 8μg/L Cu alone ($p=0.06$ & $p=0.07$, respectively), and surprisingly a significant decrease in liver Fe of zebrafish exposed to 8 μg/L Cu + high Fe diet (Fig 6.2). There was little change in expression of CTR-1 except in zebrafish treated with 8μg/L Cu + high Fe diet where there was a 13- and 7-fold significant increase in CTR-1 gene expression in both the gill and gut (Figure 6.3A, B). Likewise, we saw a 5-fold increase in DMT-1 gene expression in the gill under the same conditions (Fig 6.4A), but no changes were detected in the gut or liver. However, DMT-1 expression did increase in both the gill and gut of zebrafish fed a high Fe diet in the absence of additional Cu (Fig 6.4A,B). Interestingly, DMT-1 was upregulated in the liver when zebrafish were exposed to 8μg/L Cu (Fig 6.4C), as was

ATP7A (Fig 6.5C). Zebrafish exposed to 8μg/L Cu + high Fe diet had significant increases in ATP7A expression in both the gill and gut. There was also a significant increase in gut ATP7A expression, but no change in the gill with Cu alone (Fig 6.5A,B). Ferroportin expression increased significantly in the gut and gill of fish exposed to 8 μg/L Cu alone but the gut was not responsive to other treatments (Fig 6.6A, B). Furthermore, we saw a 2.5 - and 4-fold increase in gene expression of ferroportin in the gill and liver of fish fed a high Fe diet in the absence of Cu. However, there were no increases in ferroportin gene expression in liver of fish exposed to 8 μg/L Cu + high Fe Diet (Fig 6.6C). When we compared the expression profiles of MT1 versus MT2 in the gill and liver, we found that MT2 had a greater response to Cu than MT1, but MT1 did significantly increase when fish were exposed to both 8 μg/L Cu + Fe diet (Fig 6.7A,C). Neither MT1 nor MT2 showed changes in mRNA expression in the gut with any of the experimental treatments (Fig 6.7B).

We examined CTR-1 protein expression changes using western blotting. Band intensities were normalized to tubulin and expressed as a ratio of the control. We saw a significant decrease in protein expression of CTR-1 in zebrafish exposed to 8μg/L Cu only. Feeding zebrafish a high Fe diet resulted in a partial reversal of the Cu-induced decline in CTR-1 protein expression (Fig 6.8). Functional measurements of Cu transport revealed a significant decrease in the gill apical and whole body uptake of ^{64}Cu in zebrafish exposed for 21d of 8μg/L Cu, and only a decrease in the whole body uptake of zebrafish exposed to 8μg/L Cu + high Fe diet (Fig 6.9).

Discussion

This study shows that both chronic waterborne Cu exposure and a high Fe diet can on their own significantly alter the genetic expression pattern of Cu transporters at the level of the gill, liver, and gastrointestinal tract, but that there are also unique interaction effects. With a moderate Cu exposure (8μg/L), in confirmation of an initial pilot study at a higher Cu level (15 μg/L, unpublished), we found there was a decrease in tissue Fe levels in the gill and gut (Fig 6.2A,B). This prompted us to expose zebrafish to an elevated Fe diet, since we assumed Cu could have a competitive effect with Fe due to a shared uptake pathway (DMT-1). With the addition of Fe to the diet, we did not see any changes in Fe tissue levels, despite an increased Cu load in the gills, gut and liver (Fig 6.1). Upon examination of the gene expression profile, we found 8 μg/L Cu + Fe diet significantly increased expression of CTR-1 in the gill and gut (Fig 6.3A,B). Additional increases in DMT-1 were found in the gill and liver with waterborne Cu, with the Fe diet exacerbating the increase in gill DMT-1 expression over the 8μg/l Cu exposure (Fig 6.4A, C). Interestingly, we found that a high Fe diet without waterborne Cu significantly increased DMT-1 expression in the gut (Fig 6.4B). With respect to the basolateral Cu transporter ATP7A, we found that moderate Cu exposure increased expression levels in the gut and liver, and with the addition of an Fe diet, we saw increased expression in the gills and gut (Fig 6.5). Contrary to our gene expression profile, the protein expression of CTR-1 and the apical uptake of Cu in the gills tell a different story. Protein expression

actually decreased concurrent with a decrease in Cu uptake. This may hint at an increased protein turnover rate under stressful conditions (Fig 6.6, 6.7).

Copper homeostasis in fish is tightly regulated, and as in higher vertebrates, such as mammals, excess Cu is accumulated in the liver and excreted in the bile (Grosell et al 1998). Likewise in zebrafish, we saw an elevated Cu load in the liver of fish exposed to increased waterborne Cu (Fig 6.1C). However, we also saw increases in Cu load in the gut under these conditions (Fig 6.1B). Waterborne Cu has two possible modes of uptake, either through the gills or the gut, and excess Cu is excreted through the bile (Grosell et al 1998). Increased Cu load in the gut may be primarily due to increased biliary excretion of Cu. Furthermore, at 8μg/L waterborne Cu, we saw elevated Cu load in the gills. Grosell & Wood (Grosell & Wood 2002) demonstrated that gill Cu load rapidly peaks with waterborne Cu exposure after the first 3hr, and then gradually reaches equilibrium, which supports our findings at a moderate Cu exposure.

In our initial pilot experiment of exposing zebrafish to 15μg/L Cu (unpublished), we found evidence that fish exhibited significantly lower tissue Fe load in both the gill and liver. Although there is little prior evidence in fish to suggest Cu would have such an effect, there is the likelihood that high waterborne Cu can out-compete Fe for apical uptake at the gill, since they can use the same transporter, DMT-1 (Gunshin 1997). With zebrafish exposed to both moderate waterborne Cu (8 μg/L) and Fe diet, we were able to reverse the diminished Fe tissue levels, although a high Fe diet significantly increased the Cu load in the gills both with and without waterborne Cu (Fig 6.1A). Potentially, this is an adaptive response to an elevated Fe load, as there are Cu-essential transmembrane ferroxidases, like hephaestin, which function in addition to ferroportin, to translocate Fe across the basolateral membranes into the general circulation, although this protein has not as yet been localized in the zebrafish gill (Vulpe et al 1999).

In an effort to identify potential genetic endpoints of chronic waterborne toxicity, we examined both metal sequestering proteins and several key transporters thought to regulate the uptake of Cu at both the level of the gills and gut. Furthermore, we examined gene expression changes for these transporters in the liver, to identify any compensatory responses to toxicity and characterize detoxification methods. Upon examination of the two known MT isoforms, we found that MT2 was more responsive to Cu exposure than MT1 in both gill and liver tissue (Fig 6.7A,C), where there is the highest accumulation of Cu (Fig 6.1A, C). Previous studies examining increased zinc exposure demonstrated that MT-1 is more responsive to excessive Zn in rats (Mamdouh et al 2002), whereas MT-2 has demonstrated specificity to Cu in blue crabs, although this has yet been identified in fish species (Syring et al 2000) . We additionally saw a 9-fold increase in expression of MT1 in the liver of zebrafish exposed to both 8μg/L Cu + high Fe diet (Fig 6.7C). Aside from a role in metal sequestration, MTs have been shown to scavenge both hydroxyl radicals and superoxides in mouse and rat liver cells (Min et al 1999). Free cytoplasmic Cu^{2+} and Fe^{2+} are highly reactive ions and can result in the production of superoxide hydroxyl radicals if left unchecked. In the liver of zebrafish, there is an excessive build-up of Cu which may promote increased ROS production (Craig et al 2007), and this may be further exacerbated by Fe flux through the liver. However, the bottom line is that MT2

has been identified as a Cu related biomarker, opposite to the current thinking of examining all forms of MTs.

CTR-1 is a high affinity Cu transporter first cloned in zebrafish by Mackenzie et al. (2004). Interestingly, there is no evidence for transcriptional regulation of CTR-1 by Cu in mammals, although protein levels undergo changes in subcellular location and stabilization (Lee et al 2000, Petris et al 2002, Tennant et al 2002). Our study suggests that CTR-1 is transcriptionally regulated in the gills and gut of zebrafish by moderate Cu + high Fe diet (Fig 6.3A, B). Although increased transcription of CTR-1 in a situation of elevated waterborne Cu seems counter intuitive, it again could be a compensatory response to promote cellular assimilation and translocation of essential metals. Cu is transported from the cell into the blood stream through two potential routes, either direct secretion via ATP7A or incorporation into ceruloplasmin (Mercer & Llanos 2003, Voskoboinik & Camakaris 2002). Ceruloplasmin can act as a ferroxidase, oxidising Fe^{2+} to Fe^{3+}, which allows for Fe delivery to peripheral organs for use or detoxification (Hellman & Gitlin 2002, Kaplan 2002). Therefore, to respond to an increased Fe diet, more Cu needs to be absorbed to aid in transport and oxidization of Fe.

DMT-1 has primarily been identified as an Fe transporter, although it does have the potential to transport other divalent metals (Gunshin et al 1997, Savigni & Morgan 1998), and has been identified in numerous fish species, including zebrafish (Donovan et al 2002). In *Xenopus* oocytes, DMT-1 appears to transport a diverse range of metals such as Pb^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} (Gunshin 1997). Recently, it has been implicated in the dietary uptake of Cu and zinc in rainbow trout intestine (Neumann & Galvez 2002, Ojo et al 2008). In similar fashion to CTR-1, we saw a significant increase in DMT-1 expression in the gills and gut of zebrafish exposed to a high Fe diet (Fig 6.4A,B) , suggesting increased Fe uptake, although we did not see any substantial increase in tissue Fe load (Fig 6.2A,B). Cooper et al (2006) have shown that a low Fe diet results in increased DMT-1 expression as an adaptive response to absorb more Fe from a deficient diet. In our study, we found that DMT-1 expression increased with a high Fe diet in the gut and gills (Fig 6.4A,B). There was also significant increase in DMT-1 expression in zebrafish exposed to 8μg/l Cu + high Fe diet in the gills, although there were no changes in expression in the gut in this treatment (Fig 6.4A,B). Potentially, zebrafish exposed to high waterborne Cu are reducing the uptake of Cu in the gut by decreasing the number of uptake pathways. In the liver, we see a significant increase in DMT-1 only in zebrafish exposed to 8μg/l waterborne Cu (Fig 6.4C). In fish, Cu is transported through the blood via ceruloplasmin (Parvez et al 2003) and as seen in mammalian liver cell suspensions, Cu bound to ceruloplasmin is taken up into hepatocytes by a ceruloplasmin receptor (Tavassoli et al 1986). However, in cases of excessive Cu exposure, free Cu ion levels can increase in the blood serum, although this phenomenon has only been identified in mammals (Cauza et al 1997, DiDonato & Sarkar 1997). Although there was no change in CTR-1 expression in the liver (Fig 6.3C), the increase in DMT-1 expression (Fig 6.4C) may allow for the uptake of excessive Cu ions from the blood serum into the liver hepatocytes for sequestration and excretion.

In a manner similar to DMT-1, ferroportin, a known Fe exporter, is modulated by both Cu and Fe (Fig 6.6). The function of ferroportin as a Fe exporter has been studied in

mammalian and amphibian models, and has shown increased expression levels upon exposure to excessive exogenous Fe and Cu (Chung et al 2004, Donovan et al 2000, McKie et al 2000). Likewise, we saw increased expression in the gut when zebrafish were exposed to 8 μg/L Cu alone and a 4-fold increased expression in the liver of zebrafish fed a high Fe diet in the absence of Cu (Fig 6.6B,C). Furthermore, there were significant increases in ferroportin expression in the gills of zebrafish exposed to either 8 μg/L Cu alone or Fe diet alone (Fig 6.6A). In *Xenopus* oocytes and mammalian macrophages, excessive Fe results in increased ferroportin expression and protein levels to offload excessive Fe, which can increase levels of reactive oxygen species (Chung et al 2004, Donovan et al 2000, McKie et al 2000). However, in our fish, we saw that in the liver, there was an increased ferroportin expression when fish were exposed to a high Fe diet alone, even though there was no accumulation of Fe in the liver (Fig 6.2C). Indeed, under all experimental conditions there was a depression of Fe levels in the liver, which indicates that the liver functions to offload excessive Fe in these situations, yet we did not see the respective changes in gene expression, except under exposure of a high Fe diet (Fig 6.6C). Furthermore, we saw that Cu can stimulate ferroportin expression in the gills and gut (Fig 6.6A,B) as previously demonstrated (Chung et al 2004, Donovan et al 2000, McKie et al 2000). In mammalian macropahges, increased Cu not only increased expression levels of ferroportin gene and protein, it also produced a dose-dependent excretion of Fe (Chung et al 2004). This supports the model that Cu plays an essential role in Fe cycling and transport, and also alludes to ferroportin having the potential to transport Cu and other divalent metals as well.

Cu metabolism and export from the cell rely on important Cu-transporting ATPases. In mammals, there are two types of ATPases: ATP7A (Menkes gene) & ATP7B (Wilson's gene), which are associated with human genetic disorders of the same name (Linz & Lutsenko 2007). In mammals, ATP7A is found through all tissues, except the liver, where only ATP7B is found (Kuo et al 1997). Barnes *et al* (2005) demonstrated that ATP7A has high turnover rates and can transport more copper per minute than ATP7B. In fish, little is known about that localization and characterization of Cu-transporting ATPases, although only the ATP7A gene has been identified in the zebrafish (Mendelsohn et al 2006). Furthermore, although the ATP7B gene has been identified in the zebrafish genome through predicted computational sequencing, there is no published RNA sequence in Genbank, which hints at a functional loss of the ATP7B gene. Our study suggests that ATP7A is important in basolateral transport of Cu out of the cells of the gill and gut under conditions of moderate Cu + high Fe diet (Fig 6.5A,B). In addition, there were significant increases in ATP7A in the gut and liver under conditions of moderate Cu exposure (Fig 6.5B,C). Two plausible explanations for the increase in ATP7A are 1) Cu plays a vital role in the oxidation of Fe ions, and this relates to the increased expression of ATP7A, as this transporter also acts to shuttle Cu to the Golgi apparatus to be incorporated into secreted cuproenzymes (Mercer & Llanos 2003); 2) Increased cellular Cu is known to increase reactive oxygen species, and this results in increased oxidative damage (Craig et al 2007, DiGiulio et al 1989). Therefore increased basolateral transport of Cu would help remove Cu from the cell, and in the case of the liver, allow for the excretion of excessive Cu, although this requires further investigation.

Contrary to our findings that CTR-1 expression remained stable following exposure to 8μg/L Cu and actually increased after treatment with 8μg/L Cu + high Fe diet (Fig 6.3A), we found a significant reduction in both apical uptake (in the 8μg/L Cu + high Fe diet exposure) and whole body incorporation of waterborne Cu (in both treatments) and reduced protein expression levels of CTR-1 in the gill (Figs. 6.8, 6.9). Note also that the expression levels of the other two genes likely involved in Cu uptake at the gills (DMT1, Fig 6.4A) and ATP7A (Fig 6.5A) remained stable or increased in one or both of these treatments. This is the first study to investigate both the transcription profile and functional uptake of CTR-1, and provides significant insight into predictions of chronic endpoints of Cu toxicity, since increased expression of target genes does not necessarily translate into increased protein levels or increased function.

However, the importance of CTR-1 proteins in Cu transport cannot be disputed, as they are an essential route of Cu uptake for all vertebrates (Sharp 2004). There is obviously some post-transcriptional regulation occurring, that down-regulates the protein abundance under periods of excessive Cu; however, this requires further investigation. Outside of the above explanations for increased transcription rates of our genes of interests, an alternate explanation is that under conditions of environmental stress, there is an increased protein turnover rate, which allows for the faster production of new proteins, and faster degradation of harmful proteins (see review Hawkins 1991). Furthermore, it has been shown that growth rate is sacrificed as protein turnover rate increases in rainbow trout (McCarthy et al 1994). In all of our fish exposed to waterborne Cu, we see a significant reduction in growth rate compared to those fish under normal circumstances (Table 6.3). Increased gene expression could in part be explained by increased turnover rates, although this was not directly examined.

Perspectives

Overall, we have demonstrated that alterations in the diet can affect uptake and accumulation of Cu in zebrafish when exposed to elevated waterborne Cu, as well as the gene expression of transport and binding proteins that may be involved. This study provides further evidence that Fe can enhance the uptake of an essential metal Cu (as well as non-essential metals), which has been demonstrated in both fish and mammalian species (Cooper et al 2006, Leazer et al 2002). We have also provided evidence that MT2 can be used as a Cu-specific genetic endpoint of chronic exposure, and furthered our knowledge that Cu plays an essential role in Fe cycling and transport by modifying the expression level of ferroportin. Furthermore, this is the first study to examine the CTR-1 protein from both a transcriptional and functional point of view in zebrafish, and highlights the importance of functional studies in future research, because of the disconnect between message, protein, and functional responses seen, and this has been identified in other studies (e.g. Richards et al 2007). Our results suggest that while transcriptional responses may be of diagnostic importance, they may not relate directly to protein abundance and function.

Table 6.1: Forward (F) and reverse (R) primers used for real-time qPCR

Gene	Primer	Acession #	Amplicon size (BP)
ATP7a	F: 5'-GGCTCGACTTCTCGCAGCT-3' R: 5'-ATTCCGCATTTTCACTGCCT-3'	NM_001042720	50
CTR-1	F: 5'-GAATCAGGTGAACGTGCGCT-3' R: 5'-CCATCAGATCCTGGTACGGG-3'	AY077715	51
DMT-1	F: 5'-CAATCGACACTACCCCACGG-3' R: 5'-TCCACCATAAGCCACAGGATG-3'	AF529267	51
Ferroportin	F: 5'-ACATTCTGGCACCGATGCTT-3' R: 5'-AGTGTGAGCCAAATGCCATG-3'	NM_131629	51
MT-1	F: 5'-CGTCTAACAAAGGCTAAAGAGGGA-3' R: 5'-GCAGCAGTACAAATCAGTGCATC-3'	AY514790	51
MT-2	F: 5'-GGAGGAGGGTCAGAGGAACC-3' R: 5'-AGCAACTGAAGCTCCATCCG-3'	NM_194273	51
EF1-α	F: 5'-GTGCTGTGCTGATTGTTGCT-3' R: 5'-TGTATGCGCTGACTTCCTTG-3'	NM131263	201

Table 6.2: Concentrations of water ions (μM), copper (μg/L), and iron (μg/L) for all experimental exposures. Values presented as mean ± SEM. * indicates significant difference from respective control ($p<0.05$, $n = 21$).

Ion	Ctrl	Ctrl + Fe diet	Cu (8μg/L)	Cu (8μg/L) + Fe diet
Na^{+}	65.3 ± 3.6	53.4 ± 3.8	49.0 ± 3.0	51.6 ± 3.2
Mg^{2+}	20.5 ± 3.6	12.5 ± 0.9	12.4 ± 1.4	12.8 ± 1.9
Fe^{2+}	2.5 ± 0.7	2.2 ± 0.6	2.1 ± 0.8	2.2 ± 0.7
Ca^{2+}	49.2 ± 3.8	42.2 ± 2.9	38.0 ± 2.8	42.2 ± 2.8
Cu^{2+}	1.8 ± 0.2	1.2 ± .0.2	8.0 ± 0.4*	8.7 ± 0.3*

Table 6.3: Mean weight of fish before and after respective 21d treatment, including % change in weight. Values presented as mean ± SEM (n = 40-48 per treatment).

Treatment	Before (g)	After (g)	% Change
Ctrl	0.35 ± 0.01	0.50 ± 0.05	28.5
Ctrl + Fe Diet	0.43 ± 0.04	0.55 ± 0.13	21.6
Cu (8μg/L)	0.45 ± 0.11	0.38 ± 0.02	-17.7
Cu (8μg/L) + Fe diet	0.50 ± 0.06	0.45 ± 0.03	-10.1

Fig 6.1

Copper (Cu) load (μg/g tissue) in gill (**A**), gut (**B**), and liver (**C**) tissue from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d . Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=7 for all treatments, $p<0.05$).

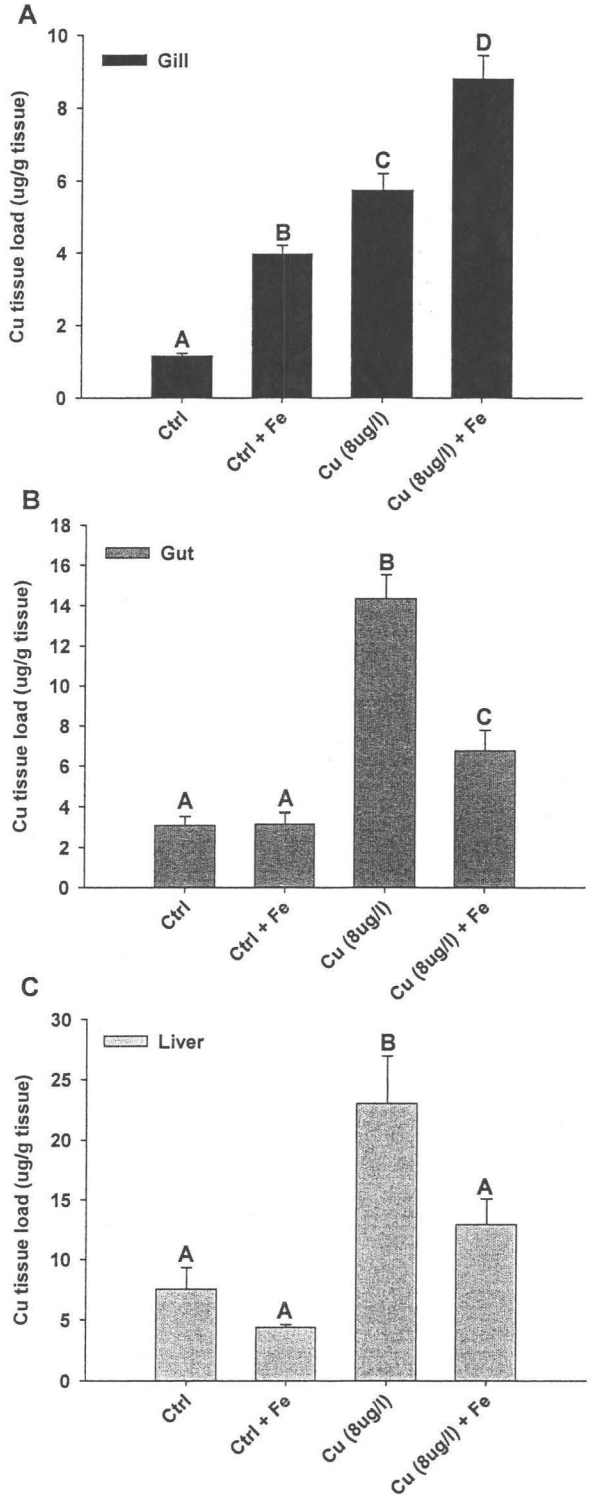

A
Gill
Cu tissue load (ug/g tissue)
0
2
4
6
8
10
A
B
C
D
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Gut
Cu tissue load (ug/g tissue)
0
2
4
6
8
10
12
14
16
18
A
A
B
C
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
C
Liver
Cu tissue load (ug/g tissue)
0
5
10
15
20
25
30
A
A
B
A
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

Fig 6.2

Iron (Fe) load (μg/g tissue) in gill (**A**), gut (**B**), and liver (**C**) tissue from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d. Values are presented as means ± SEM and treatments that do not share a common letter are significantly different from each other (n=7 for all treatments, $p<0.05$).

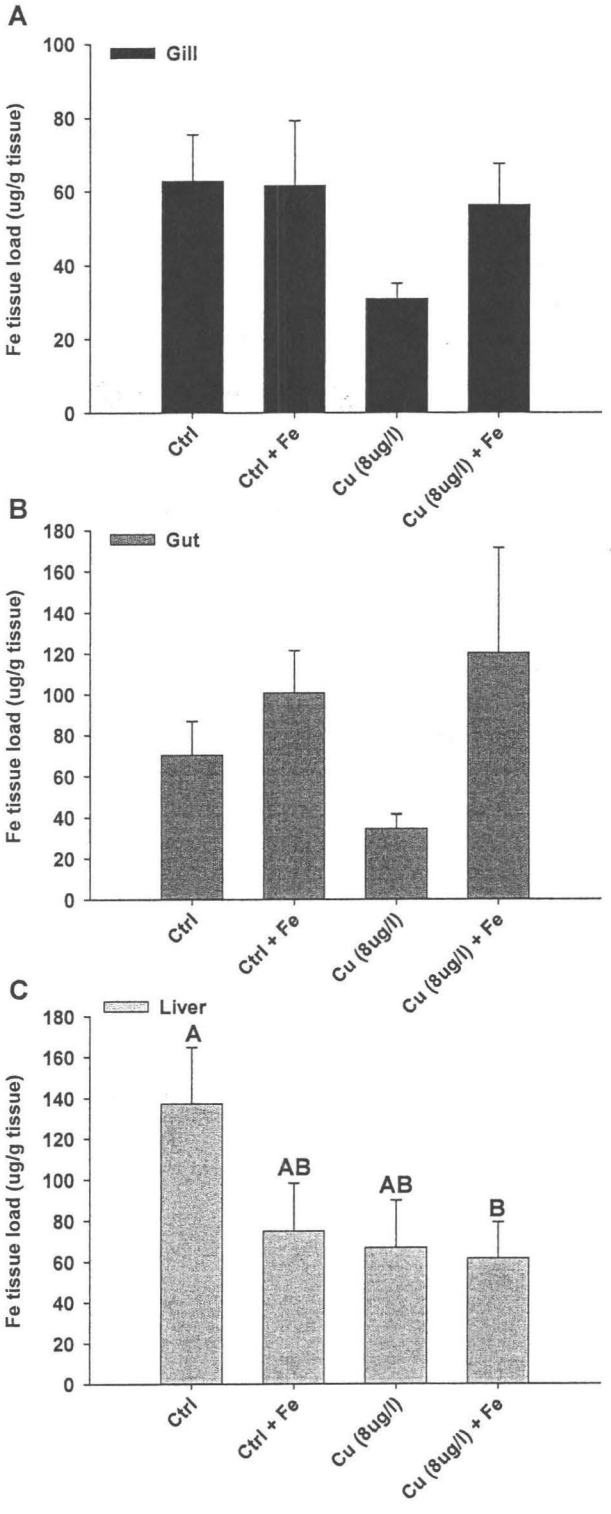

A
Gill
Fe tissue load (ug/g tissue)
100
80
60
40
20
0
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Gut
Fe tissue load (ug/g tissue)
180
160
140
120
100
80
60
40
20
0
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
C
Liver
Fe tissue load (ug/g tissue)
180
160
140
120
100
80
60
40
20
0
A
AB
AB
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

Fig 6.3

Gene expression of CTR-1 in the gill (**A**), gut (**B**), and liver (**C**) tissue from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d . Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).

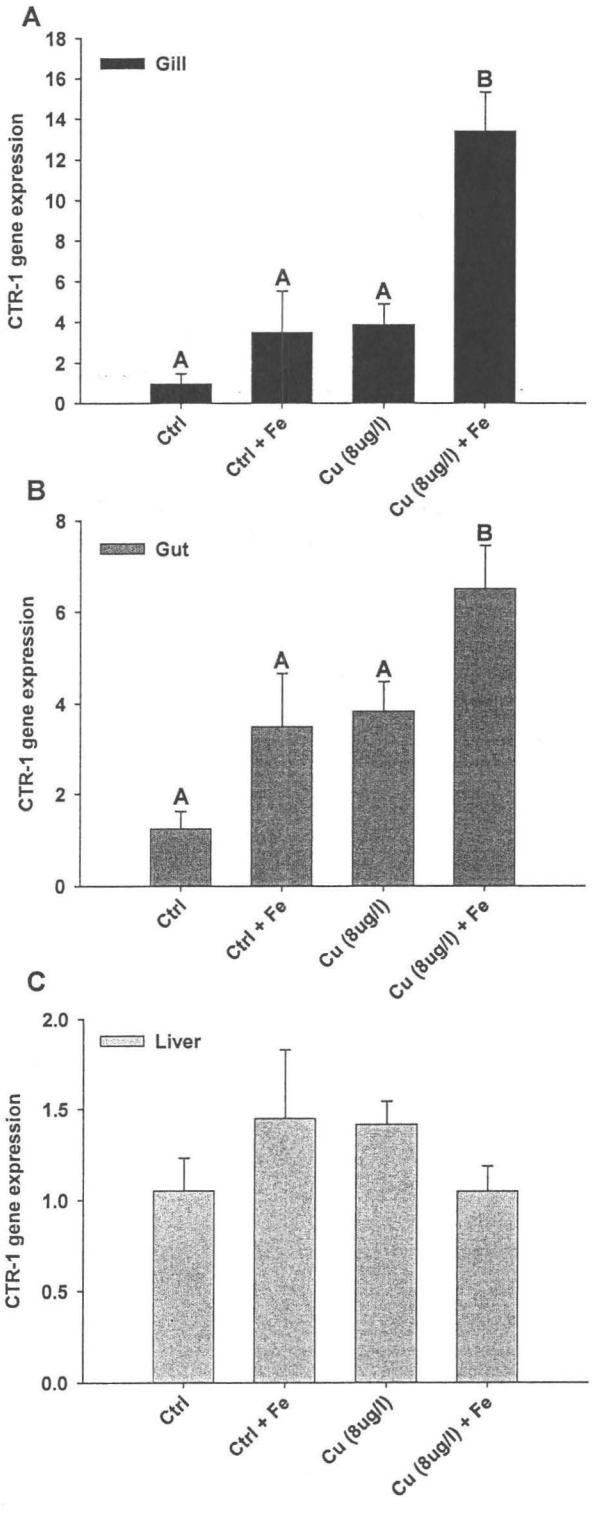

A
Gill
CTR-1 gene expression
18
16
14
12
10
8
6
4
2
0
A
A
A
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Gut
CTR-1 gene expression
8
6
4
2
0
A
A
A
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
C
Liver
CTR-1 gene expression
2.0
1.5
1.0
0.5
0.0
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

Fig 6.4

Gene expression of Divalent Metal Transporter (DMT-1) in the gill (**A**), gut (**B**), and liver (**C**) tissue from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d . Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).

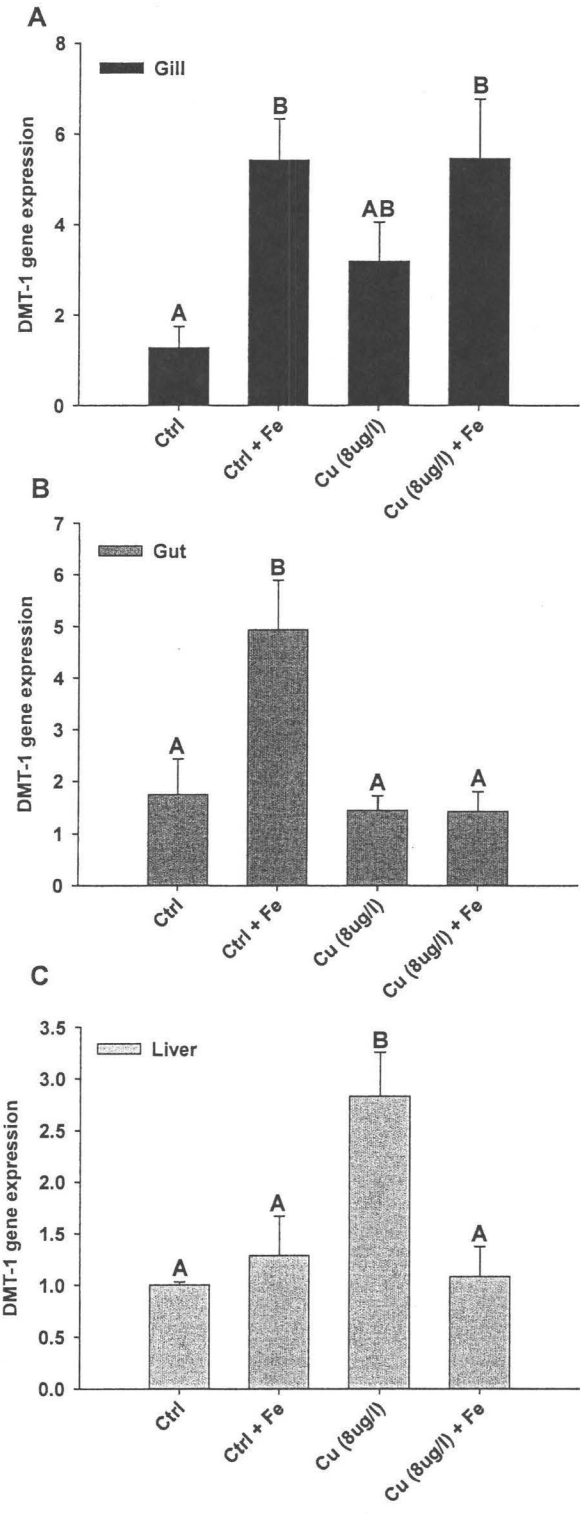

A
Gill
DMT-1 gene expression
0
2
4
6
8
A
B
AB
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Gut
DMT-1 gene expression
0
1
2
3
4
5
6
7
A
B
A
A
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
C
Liver
DMT-1 gene expression
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
A
A
B
A
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

Fig 6.5

Gene expression of Cu^{2+} transporting ATPase, alpha polypeptide (ATP7A) in the gill (**A**), gut (**B**), and liver (**C**) tissue from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d. Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).

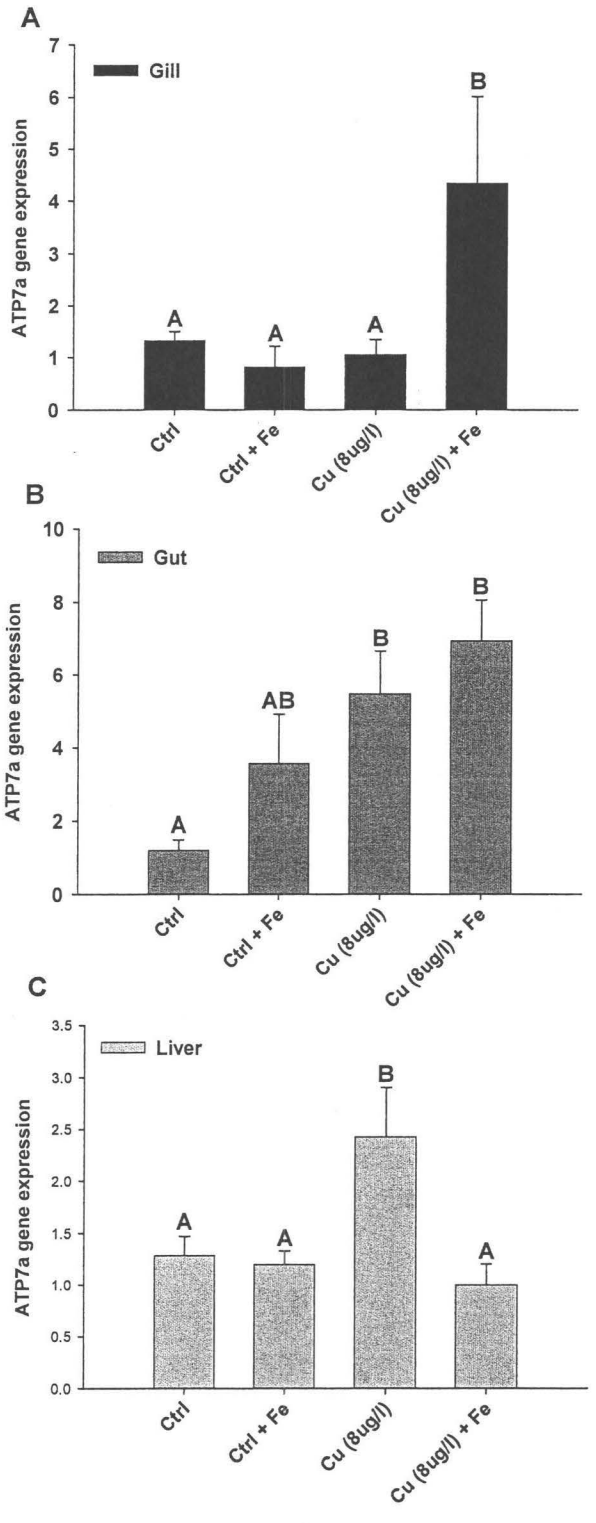

A
Gill
ATP7a gene expression
0
1
2
3
4
5
6
7
A
A
A
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Gut
ATP7a gene expression
0
2
4
6
8
10
A
AB
B
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
C
Liver
ATP7a gene expression
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
A
A
B
A
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

Fig 6.6

Gene expression of ferroportin in the gill (**A**), gut (**B**), and liver (**C**) tissue from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d. Gene expression values were normalized to EF1α, and are presented as mean ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).

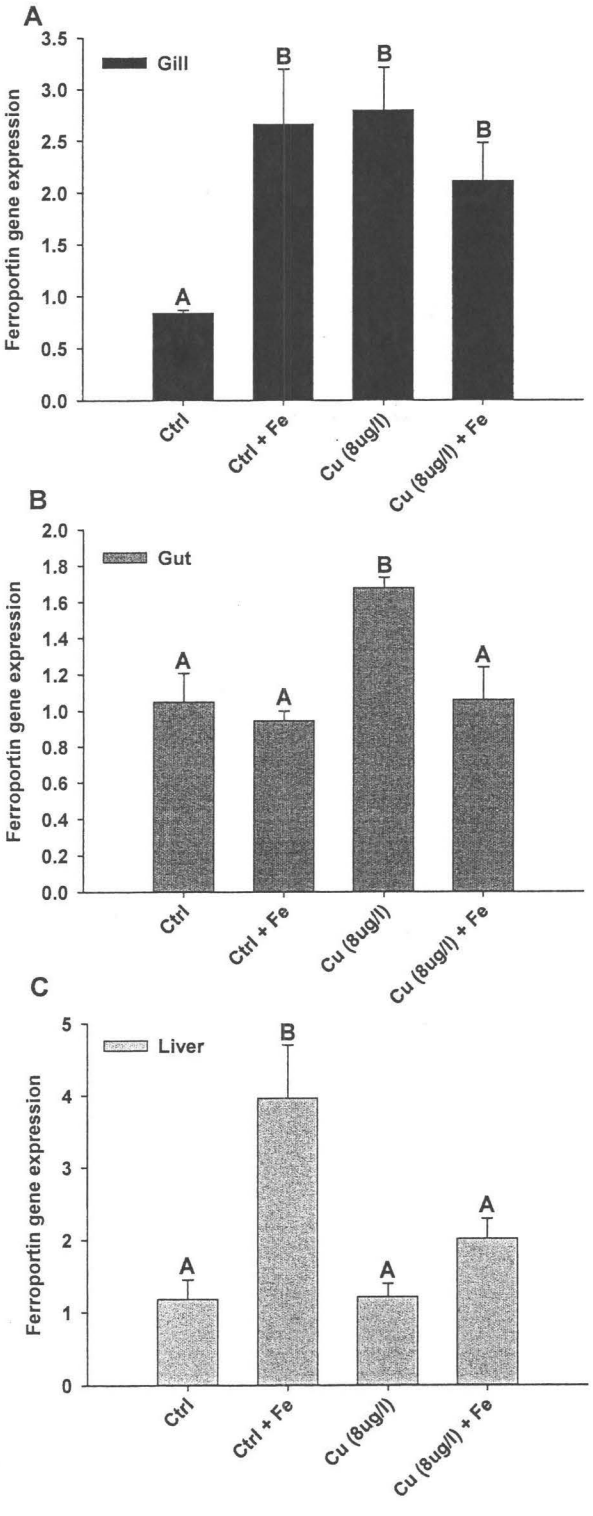

A
Gill
Ferroportin gene expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
B
B
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Gut
Ferroportin gene expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
A
B
A
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
C
Liver
Ferroportin gene expression
5
4
3
2
1
0
A
B
A
A
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

Fig 6.7

Gene expression of metallothionein 1 & 2 (MT1 & MT2) in the gill (**A**), gut (**B**), and liver (**C**) tissue from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d. MT2 expression Gene expression values were normalized to EF1α, and are presented as mean - ± SEM (arbitrary units) and treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).

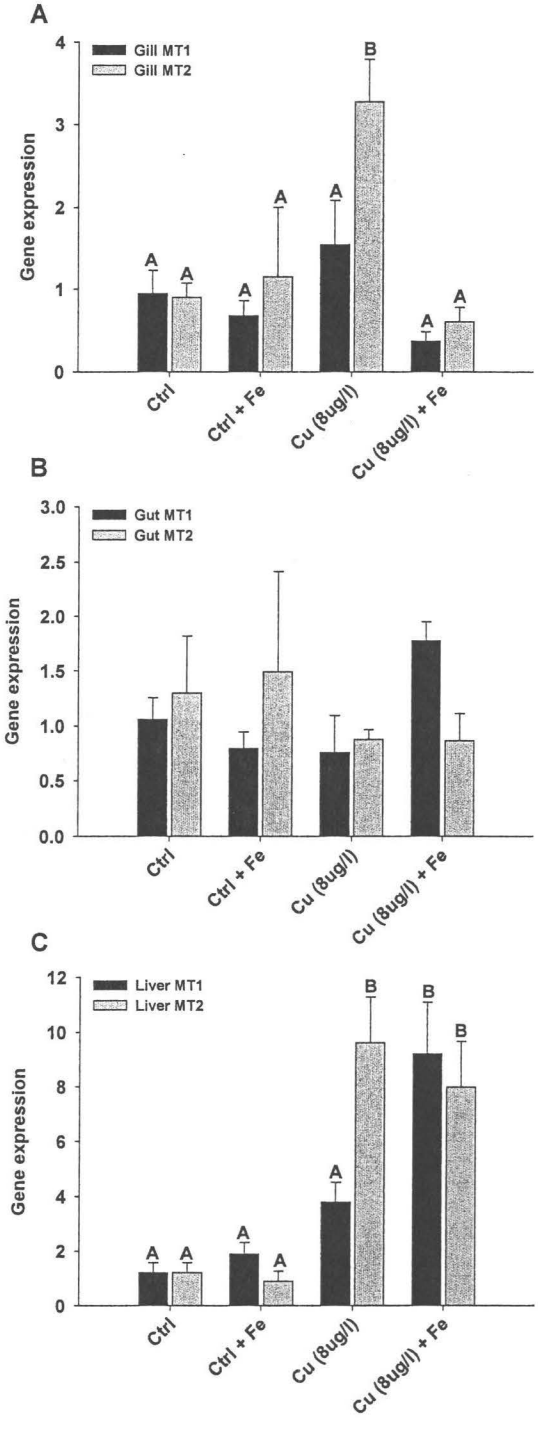

A
Gill MT1
Gill MT2
Gene expression
4
3
2
1
0
A
A
A
A
A
B
A
A
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Gut MT1
Gut MT2
Gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
C
Liver MT1
Liver MT2
Gene expression
12
10
8
6
4
2
0
A
A
A
A
A
B
B
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

Fig 6.8

(**A**) Relative protein expression of gill CTR-1 from soft-water acclimated zebrafish exposed control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d. Tubulin was measured to account for differences in protein loading. (**B**) Representative western blot picture of CTR-1 (68kDa) and protein normalizer, tubulin (60 kDa). Values are represented as means ± SEM, and treatments that do not share a common letter are significantly different from each other (n=8 for all treatments, $p<0.05$).

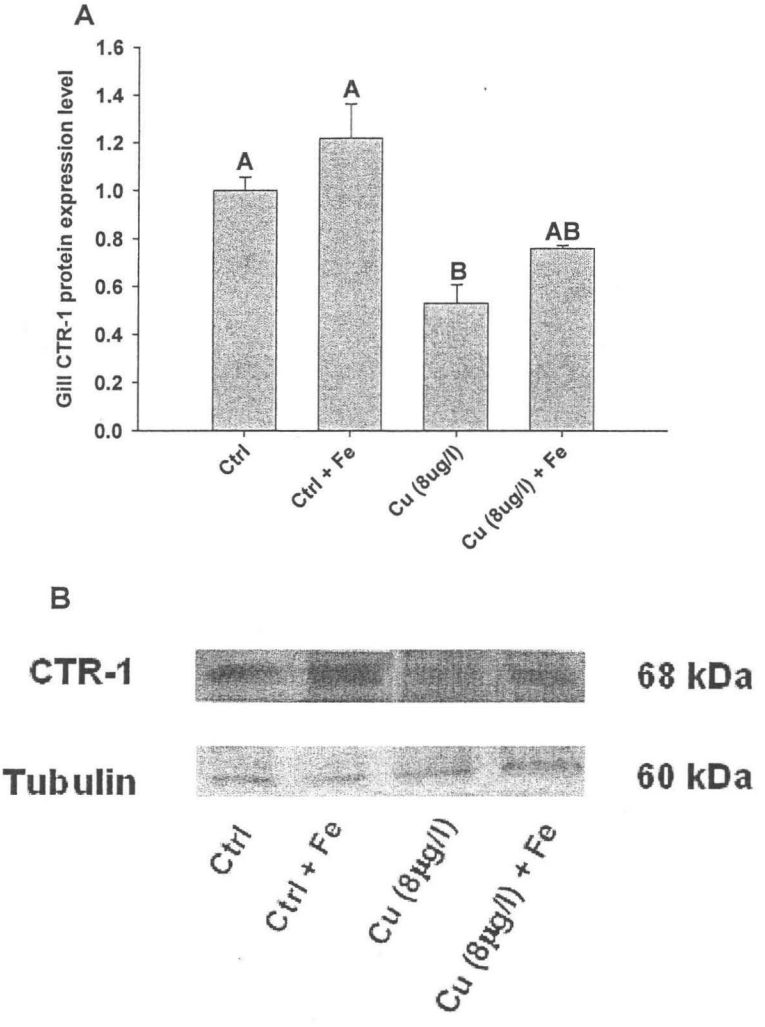
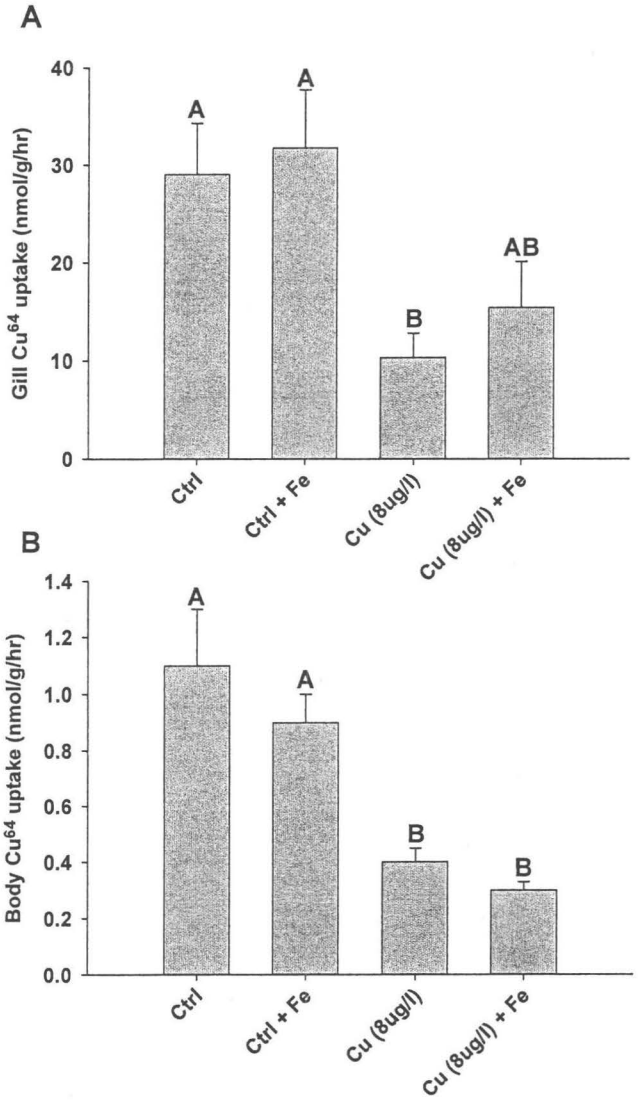

A
Gill CTR-1 protein expression level
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
A
B
AB
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
CTR-1
68 kDa
Tubulin
60 kDa
Ctrl
Ctrl + Fe
Cu (8µg/l)
Cu (8µg/l) + Fe

Fig 6.9

Gill apical (**A**) and whole body (**B**) uptake rate of ^{64}Cu (20min uptake exposure) from soft-water acclimated zebrafish exposed to control, control + Fe diet, 8μg/L water-borne Cu, and 8μg/L water-borne Cu + Fe diet for 21d . Treatments that do not share a common letter are significantly different from each other (n=6 for all treatments, $p<0.05$).


A
Gill Cu64 uptake (nmol/g/hr)
40
30
20
10
0
A
A
B
AB
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe
B
Body Cu64 uptake (nmol/g/hr)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
A
B
B
Ctrl
Ctrl + Fe
Cu (8ug/l)
Cu (8ug/l) + Fe

CHAPTER 7

GENERAL SUMMARY AND CONCLUSIONS

As outlined in the general introduction (Chapter 1), little is known about the chronic effects of waterborne metal contamination on tropical species. Moreover, most research has focused on coarse measurements of mortality, using an acute timeframe (Van den Belt et al 2000, Matz et al 2007, Griffitt et al 2007). This thesis has taken a systematic approach in characterizing specific ion channels and enzymes of interest for studying the effects of acute and chronic Cu exposure. More importantly, this work has also determined the global impact on gene expression which has allowed an unbiased assessment of gene and physiological endpoints of chronic Cu toxicity in a tropical model species. Furthermore, this novel data set can be integrated into predictive models, such as the Biotic Ligand Model (BLM), for the implementation of effective guidelines to protect aquatic organisms from the potential damaging effects of chronic, sub-lethal exposure to metal contaminants.

Zebrafish Acclimation to Soft-water

At the time this thesis began, zebrafish were already fast becoming a popular model for toxicogenomics, and a particularly useful tropical model for the study environmental pollutants, such as organic and metal contaminants (Andersson et al 2001, Cooper et al 2006, Gonzalez et al 2006). However, little research had examined basic ion homeostasis associated with various water chemistries, which has particular relevance when soft-water acclimation often precedes metal toxicological studies. To this end, **Chapter 2** set the framework for future studies by examining changes in gene and protein expression associated with Na^+ and Ca^{2+} transport (Fig 2.8; 2.11) after a 7 day acclimation to softwater, which fulfilled the goals of **objective 1**. These findings (summarized in Fig 7.1) suggested that in a soft-water acclimated state, zebrafish exposed to Cu may be predisposed to accumulate Cu at a greater rate, due to the changes seen in ion transport systems and the shared pathways Cu has with these competitive ions. Furthermore, by examining the time course of expression of a specific copper transporter (CTR-1), I discovered that the timing of future experiments after soft-water acclimation was critical, as zebrafish in an ion poor medium up-regulated CTR-1 after 6 days. However the expression recovered after 1 week in softwater (Fig 2.9) suggesting this as a minimum acclimation period before metal exposure should be done. These data provided novel insights into the phenotypic plasticity of zebrafish gills during softwater acclimation and highlighted potential mechanisms allowing zebrafish to maintain whole-body ion homeostasis. As the goals of these experiments were to establish a suitable acclimation period based on gene and protein expression, I did not pursue any further experiments to elucidate the mechanistic basis of the changes observed. However, this represents an area of fertile research.

Physiological and Gene Endpoints

The primary goal of this thesis was to establish a working short-list of physiological and gene endpoints of acute and chronic Cu toxicity. This goal was accomplished through several different experimental approaches, and **Chapters 3-6** answered **objectives 2-4**. As a first approach I examined the gene endpoints associated two environmentally relevant exposures of Cu over a chronic (21d) timeframe in softwater acclimated zebrafish. Furthermore, I examined the general stress response associated with Cu exposure and linked this to stress- and metal-specific responses of transcription through bioinformatic analysis of GREs and MREs in the promoter region of differentially expressed genes. This experiment not only identified a biphasic response to moderate and high doses of waterborne Cu (Fig 3.6), but also indicated that the changes in gene transcription seen in association with Cu exposure are predominantly regulated by a general stress response, and a very small portion of significantly regulated genes are directly regulated by increased metal concentrations (Fig 3.6; Table A.2). Despite the pattern of expression, I was able to identify several potential endpoints. For instance there was a distinct increase in gene expression of NKA subunit atp1a1, but a decrease in NKA activity, which suggests that independent of exposure concentration, these are potential gene and physiologic markers of chronic Cu exposure (Fig 3.3). Overall, the data in **Chapter 3** very importantly demonstrated Cu does not directly mediate gene expression, and identified numerous other pathways involved in transcriptional regulation. This chapter has yielded a rich data set that has already identified important gene and physiological endpoints of chronic Cu toxicity. The depth of information generated (deposited in on-line appendices) will be useful for future studies which may analyse the data for other specific pathway not directly assessed here.

Once the effects of Cu exposure solely in soft-water were established, I built on this data by examining competitive effects of the protective cations Na^+ and Ca^{2+} in the exposure water. To assess short-term versus long-term effects, these experiments were performed on both an acute and chronic timescale. Since Cu's toxic effects are most aggressive in soft water (Pagenkopf 1983, Playle et al 1992, 1993), it was important to examine physiological and gene changes resulting from competitive ions, and to identify persistent changes associated with Cu exposure, despite reduced Cu accumulation (Fig 4.3). On an acute scale, I found there was a significant positive correlation between Cu accumulation and a metal chaperone protein, COX-17, independent of the addition of protective ions (**Chapter 4**). Oxidative stress was also investigated in this chapter providing insight into the increased ROS damage induced by Cu alone, and I found that protective cations can reduce this damage (Fig 4.3). Moreover, I identified the liver as an essential target tissue for determining the gene effects of chronic Cu exposure, as the gills remained unresponsive, at least for the genes studied (Table 4.3). However, a relatively high dose of Cu was employed even on an acute scale, and I wanted to examine if these effects were persistent on a chronic scale with a lower Cu exposure, which is a more ecologically relevant platform.

Essentially the acute experiment was repeated over the course of 21d, although I matched the waterborne concentrations of Na^+ and Ca^{2+} to determine which ion had the greatest protective effect. For the most part the protective effects of Na^+ remained

consistent between the acute and chronic exposures as Cu accumulation in all tissues was reduced. In contrast, it appeared as though increased Ca^{2+} actually exacerbated the Cu load, particularly in the liver and gut (Fig 5.1). Further investigation into the gene response of the liver using microarrays indicated that when elevated waterborne Na^{+} and Ca^{2+} were applied together with Cu, they mitigated the gene response compared to Cu alone (Fig 7, Table A.3). Here I have identified a wealth of transcriptional of data (deposited in appendices online) that future studies may mine to probe specific pathways that were not pursued in this chapter. With respect to endpoints, in **Chapter 5**, I found that NKA is indeed an effective indicator of Cu only effects, as gill and liver activity showed a decrease, yet in contrast, there was an increase in activity associated with the gut (Fig 5.5). Tissue choice therefore determines the appropriate physiological endpoint. The degree of protein damage, as indicated by protein carbonyls, is also an effective endpoint of Cu only exposure, as we saw significant increases of these in the liver under both acute and chronic exposures (Fig 4.1, 5.3). Gene endpoints from this experiment proved challenging to interpret, as the effects of Na^{+} and Ca^{2+} alone resulted in significant changes in expression of particular genes of interest. This chapter identified the merits of examining multiple water chemistries on the toxic impacts of Cu, because the protective effects of Na^{+} and Ca^{2+} at the level of physiological level did not hold on a gene level. Furthermore, previous gene markers, such as COX-17 were not affected by chronic Cu exposure. This indicates that acute and chronic exposures do not share similar gene endpoints.

Lastly I examined the impacts of mixed metals exposure and a combined waterborne and dietary metal exposure. **Chapter 6** examined the effects of two essential micronutrients (Cu and Fe) and their competitive effects at toxic but sublethal levels, and effectively ascertained goals set forth by **objective 4**. What was most interesting from this chapter was the establishment of metallothionein 2 (mt 2) as a potential gene endpoint of chronic Cu exposure. Indeed looking back at **Chapter 3**, the microarray analysis indicated that mt2 mRNA expression was significantly induced in the liver tissue under moderate water-borne Cu exposure (Fig 6.7, Table A.2). Moreover, **Chapter 6** recognized that functional studies are essential in interpreting gene endpoints. It was apparent that results of transcription profiles do not always correlate with a functional response. For example increased CTR-1 expression suggested an increase capacity for Cu transport, yet there was decreased uptake of ^{64}Cu and decreased CTR-1 protein expression. As well, and seen in Chapter 3 there was a lack of correspondence in NKA activity versus expression of atp1α1 (Fig 6.9, 6.9, 3.3). These results suggest that while transcriptional profiles may be of diagnostic importance, they may not relate directly to protein abundance and ultimately function.

Microarrays

Toxicogenomics has become a popular and commonplace tool for transcriptional profiling for identifying ecotoxicological impacts from pollutants. Results from microarray experiments are invaluable as they have the ability to highlight alterations in previously unsuspected target pathways which can be beneficial or detrimental to a particular species faced with a given stressor. In this thesis I have used microarrays for

two sets of experiments, and the pattern of expression, rather than specific expression of target genes, has provided a great deal of information about chronic Cu exposure in zebrafish liver. On the one hand, I have shown that by varying the concentration of Cu alone in soft-water you can get a distinctly different pattern of expression in the liver of zebrafish (**Chapter 3**; Fig 3.5). On its own, this pattern of expression can be used to identify environmental waterborne Cu levels that would be detrimental to a fish population. Further experimentation is required to determine if these patterns of gene expression seen at this Cu level indicate a protective or detrimental response. Microarray analysis associated with varying water chemistries allowed for a different perspective, as it was surprising to see the dramatic changes in gene expression solely associated with either Na^+ or Ca^{2+} alone (**Chapter 6**; Fig 6.7). However, it was apparent that with the addition of Na^+ and Ca^{2+} coupled with Cu exposure, there was a distinct change in the pattern of gene expression from Cu alone, yet whether these changes are protective against the damaging effects of Cu still requires further investigation. In summary, microarrays provide a useful platform in understanding the global changes in gene expression associated with chronic Cu exposure, yet these results require cautious interpretation as functional studies are required to further elucidate beneficial gene expression linked to protective ions.

Perspectives & Conclusions

Chapter 1 began with a generalized model of how gene endpoints can be used to determine chronic toxicity in zebrafish (Fig 1.1). At the conclusion of this thesis, I can now provide a more detailed description of responses to chronic Cu exposure that incorporates exposures to Cu both alone and with supplementation of other competitive ions (Fig 7.2). Elevated Cu levels alone induce stress responses that encompass predominantly general and oxidative stress components, both of which appear to be the driving factor associated with increased transcription expression and resulting functional consequences (Fig 7.2A). Although there are exceptions to the following, there is a general pattern of reduced stress/oxidative damage associated with competitive ions, which effectively mutes the magnitude of response in zebrafish (Fig 7.2B). What these generalized models have demonstrated is that the stress response, particularly the generation of ROS and the subsequent oxidative damage, plays a key role in the transcriptional and physiological response to Cu exposure, rather than Cu itself. A fruitful direction of future research will be to establish links between the cellular oxidative response and the impact this has on both the individual and population level. The transcriptional and physiological consequences related to increased ROS and oxidative damage constitutes a key area for assessment of genomic and physiological endpoint of chronic Cu toxicity. Much is known about the relationship between Cu, ROS generation, and resulting cellular damage, yet few have examined the long term consequences to the individual which will be reflected in the population.

Environmental pollution is a major cost to natural ecosystems as heavily populated developing countries strive to attain global economic status. It is imperative to monitor and maintain a sustainable ecosystem in the face of these increased anthropogenic contaminants. By examining the subtle gene and associated physiological

differences resulting from waterborne Cu exposures, we can identify specific biological pathways that can act as indicators of potential detrimental effects to a population as a whole. However, this thesis highlights the complex nature involved in determining effective gene and physiological endpoints, or "fingerprints", to a particular metal, as an essential micronutrient, such as Cu, plays a significant role in numerous pathways, and can trigger a number of non-related responses when at toxic levels. Although the data are complex in nature, this thesis has taken the initial steps in determining the gene and physiological endpoints of acute and chronic Cu toxicity and can be implemented into models for prediction of chronic toxicity for other tropical species.

Fig 7.1
Gill ionocyte model based on results from softwater acclimation (**Chapter 2**). Arrows indicate direction of transcription expression at the termination of the experiment. Circular arrows indicate a transient increase in expression over the course of acclimation, whereas NC indicates no change in expression. An * indicates potential pathways of Cu absorption during waterborne Cu exposure. Abbreviations: ECaC-epithelial calcium channel, NHE-2-Na^+/H^+-exchanger 2, H^+ATP-v-type H^+-ATPase, CTR-1- copper transporter 1, NKA- Na^+K^+ATPase 1α1, CA-1 & 2- carbonic anhydrase 1 & 2. NKA activity and protein were measured as well, and demonstrated a transient change in activity, yet no distinct change in protein levels (see Fig 2.10). ECaC protein expression significantly increased over the duration of the experiment (see Fig 2.11).

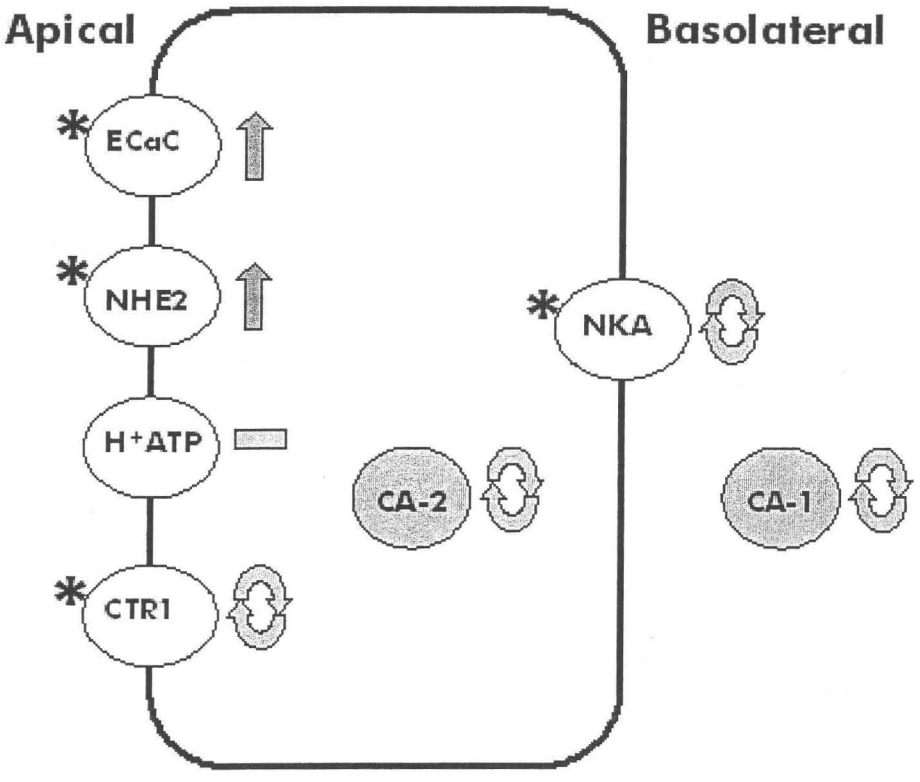
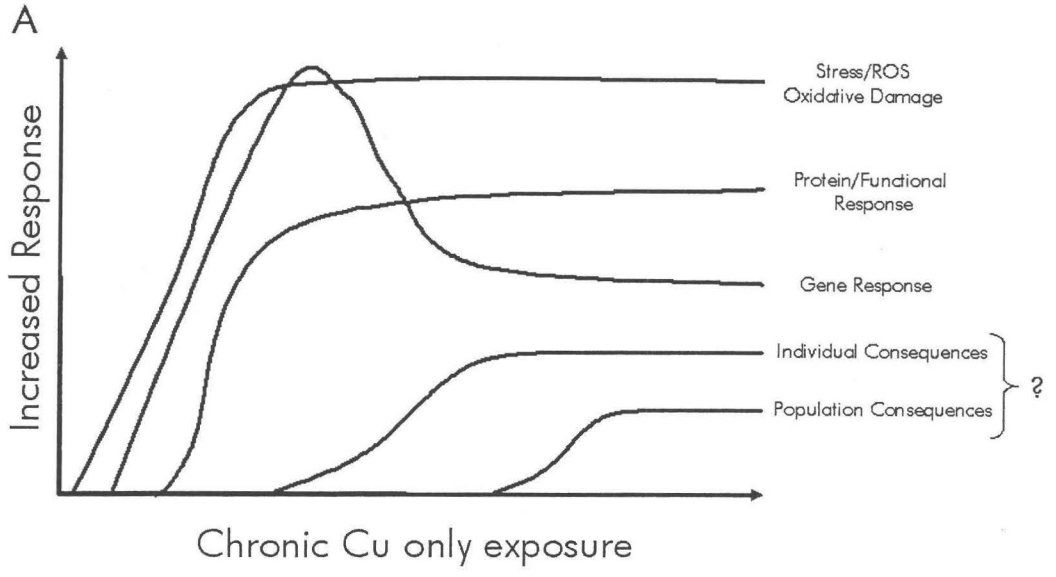
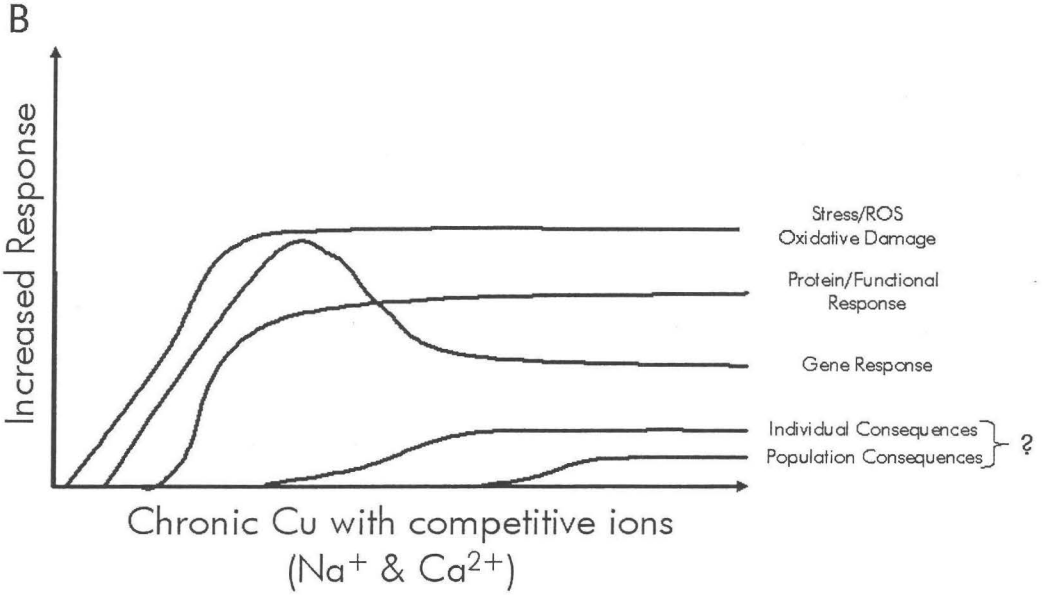

Apical
Basolateral
ECaC
NHE2
H+ATP
CTR1
NKA
CA-2
CA-1

Fig 7.2
General model of response in zebrafish to chronic Cu exposure alone (**A**) and in the presence of competitive ions (Na^+ & Ca^{2+}, **B**). Results from this thesis indicate that a combination of a general stress response associated with increased reactive oxygen species (ROS) induces a transcriptional response, which has functional consequences at the protein level. In comparing the magnitude between **A** & **B**, we see that competitive ions, with some exception, reduce the overall stress response, which in turn filters down to the respective transcript and protein responses. A fruitful area of future research will be to examine the impacts of increased ROS/oxidative damage associated with Cu exposure on the individual and population consequences, where little research has been focused to date.


A
Increased Response
Stress/ROS
Oxidative Damage
Protein/Functional
Response
Gene Response
Individual Consequences
Population Consequences
?
Chronic Cu only exposure


B
Increased Response
Stress/ROS
Oxidative Damage
Protein/Functional
Response
Gene Response
Individual Consequences
Population Consequences
?
Chronic Cu with competitive ions
(Na+ & Ca2+)

CHAPTER 8

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APPENDIX

Table A.1: Identification of genes significantly up- or down- regulated >1.5 fold upon exposure to moderate and high levels of waterborne Cu, clustered into 3 distinct groups (A,B,C). The oligonucleotide reporters were mapped at the sequence level (using megablast allowing 2 bp mismatches) to Unigene and Ensembl (mapped and predicted), and this allowed implied linkage to ZFIN, GO and human orthologues. Because of the shortage of functional information for zebrafish genes and zebrafish share similar gene set as human, the gene functional analysis of interested genes were performed using corresponding human orthologues (with sequence similarity higher than 45%) which were extracted from NCBI HomoloGene database using Unigene IDs

(A) Cluster 1 genes (231 genes)

GeneBank® ID	UniGene	UniGene Symbol	Expression Direction Moderate Cu	Expression Direction High Cu	Ensembl Zv6 Transcript ID	Human Orthologue GenPep	Human Orthologue similarity
BM187213.1	Dr.132909	si:ch211-241p10.3	Up	Down	ENSDART00000020506	Q9Y5W7	79 % / 164 aa
BG305844.1		LOC571645	Up	Down		JC5958	74.31 % / 218 aa
BE606020.1	Dr.121337	LOC793085	Up	Up			
AI106174.1	Dr.76088	zgc:153623	Up	Up			
AI721650.1	Dr.78771	zgc:100909	Up	Down	ENSDART00000008980	NP_054892.1	51.72 % / 371 aa
BM095422.1	Dr.84972	zgc:56235	Up	Up	ENSDART00000031121	B44422	70.32 % / 281 aa
BI474930.1			Up	Up			
AI626456.1	Dr.78349	LOC563963	Up	Down		NP_073612.1	89.22 % / 232 aa
AI477585.1	Dr.121695	wu:fb57f10	Up	Down		C46157	77.95 % / 127 aa
AI477589.1	Dr.11010	mhc1uea	Up	Up		P30491	33.05 % / 337 aa
AI588472.1	Dr.122917		Up	Down		O14770	68.09 % / 93 aa
AI883929.1	Dr.79138	LOC564100	Up	Up			
AW344201.1	Dr.81377	im:7140576	Up	Up		NP_060760.1	57.72 % / 492 aa
AW077323.1			Up	Up			
AI397462.1	Dr.76795	LOC557795	Up	Up		NP_001841.2	75 % / 144 aa
BI878471.1	Dr.133233		Up	Up		O60503	52 % / 51 aa
AI545576.1	Dr.75472	ccr6a	Up	Up	ENSDART00000010494	NP_008976.1	56.77 % / 174 aa
BI981281.1			Up	Up			

AW282139.1	Dr.77191	tuba2	Up	Up	ENSDART00000066177	NP_006000.2	97.78 % / 451 aa
AI667689.1	Dr.132396	pklr	Up	Up	ENSDART00000061566	KIHUPR	69.08 % / 524 aa
AW154401.1	Dr.9049	mapk4	Up	Up	ENSDART00000020621	S23429	61.37 % / 546 aa
CV481221.1	Dr.76595	ptpn2	Up	Up	ENSDART00000040340	P17706	58.54 % / 374 aa
AW826674.1	Dr.9896	zgc:154030	Up	Up		O00141	85.98 % / 214 aa
BF938013.1	Dr.82693		Up	Down			
NM_131747.1	Dr.85381	or2.7	Up	Up	ENSDART00000060120	NP_036492.1	35 % / 293 aa
AI666960.1			Up	Up			
BI980892.1			Up	Up			
AW077755.1	Dr.118935	wu:fj66b06	Up	Up			
BI672872.1			Up	Up			
BG305146.1	Dr.78284	zgc:109987	Up	Up	ENSDART00000082066	O15342	67.5 % / 80 aa
BM186155.1	Dr.113629	LOC100004167	Up	Down	ENSDART00000075353	B36429	60.67 % / 89 aa
AW058999.1	Dr.123512		Up	Down			
AW420581.1	Dr.81574		Up	Down	ENSDART00000057911	UQHUB	43 % / 103 aa
AW153708.1		pygo2	Up	Down		T08663	45 % / 120 aa
AI641589.1		wu:fc17b12	Up	Down			
AW115639.1	Dr.132719	zgc:153196	Up	Down		O15194	78.1 % / 209 aa
AF149720.1		si:ch211-45m15.2	Up	Down	ENSDART00000047308	NP_004417.1	48.57 % / 70 aa
EH578801.1	Dr.117301	LOC799860	Up	Down	ENSDART00000076161		
CR926931.1	Dr.12595	hsf2	Up	Down	ENSDART00000075070	Q03933	53.93 % / 488 aa
AA605848.1	Dr.74222	LOC565013	Up	Up		B56695	79.17 % / 186 aa
BI671542.1		glrx	Up	Down		2104198A	56.31 % / 103 aa
AI353491.1	Dr.75621	fb06f03	Up	Down		JC4760	98.44 % / 64 aa
BI983741.1	Dr.119890	LOC792144	Up	Down	ENSDART00000077131	NP_008854.2	69.85 % / 136 aa
AA497254.1		wu:fc14h11	Up	Down			
AI416326.1	Dr.136109		Up	Down			
BI846193.1	Dr.14167		Up	Down			
AI558471.1			Up	Down			
AW233684.1	Dr.81042	wu:fj40c05	Up	Down			
AW233699.1	Dr.114051	LOC794847	Up	Down		NP_002340.1	29.76 % / 176 aa
AI626448.1	Dr.140472		Up	Down			
AA605989.1	Dr.135231		Up	Down	ENSDART00000093606	810024C	74.4 % / 184 aa

BI841419.1	Dr.80729	syt4	Up	Down	ENSDART00000045479	Q9H2B2	65.38 % / 438 aa
AI793385.1			Up	Up			
BI877970.1			Up	Down			
AI666942.1	Dr.78557	zgc:110759	Up	Down		NP_115501.1	70.29 % / 174 aa
AW419644.1	Dr.106104	LOC560857	Up	Up	ENSDART00000037233		
AI545461.1	Dr.29749	eif4g2a	Up	Down	ENSDART00000027616	NP_001409.1	73.16 % / 866 aa
BI882534.1	Dr.42719	LOC553758	Up	Down	ENSDART00000024453	Q93009	92.37 % / 249 aa
AI331987.1	Dr.132176	wu:fc48a12	Up	Up		Q9Y4Z1	96.08 % / 102 aa
AA495404.1	Dr.75374	wu:fa01c11	Up	Down			
AW202589.1	Dr.134659	LOC100005948	Up	Down	ENSDART00000074501		
AW077136.1	Dr.22364	si:ch211-154o6.4	Up	Down			
AI545507.1	Dr.4955	wu:fb66d05	Up	Up			
BI671177.1	Dr.82995		Up	Down			
AW019543.1	Dr.117038	LOC555544	Up	Down	ENSDART00000086504		
BI317986.1			Up	Down			
AW128786.1	Dr.97168	nnt	Up	Down	ENSDART00000037664	2211247B	82.16 % / 1078 aa
BI880633.1			Up	Down			
AI601580.1			Up	Down			
BI670894.1	Dr.82951	guk1	Up	Down	ENSDART00000022959	S68864	63.47 % / 219 aa
BE017197.1	Dr.76012	dsg2	Up	Down	ENSDART00000084136	Q14126	46.41 % / 179 aa
AW420769.1	Dr.9543		Up	Down			
BI880189.1	Dr.125609		Up	Down			
BI842222.1	Dr.84615		Up	Down			
BE016216.1	Dr.43369	LOC562946	Up	Down		Q99715	56 % / 167 aa
BI845467.1	Dr.116789	LOC797422	Up	Down	ENSDART00000032640	NP_006454.1	71.84 % / 174 aa
BI708578.1	Dr.85029	wu:fk81d06	Up	Down			
AW233719.1	Dr.126837		Up	Down	ENSDART00000063578		
AW420564.1	Dr.128189		Up	Down			
AI722282.1	Dr.23436	tuba4l	Up	Down	ENSDART00000021765	NP_116093.1	88.47 % / 449 aa
AI105927.1	Dr.75307	atp1a1	Up	Up		P05023	89 % / 1028 aa
AF013752.1	Dr.120690	fbln1	Up	Down		C36346	44.04 % / 108 aa
AF075385.1	Dr.21021	dbh	Up	Up	ENSDART00000016547	S03020	60.56 % / 179 aa
AW076844.1			Up	Down			

AW076855.1	Dr.48159		Up	Down		Q9H346	29 % / 145 aa
AI437490.1	Dr.132277	wu:fb34c12	Up	Down			
AW116143.1	Dr.61271	zgc:152864	Up	Down	ENSDART00000085384	1814461A	67.24 % / 232 aa
BI430164.1	Dr.133689		Up	Down			
AI641218.1	Dr.78534	LOC561095	Up	Down	ENSDART00000090388	NP_038478.1	60.84 % / 163 aa
BM071628.1	Dr.133461	zgc:153663	Up	Down	ENSDART00000081040	NP_005461.1	56.46 % / 202 aa
AI544997.1			Up	Down			
AI721296.1	Dr.78680	zgc:114117	Up	Down	ENSDART00000010286	Q9UL15	66 % / 138 aa
AW171587.1		hccs	Up	Down	ENSDART00000017686	P53701	66.06 % / 269 aa
BI428856.1	Dr.115519	LOC558765	Up	Down	ENSDART00000044549		
AW059159.1	Dr.32777	btaf1	Up	Down		O14981	79.17 % / 286 aa
AI584464.1	Dr.77598	wu:fb94b05	Up	Down			
AW566816.1	Dr.35173	wu:fk19g03	Up	Down			
BI897234.1			Up	Down			
BE017666.1	Dr.33610	zgc:112077	Up	Down	ENSDART00000032118	P56554	93 % / 165 aa
BI672409.1	Dr.115695	LOC798917	Up	Down			
BI885460.1	Dr.84043	zgc:86635	Up	Down	ENSDART00000049462	Q92930	55.11 % / 175 aa
BG727471.1	Dr.116739	zgc:123177	Up	Down			
AI584201.1	Dr.100883	smarcd1	Up	Down	ENSDART00000003422		
AI601814.1	Dr.78031	whsc1	Up	Down	ENSDART00000009499	NP_075447.1	65.78 % / 259 aa
AI384436.1	Dr.52664	zgc:136942	Up	Down		A40936	80 % / 140 aa
AI601795.1	Dr.135421		Up	Down			
BI705770.1	Dr.133851		Up	Down			
AW174700.1	Dr.113583	37589723	Up	Down	ENSDART00000076519	NP_077001.1	63.93 % / 122 aa
BE017482.1			Up	Down			
AA495060.1		zgc:92842	Up	Down		NP_115875.1	95.05 % / 182 aa
AA495078.1	Dr.124952		Up	Down			
BM072363.1	Dr.123273		Up	Down			
BI891729.1	Dr.128603		Up	Down			
BG799828.1			Up	Down			
BG304480.1			Up	Down			
AW171089.1	Dr.75358	zgc:56206	Up	Down		P30154	79.38 % / 416 aa
BI428381.1	Dr.125306		Up	Down			

AW466759.1	Dr.132949	wu:fk03e08	Up	Down			
BM036424.1	Dr.14969	cln6	Up	Down	ENSDART00000011292	NP_060352.1	70.79 % / 288 aa
AI957909.1	Dr.126342	CILP	Up	Down	ENSDART00000075398		
BI844108.1			Up	Down			
AW233515.1	Dr.131758		Up	Down			
AI641239.1			Up	Down			
BI472288.1	Dr.119676	LOC557865	Up	Down		T13078	73 % / 199 aa
BM036361.1	Dr.18912		Up	Down			
BM181251.1			Up	Down			
AI957852.1			Up	Down			
AI723221.1	Dr.6242	zgc:153290	Up	Down	ENSDART00000081774	NP_078937.1	72 % / 141 aa
BI430383.1	Dr.28660	clica	Up	Down	ENSDART00000065819	NP_039234.1	63.83 % / 233 aa
BM156892.1	Dr.48854	LOC557006	Up	Down		NP_443074.1	36.75 % / 159 aa
BI475907.1	Dr.130614		Up	Down	ENSDART00000077546		
BG729255.1			Up	Down			
AI353354.1	Dr.76489	zgc:92763	Up	Down	ENSDART00000030282	NP_057001.1	49.55 % / 224 aa
AI444378.1		atrxl	Up	Down		P46100	32.79 % / 1029 aa
BI979160.1	Dr.55498	LOC100003733	Up	Down		NP_060667.1	48.89 % / 184 aa
BI533239.1	Dr.76896	zgc:64112	Up	Down	ENSDART00000013839	Q9HC24	65.03 % / 183 aa
AI793703.1	Dr.121926	wu:fc51h05	Up	Down			
BI845400.1			Up	Down			
BI979799.1	Dr.114084	LOC100003459	Up	Down	ENSDART00000036813		
AI584554.1			Up	Down			
AW826953.1	Dr.129153		Up	Down			
AW115764.1	Dr.80365	zgc:66437	Up	Down	ENSDART00000080174	T00389	39.5 % / 818 aa
AW233182.1	Dr.77869	zgc:73134	Up	Down		S24168	91.94 % / 396 aa
AW154680.1	Dr.78178	zgc:92063	Up	Down	ENSDART00000004585	NP_001285.1	35.73 % / 460 aa
BI474700.1	Dr.113704	LOC569901	Up	Down			
AW116329.1			Up	Down			
AI943009.1			Up	Down			
CT654194.2	Dr.1462	her1	Up	Down	ENSDART00000016307	NP_115969.1	29.63 % / 297 aa
AI353580.1	Dr.117216	LOC100001147	Up	Down	ENSDART00000010144	P20472	55.14 % / 107 aa
CF348671.1	Dr.75837	stom	Up	Down		P27105	83.15 % / 272 aa

BG305365.1	Dr.75801	neurod	Up	Down	ENSDART00000011837	Q13562	75 % / 349 aa
CT664110.1	Dr.6870	nr5a2	Up	Down		O00482	81.99 % / 532 aa
U62898.1	Dr.75766	chx10	Up	Down	ENSDART00000030448	P58304	67.47 % / 368 aa
AA494814.1	Dr.1307	foxa3	Up	Down	ENSDART00000010813	P55317	43.62 % / 441 aa
AA495417.1	Dr.132899	wu:fj59e04	Up	Down	ENSDART00000028087	NP_068711.1	78.98 % / 505 aa
EG586293.1	Dr.79140	smad2	Up	Down	ENSDART00000003587	Q15796	95.3 % / 468 aa
AI585160.1	Dr.77612	sema3d	Up	Down	ENSDART00000019302	O95025	63.78 % / 757 aa
AI384940.1	Dr.76690		Up	Up			
AW171502.1	Dr.80693	cuedc2	Up	Up	ENSDART00000057528	NP_076945.1	36.4 % / 219 aa
BG305576.1	Dr.82855	zgc:110344	Up	Up	ENSDART00000050086	O95475	93.12 % / 160 aa
BG727513.1	Dr.82541	hsd17b1	Up	Up		P14061	50.34 % / 294 aa
AI545682.1	Dr.75523	ubxd2	Up	Up		S27965	30.42 % / 277 aa
AI396962.1	Dr.76644	zgc:55760	Up	Down	ENSDART00000061366	JC2487	95.41 % / 218 aa
AI584351.1	Dr.28688	wu:fb92e06	Up	Down			
AW826770.1	Dr.126541		Up	Down			
AI626344.1			Up	Up			
AI444331.1	Dr.76765	zgc:153980	Up	Down		S21054	29.8 % / 192 aa
BI672612.1			Up	Down			
AI629164.1	Dr.132178	mat2a	Up	Up		S27257	91.04 % / 279 aa
AA494794.1	Dr.39150	wu:fb73f07	Up	Down	ENSDART00000058793	NP_037481.1	76.92 % / 130 aa
BG306179.1	Dr.82916		Up	Down			
AI667371.1	Dr.76424	ezh2	Up	Down		Q15910	98.24 % / 227 aa
AW826318.1	Dr.23217	wu:fk59c05	Up	Down			
BI980456.1	Dr.118733	zgc:101624	Up	Down	ENSDART00000082159	T46338	44.35 % / 115 aa
AW115534.1	Dr.79923	zgc:101700	Up	Down	ENSDART00000015277	A45207	64.13 % / 184 aa
AI588370.1	Dr.77795	fam3c	Up	Down		Q92520	65.64 % / 226 aa
BI671305.1	Dr.107659	abi1	Up	Down	ENSDART00000031245	NP_005461.1	74.15 % / 299 aa
AI601434.1	Dr.21327	wu:fc11c01	Up	Down			
AI721928.1	Dr.21601	wu:fc25c08	Up	Down			
AW077857.1			Up	Down			
AI667320.1	Dr.105763	wu:fc39c01	Up	Down			
BI430229.1	Dr.133980	LOC798716	Up	Down			
AI942582.1	Dr.128864		Up	Down			

AW280132.1		si:rp71-1p14.10	Up	Down		NP_002538.1	62.26 % / 212 aa
BI839428.1	Dr.11546	zgc:158761	Up	Down		NP_006807.1	75.44 % / 228 aa
AW420286.1	Dr.80595	pias4l	Up	Down	ENSDART00000061905	NP_006090.1	38.11 % / 400 aa
AI544932.1	Dr.118091	LOC568032	Up	Up	ENSDART00000082370	P50120	74.63 % / 134 aa
EB901399.1	Dr.266	mef2a	Up	Down	ENSDART00000078969	1EGW	71 % / 460 aa
AF246163.1	Dr.88598		Up	Down			
AW058981.1	Dr.118106	zgc:110251	Up	Down		P09917	60.89 % / 179 aa
BG305846.1	Dr.34948	id:ibd5036	Up	Down		NP_003473.1	29.65 % / 211 aa
BI980054.1	Dr.133724	LOC567939	Up	Down	ENSDART00000091180	NP_006331.1	70.29 % / 137 aa
AI931123.1	Dr.51712	LOC564598	Up	Down		NP_004710.1	66.27 % / 83 aa
AI666914.1	Dr.123027		Up	Down	ENSDART00000080120		
AW165130.1	Dr.80632	zgc:73124	Up	Down	ENSDART00000028048	NP_060560.1	59.11 % / 258 aa
AI545799.1	Dr.91161	anxa4	Up	Down	ENSDART00000052961	A42077	63.75 % / 320 aa
AW232264.1	Dr.125161		Up	Down			
AW420928.1	Dr.9441	zgc:158313	Up	Down		NP_066963.1	80.96 % / 435 aa
AI641272.1			Up	Down			
AW165042.1			Up	Down			
AI477577.1	Dr.105011	zgc:110154	Up	Down	ENSDART00000036718	P06730	70.32 % / 213 aa
AA495392.1			Up	Down			
AA495384.1			Up	Down			
BM037312.1		cdc27	Up	Down		P30260	75.75 % / 788 aa
BI881594.1			Up	Down			
AW115536.1	Dr.4244	cpa1	Up	Down	ENSDART00000005877	S29127	61.87 % / 416 aa
AI545489.1	Dr.77420	LOC565308	Up	Down	ENSDART00000042588	NP_076976.1	69.66 % / 230 aa
BI882139.1	Dr.122969		Up	Down			
BI672390.1	Dr.122808		Up	Down			
BI429693.1	Dr.120227	LOC793618	Up	Down	ENSDART00000009223	JC5193	69 % / 36 aa
BG883372.1			Up	Down			
AA606146.1	Dr.130799		Up	Down			
AI882787.1	Dr.76974	cbx1	Up	Down	ENSDART00000013016	P23197	79.35 % / 182 aa
AA495097.1		wu:fb69d01	Up	Down			
XM_701462.1	Dr.128704		Up	Down			
AW019162.1	Dr.35686	wu:fd60f10	Up	Down			

BI839302.1			Up	Down			
BI883379.1			Up	Down			
AI626745.1	Dr.77393	si:dkey-117n7.1	Up	Up	ENSDART00000067062	NP_115907.1	47.69 % / 184 aa
BM186555.1			Up	Up	ENSDART00000078485		
AW305630.1			Up	Up			
BI877842.1	Dr.118196	zgc:154057	Up	Up	ENSDART00000090940	O75478	76.22 % / 185 aa
AW170968.1	Dr.132711	mt2	Up	Up	ENSDART00000065337	NP_005724.1	39.86 % / 275 aa
BI889504.1	Dr.80044	zgc:158256	Up	Up	ENSDART00000088867		
AI396632.1	Dr.121815		Up	Up	ENSDART00000063944		
AW566862.1	Dr.79649	zgc:136847	Up	Up	ENSDART00000017427	NP_060771.1	39.84 % / 123 aa
AI793766.1	Dr.79007	zgc:153343	Up	Up	ENSDART00000091123		
BI842255.1	Dr.84618	zgc:92762	Up	Up	ENSDART00000019396	NP_001280.2	71.07 % / 236 aa
BI882797.1	Dr.119497	si:ch211-191d7.4	Up	Up	ENSDART00000080333	NP_116117.1	48 % / 107 aa
AI588406.1		wu:fc01e05	Up	Up			
AI723014.1	Dr.3182	mef2c	Up	Up		Q06413	76.2 % / 449 aa

(B) Cluster 2 Genes (201 genes)

GeneBank® ID	UniGene	UniGene Symbol	Expression Direction Moderate Cu	Expression Direction High Cu	Ensembl Zv6 Transcript ID	Human Orthologue GenPep	Human Orthologue similarity
AW115759.1	Dr.80363	zgc:66439	Up	Down	ENSDART00000036567	NP_036204.1	27.92 % / 138 aa
AI584411.1	Dr.77590	zgc:123254	Up	Down	ENSDART00000006971	O43236	69.81 % / 157 aa
BM024131.1	Dr.85991	nr1d1	Up	Down		A32608	58.13 % / 630 aa
AW117040.1	Dr.80144	exosc6	Up	Down	ENSDART00000052348	NP_478126.1	45 % / 265 aa
BI474934.1	Dr.74674	LOC100005808	Up	Down			
BG728644.1	Dr.122761		Down	Down			
AI626588.1		dedd1	Up	Down	ENSDART00000024927	NP_127491.1	45.88 % / 248 aa
Y13948.1	Dr.88617	hoxd3a	Up	Down	ENSDART00000082359	S27198	74.53 % / 105 aa
AB006104.1	Dr.120585	LOC572891	Up	Down	ENSDART00000026360	Q9H161	67.44 % / 86 aa
AI545737.1	Dr.74550	her8a	Up	Down	ENSDART00000019035	Q14469	36.84 % / 180 aa
AL715212.1	Dr.32618	hoxa3a	Up	Down	ENSDART00000052633	NP_109377.1	55.16 % / 411 aa
AW076840.1	Dr.8955	six2.1	Up	Down	ENSDART00000080805	Q9NPC8	87.07 % / 288 aa
BI845018.1	Dr.118025	LOC795873	Down	Down	ENSDART00000074671		

AF060118.1	Dr.3980	skib	Up	Down		P12755	59.36 % / 677 aa
BG738108.1	Dr.121474	LOC100007803	Up	Down	ENSDART00000017881	P06730	70.26 % / 192 aa
BE605412.1		wu:fk91b07	Up	Down	ENSDART00000090252	I55491	90 % / 222 aa
AW077407.1	Dr.106742	zgc:92804	Up	Down		NP_060679.1	72.64 % / 201 aa
AI476945.1	Dr.76975	LOC566376	Up	Down		T42674	56.02 % / 166 aa
AW077422.1	Dr.80297	LOC568465	Up	Down			
BI878053.1	Dr.115259	wu:fj80c12	Up	Down			
BG307539.1	Dr.106021	slc43a1	Up	Down		NP_003618.1	60.2 % / 363 aa
AW154129.1		wu:fi23a01	Up	Down		Q99728	47.18 % / 244 aa
AW343658.1		wdr21	Up	Down	ENSDART00000044228	T12541	55.76 % / 425 aa
AW826795.1	Dr.40048		Up	Down		NP_003535.1	100 % / 103 aa
BI896507.1	Dr.75320	hspd1	Up	Down	ENSDART00000078595	A32800	87.24 % / 572 aa
AI722079.1	Dr.75207	ncl	Up	Down	ENSDART00000017188	A35804	51.15 % / 669 aa
AI721420.1	Dr.115961	stxbp6	Up	Down			
AI793353.1		LOC100002196	Up	Down		NP_116012.1	26.91 % / 219 aa
AI477400.1	Dr.37662	rap2c	Up	Down	ENSDART00000014095	NP_066361.1	88.52 % / 183 aa
AW019834.1	Dr.80071		Up	Down			
AA605841.1	Dr.7990	srp54	Up	Down		S54143	96.03 % / 504 aa
BI672711.1	Dr.77534	zgc:55702	Up	Down	ENSDART00000030995	P11172	60.94 % / 296 aa
BM102769.1	Dr.79437	zgc:110299	Up	Down	ENSDART00000010172	Q9UI95	81.99 % / 211 aa
BI708528.1	Dr.115855	tk2	Up	Down	ENSDART00000074011	A38585	35.74 % / 205 aa
AA495483.1	Dr.48996	mdm4	Up	Down	ENSDART00000080201	NP_002384.1	49.51 % / 491 aa
AI444373.1	Dr.124992		Up	Down			
BM071667.1			Up	Down			
BI326608.1			Up	Down			
AW419509.1			Up	Down			
BI326622.1	Dr.85805		Up	Down			
AI477308.1	Dr.128740		Up	Down			
AI522692.1	Dr.12107	ndrg1	Up	Down	ENSDART00000044838	Q92597	66.83 % / 395 aa
AW232645.1	Dr.80936	wu:fj22e05	Up	Down			
AI588328.1	Dr.77776	si:ch211-59d15.5	Up	Down			
BI983945.1		LOC566307	Up	Down			
BM083108.1	Dr.42915	snai3	Up	Down	ENSDART00000073550	O43623	82.69 % / 52 aa

AW184196.1	Dr.36981	zgc:158397	Up	Down	ENSDART00000054861	1JCQ	77.25 % / 211 aa
AW281636.1	Dr.81213	LOC567576	Up	Down		T12520	47.5 % / 114 aa
BI429411.1	Dr.76277	zgc:77286	Up	Down	ENSDART00000012873	Q9Y4X4	57.43 % / 367 aa
AW566846.1	Dr.80057	zgc:91857	Up	Down			
AA605764.1	Dr.140435		Up	Down			
BF938209.1			Up	Down			
AI958865.1	Dr.132611	LOC793280	Up	Down		O76082	33 % / 165 aa
BI533946.1	Dr.121297	LOC100008455	Up	Down	ENSDART00000055152	JC4540	66.67 % / 177 aa
AI415748.1	Dr.84355	nrp2b	Up	Down	ENSDART00000056103	NP_003863.1	60.09 % / 902 aa
AW826361.1			Up	Down			
AI641026.1			Up	Down			
AW175351.1	Dr.88794	LOC572172	Up	Down	ENSDART00000086244	JE0352	44.82 % / 1226 aa
AI878162.1			Up	Down			
BI865786.1	Dr.84426		Up	Down			
AI601507.1	Dr.78388	LOC556395	Up	Down	ENSDART00000081790	T17318	54 % / 166 aa
AW171485.1	Dr.74197	zgc:153452	Up	Down			
AW826192.1	Dr.81733	rbbp5	Up	Down		Q15291	90.02 % / 510 aa
AI722818.1			Up	Down			
AW232911.1	Dr.76276	gnmt	Up	Down	ENSDART00000024379	Q14749	72.54 % / 293 aa
AI601495.1	Dr.77947	LOC100007502	Up	Down		T17272	52.58 % / 95 aa
BM184454.1	Dr.78241	tjp3	Up	Down	ENSDART00000012336	O95049	47.32 % / 177 aa
AI584761.1	Dr.132378	pls3	Up	Down	ENSDART00000054849	A34789	87.16 % / 623 aa
BI840984.1	Dr.84571	LOC569855	Up	Down		NP_006069.1	78.84 % / 189 aa
AI385049.1		naca	Up	Down	ENSDART00000014723	S49326	95.35 % / 215 aa
BI880944.1	Dr.82767		Up	Down			
BF717389.1	Dr.24956	sepx1	Up	Down	ENSDART00000034489	Q9NZV6	76.85 % / 106 aa
BI842802.1	Dr.32141	im:7162148	Down	Down	ENSDART00000054710	Q9Y2S0	66.67 % / 111 aa
BM155770.1	Dr.83576	snrpd3	Down	Down	ENSDART00000018475	P43331	93.7 % / 127 aa
BI983388.1			Down	Down			
AY050503.1	Dr.82973	gucy2f	Up	Down		P51841	69.6 % / 273 aa
AW077463.1	Dr.20261	anp32a	Up	Down	ENSDART00000008785	NP_006296.1	81.78 % / 253 aa
AI330563.1	Dr.76249	tfdp1l	Up	Down		Q14186	79.81 % / 409 aa
BI842166.1			Up	Down			

AW232841.1	Dr.80957		Up	Down			
AI959700.1	Dr.132629		Up	Down	ENSDART00000080803		
AI584375.1	Dr.2532	prkci	Up	Down	ENSDART00000015723	P41743	89.6 % / 576 aa
XM_689018.1	Dr.39929	pld2	Up	Down	ENSDART00000039698		
CK015820.1			Up	Down			
AI461367.1	Dr.76935	wu:fb44b11	Up	Down			
AI878554.1			Up	Down			
BI877511.1	Dr.117745	LOC796611	Up	Down	ENSDART00000054626	NP_003874.2	94.82 % / 193 aa
CK693730.1			Up	Down			
BI888240.1		LOC100005496	Up	Down		NP_005914.1	76 % / 215 aa
AI330531.1	Dr.76238	LOC407680	Up	Down		Q99795	34.34 % / 289 aa
BI841629.1	Dr.77340		Up	Down		NP_036338.1	37.4 % / 122 aa
AI626369.1	Dr.78342	ndrg3a	Up	Down	ENSDART00000016181	NP_114402.1	75.47 % / 362 aa
AA497164.1		col18a1	Up	Down	ENSDART00000053118	P39060	49.59 % / 360 aa
AI588560.1	Dr.128801		Up	Down			
AI558485.1	Dr.77494	vrk1	Up	Down		NP_003375.1	63.14 % / 386 aa
AI641738.1	Dr.22938	LOC793794	Up	Down		Q13118	47.23 % / 203 aa
AI558476.1		zgc:136230	Up	Down			
AI957609.1			Up	Down			
BI878851.1	Dr.122895		Up	Down			
AI444359.1	Dr.121672		Up	Down			
BI880064.1			Up	Down		NP_015566.1	87.5 % / 40 aa
AI957593.1			Up	Down			
AW280076.1	Dr.22757	wu:fj49b04	Up	Down			
AI957672.1	Dr.24428	wu:fc96h04	Up	Down			
BE016416.1	Dr.118834	LOC566639	Up	Down		A45259	34.86 % / 168 aa
BG308367.1	Dr.76453	zgc:77741	Up	Down	ENSDART00000066269	P49703	75.12 % / 200 aa
AW116368.1			Up	Down			
BG303704.1	Dr.75737	mtr	Up	Down		Q99707	78.25 % / 1255 aa
AI943021.1	Dr.74015	daam1l	Up	Down		NP_006720.1	35.07 % / 205 aa
AI878773.1	Dr.136437		Up	Down			
AF160691.2	Dr.17314	evla	Up	Down		NP_057421.1	59.91 % / 386 aa
BM070637.1	Dr.84757	gnrh2	Up	Down	ENSDART00000065779		

BG305908.1	Dr.82887		Up	Down			
AW117085.1	Dr.123184		Up	Down			
AB055670.1	Dr.88602		Up	Down			
DN836570.1			Up	Down			
NM_131436.1	Dr.85386	npyryb	Up	Down	ENSDART00000015833	P25929	48 % / 372 aa
NM_131737.1	Dr.85378	or102-5	Up	Down	ENSDART00000060081	Q9H207	29 % / 303 aa
BF717829.1	Dr.119569	esr1	Up	Down	ENSDART00000024518	P03372	53.32 % / 531 aa
AF170065.1	Dr.2746	meis2.1	Up	Down		O14770	76.48 % / 451 aa
CO924448.1	Dr.83043	cbfb	Up	Down	ENSDART00000059961	NP_074036.1	87.17 % / 187 aa
BG727168.1	Dr.123271		Up	Down	ENSDART00000080489	P00540	50.6 % / 83 aa
CR927378.1	Dr.92960	LOC570077	Up	Down	ENSDART00000066851		
XM_693888.1	Dr.135664	LOC570409	Up	Down	ENSDART00000056441		
AW154456.1	Dr.76576	actr2	Up	Down	ENSDART00000074294	O15142	96.95 % / 394 aa
AW078017.1	Dr.8984	LOC793114	Down	Down			
CA471293.1	Dr.118667	LOC792002	Down	Down	ENSDART00000050678	NP_060291.1	65 % / 200 aa
AW422373.1		LOC794025	Down	Down		NP_476502.1	33 % / 105 aa
BG303805.1	Dr.132523	zgc:158612	Up	Down	ENSDART00000089017		
AI957828.1	Dr.32839	rnd3b	Up	Down	ENSDART00000022392	P52199	76.09 % / 224 aa
BM036330.1	Dr.84864		Up	Down			
BI704967.1	Dr.28227	im:7148063	Down	Down	ENSDART00000029797	T43466	26.99 % / 329 aa
AW279682.1	Dr.81058	LOC567837	Up	Down			
AI332238.1	Dr.76116	zgc:112318	Down	Down	ENSDART00000008453	NP_036226.1	57.14 % / 175 aa
AI584243.1		wu:fi15b05	Down	Down		NP_065783.1	64.79 % / 71 aa
AI545842.1	Dr.15212	zgc:92140	Up	Down	ENSDART00000063281	NP_110433.1	63.03 % / 113 aa
AI397436.1		LOC795324	Up	Down			
BI430036.1	Dr.120479	LOC794314	Up	Down	ENSDART00000058364	NP_002120.1	70.37 % / 214 aa
AW077289.1		zgc:56140	Up	Down	ENSDART00000014098	NP_076956.1	59.2 % / 173 aa
BG304701.1			Up	Down			
AI958952.1	Dr.104586	LOC557328	Up	Down	ENSDART00000092793		
BM023805.1			Up	Down			
BI325659.1		zgc:92411	Up	Down		A35649	60.25 % / 238 aa
BM153934.1	Dr.139951	LOC794092	Up	Down	ENSDART00000064565		
AI385114.1	Dr.113635	LOC100003479	Up	Down			

BG985725.1	Dr.2413	dusp1	Up	Down	ENSDART00000014515	P28562	70.46 % / 359 aa
BI672146.1	Dr.83425	LOC553260	Up	Down	ENSDART00000055308	NP_060386.1	40 % / 264 aa
AI957441.1	Dr.137872		Up	Down			
AI544472.1			Up	Down			
AI558441.1	Dr.118555	LOC567390	Up	Down		NP_077303.1	36.31 % / 157 aa
BG727211.1	Dr.83060		Up	Down			
BI864881.1	Dr.123124		Up	Down			
AI793434.1	Dr.6952	zgc:158650	Up	Down	ENSDART00000086603	NP_005484.1	91.67 % / 119 aa
AW466774.1	Dr.122483	tnc	Up	Down			
AW116857.1	Dr.14309	zgc:55855	Up	Down	ENSDART00000004546	P11940	72.99 % / 620 aa
AI437014.1	Dr.105249	wu:fb77h12	Up	Down		NP_065703.1	44.68 % / 182 aa
BI671366.1		LOC100006212	Up	Down		NP_060039.1	86 % / 217 aa
AI601471.1	Dr.77937	LOC562019	Up	Down		P34932	44.05 % / 212 aa
AW019627.1	Dr.133023	zgc:153972	Up	Down		O94876	72.93 % / 133 aa
BI533460.1	Dr.80032	LOC562319	Up	Down		NP_006451.1	59.73 % / 220 aa
BM184314.1	Dr.123315		Up	Down			
BF937070.1			Up	Down		I77403	97.73 % / 44 aa
BG737500.1			Up	Down			
AI959115.1	Dr.121552		Up	Down			
BI841868.1	Dr.133404	zgc:158245	Up	Down		P22304	71.27 % / 181 aa
AI588355.1	Dr.77264	arcn1	Up	Down		A56750	82.58 % / 509 aa
BI841705.1	Dr.13903		Up	Down			
BE016176.1	Dr.76581	sec6l1	Up	Down	ENSDART00000009347	I56134	23.64 % / 594 aa
AI959095.1	Dr.132379	LOC557376	Up	Down	ENSDART00000087502	NP_055705.1	50.44 % / 226 aa
AW078366.1	Dr.121549	LOC798137	Up	Down		O15127	64.29 % / 70 aa
BI889302.1	Dr.36545	pacsin1	Up	Down	ENSDART00000061746	NP_009160.1	53.06 % / 317 aa
BI673858.1	Dr.120759	LOC100000098	Up	Down	ENSDART00000067290		
AW281911.1	Dr.76148	si:dkey-18o7.1	Up	Down	ENSDART00000014257	I55491	78.78 % / 306 aa
BG303529.1	Dr.83024	zgc:153293	Up	Down		NP_542385.1	29 % / 163 aa
AA497306.1	Dr.75435	gtf2f1	Up	Down	ENSDART00000026090	1804353A	56.84 % / 440 aa
AI721498.1	Dr.161	eif3s4	Up	Down	ENSDART00000018071	NP_003746.1	79.5 % / 293 aa
BI897906.1	Dr.113555	hip2	Up	Down		P27924	99.05 % / 105 aa
BG729502.1	Dr.83155	zgc:73350	Up	Down		NP_068578.1	67.4 % / 225 aa

AI722406.1	Dr.117293	LOC100001787	Up	Down	ENSDART00000011122	S28942	98.24 % / 170 aa
AI657823.1	Dr.88	LOC563915	Up	Down		NP_079417.1	70.65 % / 184 aa
BI472488.1	Dr.123592		Up	Down	ENSDART00000032392	NP_055290.1	42.86 % / 154 aa
AA497156.1	Dr.119814	LOC100003460	Up	Down	ENSDART00000079340		
AW778516.1			Up	Down			
AI721768.1	Dr.75211	zgc:63728	Up	Down	ENSDART00000019252	NP_387448.1	89.1 % / 530 aa
BG303448.1	Dr.122597		Up	Down		A41060	33 % / 227 aa
AI641439.1	Dr.206	zgc:55573	Up	Down	ENSDART00000015932	JC4636	46.32 % / 487 aa
BI984062.1	Dr.106940	rgl1	Up	Down	ENSDART00000010604	Q9NZL6	66.97 % / 759 aa
AW175561.1	Dr.74557	LOC100003734	Up	Down	ENSDART00000082909	NP_076996.1	57.14 % / 200 aa
AI942997.1	Dr.38804	LOC558556	Up	Down		Q13409	80 % / 86 aa
AW777642.1	Dr.79162	si:dkey-25e12.6	Up	Down		P11586	83.93 % / 56 aa
BI979868.1		zgc:77312	Up	Down			
BI865355.1		bcor	Up	Down		Q99728	31.33 % / 245 aa
BI428214.1	Dr.124022		Up	Down	ENSDART00000026362		
AW421020.1	Dr.74575	wu:fd49b10	Up	Down		NP_000766.2	38.89 % / 232 aa
AW344325.1	Dr.78482	abcb5	Up	Down		P21439	64.73 % / 241 aa
AI444467.1	Dr.120742	LOC561841	Up	Down		NP_055716.1	47 % / 107 aa
BI891799.1		gpd1l	Up	Down		2113206A	71.63 % / 349 aa
AW154271.1	Dr.559	zgc:158535	Up	Down		NP_077726.1	59.06 % / 685 aa
BM155714.1	Dr.133500		Up	Down			
EB965226.1	Dr.83047	hoxc1a	Down	Down	ENSDART00000077428	P14653	34.5 % / 262 aa
BM861732.1	Dr.119962	LOC100002634	Down	Down	ENSDART00000063982	NP_066926.1	78.57 % / 42 aa

(c) Cluster 3 genes (141 genes)

GeneBank® ID	UniGene	UniGene Symbol	Expression Direction Moderate Cu	Expression Direction High Cu	Ensembl Zv6 Transcript ID	Human Orthologue GenPep	Human Orthologue similarity
AI601693.1			Down	Up			
BM035033.1	Dr.118987	zgc:101628	Down	Down		NP_060845.1	82.11 % / 95 aa
AW128245.1	Dr.80492	zgc:92241	Down	Up	ENSDART00000022839	JC4760	98.92 % / 93 aa
AI522608.1	Dr.77169	wu:fb60c02	Down	Up			
BI326539.1	Dr.118115		Down	Up			

AI584257.1	Dr.21171	si:ch211-233a1.1	Down	Up			
BI888384.1	Dr.105295	LOC797547	Down	Up		NP_055318.1	52 % / 193 aa
AI397223.1	Dr.75834	hsp90a	Down	Up		NP_005339.1	85.11 % / 726 aa
AI522468.1	Dr.24364	wu:fb19b08	Down	Up			
BI671167.1			Down	Up			
AI877575.1	Dr.7422	stau1	Down	Up	ENSDART00000064865	O95793	74.41 % / 510 aa
BI845382.1	Dr.123219		Down	Up			
BI430171.1		LOC572585	Down	Down		NP_055490.1	43 % / 218 aa
AW826238.1			Down	Up			
AW058948.1	Dr.123003		Down	Down			
AW171235.1	Dr.116102	zgc:63744	Down	Up	ENSDART00000061481	P08217	65.67 % / 266 aa
BM103450.1			Down	Up			
AW018997.1	Dr.25202	znf259	Down	Up	ENSDART00000063923	NP_003895.1	72.3 % / 426 aa
AI330524.1		ube2d1	Down	Up		NP_057067.1	95.92 % / 147 aa
BM181765.1	Dr.33963	zgc:100903	Down	Up		P55058	55.05 % / 464 aa
AI601661.1	Dr.77660	zgc:100963	Down	Up	ENSDART00000020638	NP_005813.1	55.67 % / 187 aa
AI545173.1	Dr.131930		Down	Up			
AA658676.1	Dr.132182	bactin2	Down	Up	ENSDART00000055194	P02570	99.2 % / 375 aa
BI708458.1	Dr.16561	zgc:153259	Down	Up		A54869	36.09 % / 131 aa
BG302604.1	Dr.77467	LOC556400	Down	Up		NP_067640.1	57.05 % / 149 aa
BI706477.1			Down	Up			
AI588458.1	Dr.77832	LOC560168	Down	Up		P31146	63.9 % / 200 aa
BE015932.1	Dr.123183		Down	Up	ENSDART00000030579	S13640	62.3 % / 61 aa
BI673959.1	Dr.118558	LOC100007489	Down	Up	ENSDART00000077420		
BI883526.1	Dr.115558	LOC799805	Down	Up	ENSDART00000073769	A33851	72.14 % / 262 aa
BI842325.1	Dr.84627		Down	Up			
AI965289.1	Dr.122070		Down	Up			
AI545295.1	Dr.19509	LOC571280	Down	Up	ENSDART00000052272	T12496	62.14 % / 227 aa
BI883698.1	Dr.108000	traf3ip2	Down	Down		T08794	58.02 % / 81 aa
AI641121.1	Dr.78494	wu:fc19d12	Down	Up			
AF210643.1	Dr.81979	rds2	Down	Up	ENSDART00000055415	A40308	65.22 % / 344 aa
BI475215.1			Down	Up			
BG305386.1	Dr.120152	rlbp1l	Down	Down		P12271	65.8 % / 307 aa

AI332150.1	Dr.76381	ripk5	Down	Up		NP_001996.1	31.82 % / 267 aa
AA605880.1		zgc:158387	Down	Down	ENSDART00000045329	NP_004519.1	45.26 % / 134 aa
AI415824.1	Dr.114332		Down	Up			
BI673846.1			Down	Up			
AI331778.1	Dr.2860	zgc:73293	Down	Up	ENSDART00000078423	Q06055	72.34 % / 138 aa
AI584905.1	Dr.76846	wu:fb30e06	Down	Down		1211338A	43.95 % / 248 aa
AI721527.1		LOC797924	Down	Up		NP_004263.1	36 % / 81 aa
AI666880.1			Down	Up			
AI626645.1	Dr.67647	klhl31	Down	Up	ENSDART00000057049	Q9P2J3	36.44 % / 578 aa
AW173989.1	Dr.75838	zorba	Down	Down	ENSDART00000024062	NP_085097.1	67 % / 483 aa
AI415874.1	Dr.76827	wu:fb36b09	Down	Up			
AW128338.1	Dr.12720	zdhhc5	Down	Up	ENSDART00000014252	T47144	38.56 % / 295 aa
BI867043.1	Dr.125469		Down	Up			
BM104128.1	Dr.48052	zgc:136545	Down	Up	ENSDART00000036580	NP_004524.1	67 % / 161 aa
BM103081.1	Dr.94477		Down	Up			
BE016444.1	Dr.122560		Down	Up			
AI667571.1	Dr.139134		Down	Up	ENSDART00000031574		
CK027424.1	Dr.54908	LOC407625	Down	Up	ENSDART00000033970	T00076	74.77 % / 107 aa
AW826304.1			Down	Up			
AW233084.1	Dr.80987	zgc:153628	Down	Up			
BI880107.1			Down	Up			
BI887504.1		zgc:65772	Down	Down		NP_473357.1	59.54 % / 248 aa
AI461327.1	Dr.118190	LOC797198	Down	Up		NP_056139.1	59.79 % / 187 aa
AI658309.1	Dr.78540	LOC557290	Down	Down	ENSDART00000090098	NP_060632.1	77 % / 199 aa
BM036395.1	Dr.73909	si:ch211-154o6.6	Down	Up	ENSDART00000078858	NP_055173.1	38.1 % / 142 aa
BI879986.1	Dr.77119	cyp51	Down	Up		JC4759	76.66 % / 492 aa
BE016086.1	Dr.74614	LOC571880	Down	Up		T46917	48.46 % / 227 aa
AI641369.1			Down	Up			
AI601440.1	Dr.121867		Down	Up			
AI641113.1			Down	Up			
BI846691.1	Dr.20096	zgc:66323	Down	Up	ENSDART00000048853	NP_057067.1	93.2 % / 147 aa
AI397344.1	Dr.121648	wu:fb10c10	Down	Up			
BG738407.1	Dr.133491		Down	Down	ENSDART00000047645	NP_002426.1	58.06 % / 123 aa

AW281977.1			Down	Up			
AW203034.1	Dr.78214	LOC566384	Down	Up		Q9BXS4	61.42 % / 267 aa
AI397339.1		zgc:152808	Down	Up			
X85734.1		wt1a	Down	Up		NP_077743.1	73.52 % / 419 aa
DV591371.1	Dr.88588	igiv1s7	Down	Up	ENSDART00000017807	S49531	49.11 % / 109 aa
AI416365.1	Dr.76029	sfrs5	Down	Down	ENSDART00000043312	S59042	65.77 % / 257 aa
AA497258.1	Dr.20193	zgc:85725	Down	Down		P01118	96.28 % / 188 aa
BI890149.1	Dr.36786	zgc:113841	Down	Up			
AW019653.1	Dr.125603		Down	Up	ENSDART00000027072		
BI710504.1			Down	Down			
AW777920.1		wu:fk52g02	Down	Up			
AI522753.1		zgc:112368	Down	Up		S70439	56.85 % / 241 aa
BE201733.1	Dr.82162	zgc:101098	Down	Up	ENSDART00000035670	NP_002686.2	94.76 % / 210 aa
BF937081.1	Dr.119965	LOC100004041	Down	Down	ENSDART00000042789	NP_060517.1	74.42 % / 214 aa
AA497183.1	Dr.122044		Down	Up	ENSDART00000075484		
AW077421.1			Down	Up			
AI106115.1	Dr.76051		Down	Up	ENSDART00000079833		
BI880667.1			Down	Up			
BM101834.1	Dr.133777	LOC100002789	Down	Up		I38182	36.36 % / 219 aa
AI878581.1		wu:fc62b05	Down	Down			
AI957859.1	Dr.124757		Down	Down			
AI721411.1	Dr.78708	LOC566581	Down	Up	ENSDART00000055003	TGHUM1	39 % / 122 aa
AI722567.1	Dr.33371	zgc:86902	Down	Up		O14647	37 % / 152 aa
BM082504.1	Dr.18807	bokb	Down	Up	ENSDART00000021141	NP_115904.1	56.94 % / 209 aa
AI878392.1	Dr.79199	si:dkey-153k10.9	Down	Down			
BM154212.1		crygm6	Down	Down	ENSDART00000040744	P07316	57.89 % / 171 aa
BI882464.1	Dr.81197	LOC100000715	Down	Up			
AI477082.1	Dr.114529	wu:fb55d12	Up	Up		NP_002464.1	74.44 % / 223 aa
BG307388.1		sst3	Down	Up			
BI886790.1	Dr.31864	nup205	Down	Up	ENSDART00000062216		
AF132085.2	Dr.81301	pthr3	Down	Up	ENSDART00000003805	2119172A	57.54 % / 444 aa
AI667215.1	Dr.78610	slc26a11	Down	Up	ENSDART00000063157	NP_075062.1	30.92 % / 440 aa
AI496745.1	Dr.77109	amy2a	Down	Up	ENSDART00000003035	P04746	73.83 % / 512 aa

AI722393.1		hapln1a	Down	Up			
AI584306.1			Down	Up			
AW116490.1	Dr.7346	LOC563289	Down	Up			
AA605846.1	Dr.16227	LOC571153	Down	Up	ENSDART00000039970	NP_006022.1	35 % / 142 aa
AW019025.1	Dr.79887	slc6a19	Down	Up		NP_064593.1	49.06 % / 576 aa
BI889842.1	Dr.141	tmem46	Down	Up	ENSDART00000045574		
BE201712.1			Down	Up			
AI878541.1	Dr.131979		Down	Up	ENSDART00000040804		
AI416037.1	Dr.121917	wu:fc48e01	Down	Up			
AI601672.1	Dr.77987	zgc:113026	Down	Up	ENSDART00000052127	Q9Y2X9	44 % / 219 aa
BI672607.1	Dr.83482	DKEY-270E21.3	Down	Up		NP_036434.1	46 % / 91 aa
BM857883.1	Dr.30340	slc39a1	Down	Up	ENSDART00000062257	NP_055394.1	38.2 % / 296 aa
BI983119.1	Dr.125490		Down	Up			
AF137535.1	Dr.73516	daxx	Down	Up	ENSDART00000046620	NP_001341.1	45.37 % / 407 aa
AW233384.1	Dr.597	vegfa	Down	Up		A41551	48.48 % / 188 aa
BE202082.1	Dr.82190	wu:fl05f04	Down	Up	ENSDART00000050229	NP_005808.1	41.88 % / 402 aa
BI983579.1	Dr.85524	LOC792677	Down	Up			
AL912380.1	Dr.21063	bapx1	Down	Up	ENSDART00000054822	NP_001180.1	49.1 % / 245 aa
AA605887.1	Dr.118648	LOC100007702	Down	Up	ENSDART00000058572		
AF288211.1	Dr.82517	hmx3	Down	Up	ENSDART00000035373	A47234	86.67 % / 75 aa
AI545120.1	Dr.75296	zgc:55404	Down	Up	ENSDART00000024595		
AI666883.1	Dr.78549	lima1	Down	Up		NP_057441.1	36.13 % / 627 aa
AI588610.1	Dr.77727	zgc:77793	Down	Up	ENSDART00000052368	NP_060409.1	51.79 % / 580 aa
BM095853.1	Dr.84745	LOC563801	Down	Up	ENSDART00000088298	NP_060579.1	46 % / 168 aa
BE016400.1	Dr.107525	zgc:153953	Down	Up		NP_060735.1	56 % / 145 aa
AW420532.1	Dr.113877	zgc:111878	Down	Up		P16260	30.69 % / 282 aa
AI331461.1	Dr.76375	zgc:136962	Down	Up		NP_036465.1	62 % / 102 aa
AA495415.1	Dr.105027	cxcl12b	Down	Up			
AW171484.1	Dr.37483	LOC565025	Down	Up		T46421	59.28 % / 220 aa
BE693136.1	Dr.74453	LOC100000597	Down	Up	ENSDART00000074575	Q16181	88.07 % / 285 aa
BG892168.1	Dr.81746	LOC563864	Down	Up		O95967	64 % / 48 aa
BI980500.1		hic2	Down	Up		NP_006488.1	43.9 % / 556 aa
BI864878.1	Dr.96460		Down	Up			

BG882996.1	Dr.80613	hsd11b2	Down	Down	ENSDART00000025665	2206292A	45.14 % / 380 aa
BI673714.1	Dr.83521	LOC792686	Down	Up		P51452	31.79 % / 149 aa
AW077765.1	Dr.107078	reverbb1	Down	Up		Q14995	56.56 % / 403 aa
AI330794.1	Dr.75574	pfn2l	Down	Up		NP_444252.1	78.1 % / 137 aa

Table A.2: A GRE analysis of the promoter region of up- and down-regulated cu responsive genes. Using the zebrafish Ensembl ID, we were able to extract a 2KB region upstream of the start codon using the BioMart tool from the e!Ensembl zebrafish Genome server Zv7. Using consensus sequence matrices found in NubiScan Software, we analysed the 2kb promoter region for significant GRE hits. There were a total of 257 genes that contained at least 1 consensus GRE, and significant hits were ranked based on the number of hits and the proximity to the start codon, which increases the likelihood that the gene is in part regulated by glucocorticoids. Genes highlighted in grey are unique genes (172) that do not contain one consensus MRE promoter sequence (See Supplemental Table 3).

Ensembl ID	Gene Name	Human Ensembl ID	% Identity	Expression Direction Low Cu	Expression Direction High Cu	Hit 1	Hit 2	Hit 3	Hit 4	Hit 5	Hit 6	Hit 7
ENSDARG00000059467	LOC565025*			Down	Up	1371	819	593	348	208	92	
ENSDARG00000018740	LOC567576			Up	Down	1123	963	828	677	542	153	101
ENSDARG00000011247	zgc:136847*	ENSG00000184302	86	Up	Up	1777	1294	533	473	355		
ENSDARG00000061048	zgc:158650	ENSG00000138071	95	Up	Down	1967	1445	730	602	588	262	156
ENSDARG00000070038	LOC568032*			Up	Up	1909	1531	1245	881	498	85	
ENSDARG00000060541	zgc:152864*	ENSG00000184787	93	Up	Down	1774	1339	705	627	537	154	
ENSDARG00000005776	guk1*			Up	Down	1137	1034	777	453	378		
ENSDARG00000001975	hsd11b2*	ENSG00000176387	46	Down	Down	1603	1399	607	475	72		
ENSDARG00000071353	LOC100002196*	ENSG00000140350	77	Up	Down	1616	1466	1414	265	128		
ENSDARG00000057121	LOC100003460*			Up	Down	438	342	296				
ENSDARG00000018508	zdhhc5*	ENSG00000099904	39	Down	Up	1982	1813	1687	1019	899	693	301
ENSDARG00000008807	bokb*	ENSG00000176720	55	Down	Up	1214	731	253	219			
ENSDARG00000003058	ccr6a			Up	Up	1350	1263	519	372			
ENSDARG00000062799	LOC567939*			Up	Down	1902	1212	73	58			
ENSDARG00000054030	LOC799860	ENSG00000150593	69	Up	Down	1039	992	130	20			
ENSDARG00000029764	mef2c*			Up	Up	1351	1301	1223	900	729	429	
ENSDARG00000069979	zgc:73350			Up	Down	1809	1508	1129	938	853	29	
ENSDARG00000002791	atp1a1*			Up	Up	1966	1574	647				
ENSDARG00000013687	CILP	ENSG00000119614	65	Up	Down	1599	1362	1053	636	55		
ENSDARG00000039365	cuedc2*	ENSG00000091704	62	Up	Up	1450	989	378	257	57		
ENSDARG00000004111	esr1*	ENSG00000067955	79	Up	Down	1690	1345	1046	994	17		

ENSDARG00000036060	exosc6*	ENSG00000182871	44	Up	Down	1953	1908	1454	950	119		
ENSDARG00000040024	gpd1l			Up	Down	1445	1161	997	745	392		
ENSDARG00000056160	hspd1	ENSG00000160570	41	Up	Down	1971	1642	1518	629	69		
ENSDARG00000009621	LOC569855	ENSG00000120129	67	Up	Down	1884	1838	1583	1463	1332	1184	1096
ENSDARG00000013004	wu:fb44b11	ENSG00000130811	88	Up	Down	1870	1304	957	727	513		
ENSDARG00000040192	wu:fb73f07			Up	Down	1483	89	68				
ENSDARG00000059057	zgc:109987*			Up	Up	1915	1644	1377	1309	531		
ENSDARG00000071673	zgc:153196*	ENSG00000166170	50	Up	Down	913	243	131				
ENSDARG00000012215	zgc:55702*	ENSG00000091831	47	Up	Down	893	208	36				
ENSDARG00000055846	zgc:73293*	ENSG00000135390	73	Down	Up	1497	930	499	337	66		
ENSDARG00000040917	cbfb*			Up	Down	477	274					
ENSDARG00000059280	hoxc1a	ENSG00000157344	45	Down	Down	492	101					
ENSDARG00000027469	hsd17b1*	ENSG00000143774	37	Up	Up	139	67					
ENSDARG00000037570	im:7162148*	ENSG00000124713	72	Down	Down	1484	1209	935	469			
ENSDARG00000070081	LOC100007702*	ENSG00000146374	40	Down	Up	1422	1031	678	385			
ENSDARG00000009549	LOC407625*	ENSG00000127481	76	Down	Up	1902	1195	864	456			
ENSDARG00000057212	LOC560857*	ENSG00000133065	81	Up	Up	1771	1173	1033	114			
ENSDARG00000039134	mtr*			Up	Down	1533	1097	1008	374			
ENSDARG00000069717	ncl*			Up	Down	151	106					
ENSDARG00000025600	or102-5*	ENSG00000125651	67	Up	Down	1438	867	551	396			
ENSDARG00000008034	skib*			Up	Down	1820	1556	1271	83			
ENSDARG00000031243	snai3*			Up	Down	453	153					
ENSDARG00000019293	tfdp1l			Up	Down	1599	1356	715	137			
ENSDARG00000068588	ubxd2	ENSG00000066117	84	Up	Up	784	688	583	529			
ENSDARG00000068290	wu:fd49b10	ENSG00000105997	59	Up	Down	1890	1616	1281	118			
ENSDARG00000069966	zgc:110251	ENSG00000105393	48	Up	Down	1965	1528	573	179			
ENSDARG00000016623	zgc:154030*	ENSG00000164051	58	Up	Up	1391	1059	991	145			
ENSDARG00000035075	zgc:92763*	ENSG00000089818	68	Up	Down	1824	1779	1458	505			
ENSDARG00000067809	zgc:92804			Up	Down	1705	1574	747	139			
ENSDARG00000010155	abi1	ENSG00000136754	78	Up	Down	1686	1312	136				
ENSDARG00000037639	bapx1	ENSG00000109705	64	Down	Up	1109	533	400				
ENSDARG00000069446	dbh*	ENSG00000107874	42	Up	Up	1969	1147	149				
ENSDARG00000071245	DKEY-270E21.3			Down	Up	1599	1517	121				

ENSDARG00000016889	eif3s4			Up	Down	1817	1256	469		
ENSDARG00000035650	evla*	ENSG00000144381	86	Up	Down	915	799	255		
ENSDARG00000037955	LOC553260	ENSG00000113194	83	Up	Down	1652	1236	401		
ENSDARG00000070214	LOC555544*	ENSG00000105248	60	Up	Down	1711	1560	22		
ENSDARG00000070040	LOC563289*			Down	Up	1910	1462	1258	720	669
ENSDARG00000019033	LOC566384*			Down	Up	1711	1267	971	778	715
ENSDARG00000004114	LOC570077*	ENSG00000186184	64	Up	Down	1345	1269	159		
ENSDARG00000041022	LOC793618*			Up	Down	1329	1135	208		
ENSDARG00000069685	LOC793794*	ENSG00000145337	48	Up	Down	1900	1512	1384	1281	532
ENSDARG00000071048	LOC794092			Up	Down	981	521	84		
ENSDARG00000005513	naca*			Up	Down	1469	1251	336		
ENSDARG00000042215	pias4l*			Up	Down	961	898	53		
ENSDARG00000034262	pld2*	ENSG00000034510	73	Up	Down	1671	793	359		
ENSDARG00000005989	rgl1*	ENSG00000112936	44	Up	Down	1906	703	130		
ENSDARG00000000853	ripk5			Down	Up	1350	613	171		
ENSDARG00000004386	si:dkey-153k10.9			Down	Down	1805	1400	120		
ENSDARG00000002003	wu:fb55d12			Up	Up	1736	1336	1272	1101	866
ENSDARG00000031720	wu:fk52g02*			Down	Up	1345	1284	477		
ENSDARG00000069589	zgc:110759*	ENSG00000197582	66	Up	Down	1786	1689	1468	1284	1053
ENSDARG00000038942	zgc:111878*			Down	Up	1523	1376	1290	1201	1083
ENSDARG00000043175	zgc:112368*			Down	Up	1450	974	138		
ENSDARG00000041481	zgc:113841*			Down	Up	1356	928	235		
ENSDARG00000004169	zgc:136942*			Up	Down	1847	1530	191		
ENSDARG00000058666	zgc:153628*			Down	Up	1982	1159	889	771	542
ENSDARG00000033364	zgc:158387			Down	Down	1576	1511	65		
ENSDARG00000037665	zgc:158397*			Up	Down	1974	1638	304		
ENSDARG00000017230	zgc:63728	ENSG00000124279	43	Up	Down	1582	1479	117		
ENSDARG00000021488	zgc:73134*			Up	Down	1372	848	156		
ENSDARG00000022437	zgc:92411*			Up	Down	1401	1312	1067	1012	660
ENSDARG00000017798	bcor			Up	Down	806	18			
ENSDARG00000044776	clica	ENSG00000138615	73	Up	Down	1586	142			
ENSDARG00000056152	fam3c			Up	Down	1915	54			
ENSDARG00000028106	glrx*			Up	Down	685	246			

ENSDARG00000025504	gucy2f*			Up	Down	1359	511			
ENSDARG00000037855	LOC100008455			Up	Down	1773	1723	1498	1177
ENSDARG00000040543	LOC407680*	ENSG00000175766	71	Up	Down	1224	1099	987	888
ENSDARG00000014624	LOC794847*			Up	Down	1895	1751	905	825
ENSDARG00000017681	mapk4*			Up	Up	1013	41		
ENSDARG00000057513	mdm4	ENSG00000064995	62	Up	Down	671	165		
ENSDARG00000042361	meis2.1			Up	Down	585	142		
ENSDARG00000041026	or2.7*			Up	Up	1560	1392	753	603
ENSDARG00000037655	pls3*	ENSG00000104872	35	Up	Down	835	151		
ENSDARG00000042221	si:dkey-25e12.6*	ENSG00000180357	60	Up	Down	1784	124		
ENSDARG00000058004	six2.1*	ENSG00000111602	54	Up	Down	1633	264		
ENSDARG00000003835	stom*			Up	Down	1133	516		
ENSDARG00000034138	tmem46*			Down	Up	1764	92		
ENSDARG00000026225	whsc1			Up	Down	1036	189		
ENSDARG00000031420	wt1a*			Down	Up	1285	217		
ENSDARG00000070447	zgc:101628*			Down	Down	1462	193		
ENSDARG00000007096	zgc:110299*	ENSG00000118407	66	Up	Down	1825	1364	1044	649
ENSDARG00000031316	zgc:110344*			Up	Up	1847	383		
ENSDARG00000031562	zgc:154057	ENSG00000183208	56	Up	Up	904	34		
ENSDARG00000071699	zgc:158245*			Up	Down	1967	1618	1390	1046
ENSDARG00000020798	zgc:73124*			Up	Down	884	159		
ENSDARG00000015312	zgc:77286*			Up	Down	1060	307		
ENSDARG00000003573	zgc:92842*			Up	Down	1423	463		
ENSDARG00000008454	zorba			Down	Down	1216	516		
ENSDARG00000052438	actr2*			Up	Down	1395	802	512	
ENSDARG00000060089	btaf1*			Up	Down	379			
ENSDARG00000036558	col18a1*			Up	Down	1640	1526	594	
ENSDARG00000055100	cxcl12b*			Down	Up	413			
ENSDARG00000042641	cyp51			Down	Up	1964	1755	1432	
ENSDARG00000007628	dusp1			Up	Down	1723	1592	1552	
ENSDARG00000044754	gnrh2			Up	Down	1792	1377	977	
ENSDARG00000003051	hccs*	ENSG00000170608	28	Up	Down	1845	590	552	
ENSDARG00000016363	her8a*			Up	Down	325			

ENSDARG00000038298	hic2			Down	Up	497		
ENSDARG00000010478	hsp90a*			Down	Up	120		
ENSDARG00000052374	im:7148063			Down	Down	436		
ENSDARG00000055115	LOC100007489			Down	Up	53		
ENSDARG00000038538	LOC558765			Up	Down	36		
ENSDARG00000056836	LOC566639			Up	Down	195		
ENSDARG00000059812	LOC795324			Up	Down	1811	1437	1002
ENSDARG00000052765	LOC795873*			Down	Down	1433	1016	624
ENSDARG00000018847	LOC797547			Down	Up	1729	1289	1054
ENSDARG00000040334	mat2a*	ENSG00000124356	64	Up	Up	1928	1430	1270
ENSDARG00000013087	ndrg3a*			Up	Down	1929	1401	1285
ENSDARG00000005780	npyryb*			Up	Down	75		
ENSDARG00000033160	nr1d1*	ENSG00000101407	54	Up	Down	1695	1328	141
ENSDARG00000042128	pacsin1*	ENSG00000066697	49	Up	Down	307		
ENSDARG00000036772	pygo2*			Up	Down	1963	1611	1316
ENSDARG00000038018	rds2	ENSG00000112619	64	Down	Up	1424	1195	617
ENSDARG00000014582	sec6l1	ENSG00000196292	14	Up	Down	436		
ENSDARG00000025436	sepx1*	ENSG00000004848	67	Up	Down	65		
ENSDARG00000029818	sfrs5			Down	Down	1657	1213	1016
ENSDARG00000002909	tjp3*	ENSG00000158315	63	Up	Down	1998	1099	741
ENSDARG00000018006	vrk1*			Up	Down	1570	1320	1135
ENSDARG00000060679	wu:fj66b06*			Up	Up	1727	1520	1149
ENSDARG00000045003	zgc:100909*			Up	Down	1135	971	843
ENSDARG00000056723	zgc:136230*			Up	Down	491		
ENSDARG00000030157	zgc:136545*	ENSG00000086967	62	Down	Up	1720	1096	889
ENSDARG00000014008	zgc:153452*			Up	Down	1424	1361	1289
ENSDARG00000059794	zgc:158313	ENSG00000138443	74	Up	Down	1010	719	578
ENSDARG00000014373	zgc:158535*			Up	Down	1090	993	560
ENSDARG00000061854	zgc:158761*			Up	Down	502		
ENSDARG00000002597	zgc:55855			Up	Down	1813	872	708
ENSDARG00000021564	zgc:56235*	ENSG00000161091	64	Up	Up	1600	1489	721
ENSDARG00000014361	zgc:64112			Up	Down	1657	1033	971
ENSDARG00000045074	zgc:77741			Up	Down	1075	942	748

ENSDARG00000035993	zgc:86902*			Down	Up	1977	1623	1491
ENSDARG00000043100	zgc:92140*			Up	Down	312		
ENSDARG00000046062	zgc:92241*			Down	Up	1935	1743	1111
ENSDARG00000010625	zgc:92762*	ENSG00000102921	31	Up	Up	354		
ENSDARG00000006487	anp32a	ENSG00000120251	81	Up	Down	1297	1014	
ENSDARG00000002792	arcn1*	ENSG00000171720	92	Up	Down	1429	559	
ENSDARG00000005574	chx10*			Up	Down	1215	951	
ENSDARG00000009689	daam1l*			Up	Down	1858	886	
ENSDARG00000002758	dedd1*	ENSG00000198625	46	Up	Down	1727	1102	
ENSDARG00000006840	gnmt*			Up	Down	1975	1492	
ENSDARG00000032129	gtf2f1			Up	Down	1630	547	
ENSDARG00000014722	her1*			Up	Down	1282	1116	
ENSDARG00000070955	hmx3*	ENSG00000188620	74	Down	Up	1741	614	
ENSDARG00000036231	hoxa3a	ENSG00000196531	95	Up	Down	1554	1028	
ENSDARG00000014704	im:7140576			Up	Up	1970	1366	
ENSDARG00000002768	LOC100001147*	ENSG00000025156	57	Up	Down	1296	861	
ENSDARG00000003008	LOC100001787*	ENSG00000115053	51	Up	Down	1552	1112	
ENSDARG00000061147	LOC100003733*			Up	Down	1620	680	
ENSDARG00000010238	LOC100004041*	ENSG00000086589	80	Down	Down	1564	855	
ENSDARG00000014565	LOC100007803*	ENSG00000104419	69	Up	Down	1634	752	
ENSDARG00000053544	LOC562019*	ENSG00000101079	71	Up	Down	1119	504	
ENSDARG00000024026	LOC792144*	ENSG00000100711	69	Up	Down	1344	1123	
ENSDARG00000052030	LOC799805	ENSG00000117448	63	Down	Up	1212	1093	
ENSDARG00000023536	nnt*	ENSG00000068305	76	Up	Down	1499	1030	
ENSDARG00000042556	nr5a2*			Up	Down	1691	1097	
ENSDARG00000038446	nrp2b	ENSG00000204174	48	Up	Down	1953	1679	
ENSDARG00000042530	nup205*	ENSG00000155561	70	Down	Up	1860	786	
ENSDARG00000012682	pfn2l*			Down	Up	1910	1775	
ENSDARG00000018418	pthr3*			Down	Up	1912	1581	
ENSDARG00000016481	ptpn2	ENSG00000112992	82	Up	Up	1350	1159	
ENSDARG00000003820	reverbb1			Down	Up	1637	1317	
ENSDARG00000043680	si:ch211-59d15.5*			Up	Down	1886	1548	
ENSDARG00000043021	slc26a11*	ENSG00000181045	60	Down	Up	1464	1404	

ENSDARG00000013800	snrpd3*	ENSG00000118257	59	Down	Down	942	743
ENSDARG00000044182	stau1	ENSG00000124214	59	Down	Up	1378	1036
ENSDARG00000036505	syt4*			Up	Down	1835	695
ENSDARG00000029107	ube2d1*			Down	Up	1950	795
ENSDARG00000018935	wu:fj59e04*			Up	Down	1433	1254
ENSDARG00000006639	wu:fj80c12*			Up	Down	1918	1354
ENSDARG00000013711	wu:fl05f04*	ENSG00000105355	34	Down	Up	1380	888
ENSDARG00000003109	zgc:100963*	ENSG00000159200	55	Down	Up	1183	715
ENSDARG00000059131	zgc:101624*			Up	Down	1075	711
ENSDARG00000055064	zgc:112318*	ENSG00000124507	73	Down	Down	1264	1220
ENSDARG00000017316	zgc:114117*			Up	Down	1910	1098
ENSDARG00000043287	zgc:123177*			Up	Down	1493	661
ENSDARG00000010385	zgc:123254*			Up	Down	1930	1514
ENSDARG00000071881	zgc:153259*			Down	Up	1701	620
ENSDARG00000003582	zgc:153293*	ENSG00000102024	86	Up	Down	1894	1723
ENSDARG00000070217	zgc:153623*			Up	Up	1810	1433
ENSDARG00000060954	zgc:153972	ENSG00000163558	88	Up	Down	1647	1025
ENSDARG00000061929	zgc:153980*			Up	Down	1762	662
ENSDARG00000041878	zgc:55760*			Up	Down	1704	849
ENSDARG00000029107	zgc:66323*			Down	Up	1950	795
ENSDARG00000057491	zgc:66437			Up	Down	1244	521
ENSDARG00000026751	zgc:66439*	ENSG00000123728	92	Up	Down	1227	395
ENSDARG00000036076	zgc:77793*	ENSG00000155393	52	Down	Up	1546	728
ENSDARG00000043542	znf259	ENSG00000109917	69	Down	Up	1104	1024
ENSDARG00000013856	amy2a*	ENSG00000197839	73	Down	Up	1771	
ENSDARG00000037870	bactin2	ENSG00000075624	99	Down	Up	1112	
ENSDARG00000020377	eif4g2a			Up	Down	1163	
ENSDARG00000010571	ezh2*	ENSG00000110321	83	Up	Down	670	
ENSDARG00000029738	fbln1			Up	Down	935	
ENSDARG00000013505	hip2*			Up	Down	1361	
ENSDARG00000053097	hsf2*	ENSG00000004961	66	Up	Down	1780	
ENSDARG00000039066	klhl31*	ENSG00000124743	75	Down	Up	1293	
ENSDARG00000052673	LOC100000597			Down	Up	1518	

ENSDARG00000054911	LOC100002634	ENSG00000143344	59	Down	Down	1617
ENSDARG00000069946	LOC100004167			Up	Down	1323
ENSDARG00000041978	LOC553758			Up	Down	1492
ENSDARG00000057782	LOC561095*			Up	Down	1116
ENSDARG00000027887	LOC565308*			Up	Down	1771
ENSDARG00000058011	LOC572891	ENSG00000115963	78	Up	Down	844
ENSDARG00000069833	LOC792002	ENSG00000180104	74	Down	Down	1331
ENSDARG00000070788	LOC792686*			Down	Up	523
ENSDARG00000071652	LOC797198*			Down	Up	1722
ENSDARG00000032849	ndrg1	ENSG00000198736	67	Up	Down	1526
ENSDARG00000019566	neurod*			Up	Down	1232
ENSDARG00000021225	prkci*			Up	Down	1393
ENSDARG00000015649	rap2c*	ENSG00000070961	82	Up	Down	1636
ENSDARG00000042147	rbbp5*			Up	Down	449
ENSDARG00000012504	rlbp1l			Down	Down	1754
ENSDARG00000007396	rnd3b	ENSG00000170577	85	Up	Down	829
ENSDARG00000056379	si:ch211-154o6.6*			Down	Up	1329
ENSDARG00000012684	si:dkey-18o7.1*			Up	Down	1787
ENSDARG00000056719	slc6a19*			Down	Up	1019
ENSDARG00000006389	smad2*	ENSG00000205808	61	Up	Down	807
ENSDARG00000019004	smarcd1*	ENSG00000135317	70	Up	Down	732
ENSDARG00000003813	srp54*			Up	Down	650
ENSDARG00000055478	tnc	ENSG00000185669	46	Up	Down	978
ENSDARG00000045014	tuba2*			Up	Up	1064
ENSDARG00000071039	wu:fc39c01			Up	Down	580
ENSDARG00000026183	zgc:101098*	ENSG00000099817	93	Down	Up	1636
ENSDARG00000012274	zgc:110154*			Up	Down	800
ENSDARG00000025404	zgc:112077*			Up	Down	1217
ENSDARG00000035910	zgc:113026*	ENSG00000162702	44	Down	Up	1501
ENSDARG00000062760	zgc:153343*	ENSG00000185946	7	Up	Up	1197
ENSDARG00000061908	zgc:158256*			Up	Up	1133
ENSDARG00000011637	zgc:55573*	ENSG00000100028	93	Up	Down	1958
ENSDARG00000007034	zgc:56206	ENSG00000108264	80	Up	Down	1706

ENSDARG00000056765	zgc:63744*	ENSG00000162438	62	Down	Up	1635
ENSDARG00000069448	zgc:65772			Down	Down	929
ENSDARG00000010844	zgc:85725			Down	Down	1632
ENSDARG00000016048	zgc:91857*			Up	Down	649
ENSDARG00000021048	zgc:92063*	ENSG00000155957	64	Up	Down	1656

Table A.3: A MRE analysis of the promoter region of up- and down-regulated cu responsive genes. Using the zebrafish Ensembl ID, we were able to extract a 2KB region upstream of the start codon using the BioMart tool from the e!Ensembl zebrafish Genome server Zv7. Sequences were searched for the MRE core consensus, TGCRCNC, as well as other MREs contained within the Transfac database (2007) using GCG version 11.1 (Accelrys) software. A total of 99 sequences contained at least one consensus MRE, and significant hits were ranked based on the number of hits and the proximity to the start codon, increasing the likelihood that the gene is in part regulated by an MRE. Genes highlighted in grey are unique genes (14) that do not contain one consensus GRE promoter sequence (See Supplemental Table 2).

Ensembl ID	Gene Name	Human Ensembl ID	% Identity	Expression Direction Low Cu	Expression Direction High Cu	Hit 1	Hit 2	Hit 3	Hit 4	Hit 5	Hit 6	Hit 7
ENSDARG00000035768	zgc:100903*			Down	Up	923	273	261	416	9	71	175
ENSDARG00000014704	im:7140576			Up	Up	277	585	219	261			
ENSDARG00000042361	meis2.1			Up	Down	145	489	800	144			
ENSDARG00000056160	hspd1	ENSG00000160570	41	Up	Down	132	632	989	201	669		
ENSDARG00000037955	LOC553260	ENSG00000113194	83	Up	Down	380	319	421				
ENSDARG00000000853	ripk5			Down	Up	218	671	705	758	785	914	939
ENSDARG00000010155	abi1	ENSG00000136754	78	Up	Down	522	269	394	576			
ENSDARG00000044754	gnrh2			Up	Down	584	904	254	301			
ENSDARG00000006487	anp32a	ENSG00000120251	81	Up	Down	327	338	629				
ENSDARG00000018740	LOC567576			Up	Down	649	283	324				
ENSDARG00000071048	LOC794092			Up	Down	360	812	326				
ENSDARG00000012504	rlbp1l			Down	Down	68	337	31				
ENSDARG00000019293	tfdp1l			Up	Down	31	81	879				
ENSDARG00000014361	zgc:64112			Up	Down	118	62	881				
ENSDARG00000020377	eif4g2a			Up	Down	836	219	848	879			
ENSDARG00000036231	hoxa3a	ENSG00000196531	95	Up	Down	71	9					
ENSDARG00000038018	rds2	ENSG00000112619	64	Down	Up	319	421					
ENSDARG00000069966	zgc:110251	ENSG00000105393	48	Up	Down	336	65					

ENSDARG00000061048	zgc:158650	ENSG00000138071	95	Up	Down	252	725	728	595
ENSDARG00000008454	zorba			Down	Down	334	495		
ENSDARG00000030915	cpa1*			Up	Down	615	100	510	
ENSDARG00000007396	rnd3b	ENSG00000170577	85	Up	Down	564	654	243	
ENSDARG00000040192	wu:fb73f07			Up	Down	857	447	927	
ENSDARG00000017230	zgc:63728	ENSG00000124279	43	Up	Down	258	946	900	
ENSDARG00000045074	zgc:77741			Up	Down	237	608	554	
ENSDARG00000004189	cbx1*			Up	Down	528	277		
ENSDARG00000012788	foxa3*			Up	Down	473	567		
ENSDARG00000032129	gtf2f1			Up	Down	973	278		
ENSDARG00000037855	LOC100008455			Up	Down	497	268		
ENSDARG00000009621	LOC569855	ENSG00000120129	67	Up	Down	427	816		
ENSDARG00000058011	LOC572891	ENSG00000115963	78	Up	Down	328	655		
ENSDARG00000044182	stau1	ENSG00000124214	59	Down	Up	981	131		
ENSDARG00000004169	zgc:136942			Up	Down	130	505		
ENSDARG00000069444	zgc:136962*			Down	Up	151	905		
ENSDARG00000031562	zgc:154057	ENSG00000183208	56	Up	Up	100	510		
ENSDARG00000055563	zgc:158612*			Up	Down	922	319		
ENSDARG00000037870	bactin2	ENSG00000075624	99	Down	Up	422			
ENSDARG00000042641	cyp51			Down	Up	166			
ENSDARG00000071245	DKEY-270E21.3			Down	Up	326			
ENSDARG00000007628	dusp1			Up	Down	243			
ENSDARG00000016889	eif3s4			Up	Down	267			
ENSDARG00000029738	fbln1			Up	Down	257			
ENSDARG00000038298	hic2			Down	Up	444			
ENSDARG00000052374	im:7148063			Down	Down	491			
ENSDARG00000050405	lima1*	ENSG 00000050405		Down	Up	494			
ENSDARG00000052673	LOC100000597			Down	Up	191			
ENSDARG00000054911	LOC100002634	ENSG00000143344	59	Down	Down	157			
ENSDARG00000055115	LOC100007489			Down	Up	388			

ENSDARG00000041978	LOC553758			Up	Down	248		
ENSDARG00000042484	LOC565013*			Up	Down	166		
ENSDARG00000056836	LOC566639			Up	Down	201		
ENSDARG00000069833	LOC792002	ENSG00000180104	74	Down	Down	264		
ENSDARG00000018847	LOC797547			Down	Up	283		
ENSDARG00000054030	LOC799860	ENSG00000150593	69	Up	Down	128		
ENSDARG00000057513	mdm4	ENSG00000064995	62	Up	Down	360		
ENSDARG00000005513	naca			Up	Down	311		
ENSDARG00000032849	ndrg1	ENSG00000198736	67	Up	Down	278		
ENSDARG00000003820	reverbb1			Down	Up	103		
ENSDARG00000029818	sfrs5			Down	Down	257		
ENSDARG00000004386	si:dkey-153k10.9			Down	Down	277		
ENSDARG00000012684	si:dkey-18o7.1			Up	Down	31		
ENSDARG00000055478	tnc	ENSG00000185669	46	Up	Down	34		
ENSDARG00000002003	wu:fb55d12			Up	Up	671	758	914
ENSDARG00000071039	wu:fc39c01			Up	Down	487		
ENSDARG00000003701	wu:fc48a12*			Up	Up	114		
ENSDARG00000060954	zgc:153972	ENSG00000163558	88	Up	Down	308	827	935
ENSDARG00000059794	zgc:158313	ENSG00000138443	74	Up	Down	432		
ENSDARG00000057491	zgc:66437			Up	Down	360		
ENSDARG00000026484	zgc:86635*			Up	Down	304		
ENSDARG00000043542	znf259	ENSG00000109917	69	Down	Up	229		
ENSDARG00000017798	bcor			Up	Down	946	900	
ENSDARG00000055100	cxcl12b			Down	Up	584	596	
ENSDARG0000070309	hoxc1a	ENSG00000157344	45	Down	Down	601	962	
ENSDARG00000052030	LOC799805	ENSG00000117448	63	Down	Up	608	554	
ENSDARG00000041623	mt2*			Up	Up	576	648	
ENSDARG00000112715	vegfa*	ENSG00000112715		Down	Up	942	918	
ENSDARG00000007034	zgc:56206	ENSG00000108264	80	Up	Down	592	790	
ENSDARG00000036456	anxa4*			Up	Down	524		

ENSDARG00000003058	ccr6a			Up	Up	699
ENSDARG00000013687	CILP	ENSG00000119614	65	Up	Down	731
ENSDARG00000044776	clica	ENSG00000138615	73	Up	Down	904
ENSDARG00000056152	fam3c			Up	Down	922
ENSDARG00000040024	gpd1l			Up	Down	701
ENSDARG00000069946	LOC100004167			Up	Down	625
ENSDARG00000038538	LOC558765			Up	Down	938
ENSDARG00000059812	LOC795324			Up	Down	639
ENSDARG00000038446	nrp2b	ENSG00000204174	48	Up	Down	938
ENSDARG00000016481	ptpn2	ENSG00000112992	82	Up	Up	904
ENSDARG00000014582	sec6l1	ENSG00000196292	14	Up	Down	881
ENSDARG00000068588	ubxd2	ENSG00000066117	84	Up	Up	553
ENSDARG00000026225	whsc1			Up	Down	879
ENSDARG00000013004	wu:fb44b11	ENSG00000130811	88	Up	Down	567
ENSDARG00000068290	wu:fd49b10	ENSG00000105997	59	Up	Down	553
ENSDARG00000014008	zgc:153452*			Up	Down	827
ENSDARG00000033364	zgc:158387			Down	Down	910
ENSDARG00000002597	zgc:55855			Up	Down	677
ENSDARG00000069448	zgc:65772			Down	Down	905
ENSDARG00000069979	zgc:73350			Up	Down	886
ENSDARG00000010844	zgc:85725			Down	Down	576
ENSDARG00000067809	zgc:92804			Up	Down	595

Table A.4: Results of K-means clustering showing 4 distinct cluster patterns (A,B,C,D) of expression containing genes significantly different from control treatment ($p<0.05$) with an expressional direction greater than 2-fold. Complete results (all significantly regulated genes) of this microarray experiment were submitted to the public archive ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) in accordance with Microarray Gene Expression Data Society (MGED) recommendations.

(A) Cluster-1

Probe Set ID	Unigene ID	Gene Title	Cu	Ca	Na	CaCu	NaCu
Dr.813.1.S1_at	Dr.813	acetyl-CoA acetyltransferase 2	-	down	down	down	down
Dr.3267.1.S1_at	Dr.3267	claudin h	up	-	-	-	-
Dr.3099.1.A1_at	Dr.77826	coronin, actin binding protein, 2A	up	-	-	-	-
Dr.1603.1.A1_at	Dr.77119	Cytochrome P450, family 51	down	down	down	down	down
Dr.1603.1.A1_x_at	Dr.77119	Cytochrome P450, family 51	down	down	down	down	down
Dr.20850.1.S1_at	Dr.20850	fatty acid binding protein 7, brain, a	down	down	down	down	-
Dr.1041.1.S1_at	Dr.76172	fucosidase, alpha-L- 1, tissue	up	-	-	-	-
Dr.20230.1.S1_at	Dr.76342	glia maturation factor, beta	-	-	down	down	-
Dr.7311.1.S1_at	Dr.76519	guanine nucleotide binding protein-like 3 (nucleolar)	-	-	down	-	-
Dr.8000.1.S1_at	Dr.79923	glutathione peroxidase 1b	up	-	-	-	-
Dr.13321.1.S2_at	Dr.76367	hypoxia induced gene 1	-	-	down	down	down
Dr.13321.1.S1_at	Dr.76367	hypoxia induced gene 1 /// similar to putative growth hormone like protein-1	-	-	down	down	-
Dr.2051.1.S1_at	Dr.77183	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	down	down	down	down	down
Dr.25191.1.S1_at	Dr.75844	isocitrate dehydrogenase 1 (NADP+), soluble	-	-	down	-	-
Dr.6064.1.A1_at	Dr.6064	hypothetical protein LOC100000433	up	up	-	up	-
Dr.13618.1.A1_at	Dr.23709	similar to bloodthirsty	-	down	down	-	-
Dr.3310.1.A1_at	Dr.78055	similar to LOC495252 protein	up	-	-	-	-
Dr.17283.1.A1_at		hypothetical LOC558794	down	down	down	down	down
Dr.4267.1.A1_at		hypothetical LOC558794	down	down	down	down	down
Dr.6321.1.A1_at	Dr.6321	hypothetical LOC559001	-	down	down	down	down
Dr.2871.1.A1_at	Dr.132256	similar to Tmc4 protein	up	-	-	-	-
Dr.17419.1.A1_at	Dr.75992	hypothetical LOC564534	-	-	-	down	down

Dr.23441.1.S1_at	Dr.78880	zgc:85644 /// hypothetical protein LOC791831	up	-	-	-	-
Dr.16696.1.S1_at	Dr.121313	hypothetical protein LOC796134 /// si:dkey-286j15.3	-	-	down	-	down
Dr.11310.1.S1_at	Dr.11310	tubulin, alpha 1 /// similar to alpha-tubulin isotype M-alpha-2	up	-	-	-	-
Dr.6695.1.S1_at	Dr.6695	3'-phosphoadenosine 5'-phosphosulfate synthase 2	down	-	down	-	down
Dr.13544.1.S1_at	Dr.84415	programmed cell death 6	-	-		-	down
Dr.6619.1.S1_at	Dr.77295	phosphogluconate hydrogenase	-	-	down	-	down
Dr.12110.1.S1_at	Dr.12110	sterol-C4-methyl oxidase-like	-	down	down	down	down
Dr.11087.1.A1_at	Dr.80934	secretogranin III	up	-	-	-	-
Dr.22702.1.A1_at	Dr.81058	si:dkey-193c22.1	-	-	down	-	down
Dr.22146.1.A1_at	Dr.79887	solute carrier family 6 (neurotransmitter transporter), member 19	up	down	down	down	down
Dr.7815.1.S1_at	Dr.79639	synaptosome-associated protein 25a	up	-	-	-	-
Dr.24558.1.S1_at	Dr.84122	signal recognition particle 9	-	down	down	down	down
Dr.24214.1.S1_at	Dr.132384	sulfotransferase family, cytosolic sulfotransferase 1	down	down	down	down	down
Dr.4767.1.A1_a_at	Dr.76728	transmembrane emp24 protein transport domain containing 3	-	-	down	-	-
Dr.3773.1.S1_at	Dr.77112	UDP-N-acteylglucosamine pyrophosphorylase 1	up	-	down	-	-
Dr.1129.1.A1_at		wu:fa99h02	-	-	down	-	-
Dr.23582.1.A1_at		wu:fb13f03	up	-	-	-	-
Dr.25291.4.S1_at	Dr.104591	wu:fb18f06	up	-	-	-	-
Dr.1659.1.A1_at		wu:fc02a12	-	-	-	down	down
Dr.4920.1.A1_at		wu:fc27g01	up	-	-	-	-
Dr.3698.1.A1_at		wu:fd02f07	down	down	down	down	down
Dr.7168.1.S1_at		wu:fd56d05	-	down	down	down	down
Dr.6154.1.A1_at		wu:fj19d05	-	-	down	down	down
Dr.7862.1.A1_at		wu:fj55d04	-	down	down	down	down
Dr.20787.2.A1_a_at	Dr.84956	zgc:103549	-	-	down	-	down
Dr.21447.1.A1_at	Dr.44087	zgc:112255	-	-	-	-	down
Dr.13622.1.A1_at	Dr.84494	zgc:112474	-	down	down	-	down
Dr.4218.1.A1_at	Dr.74233	zgc:162396	-	-	-	-	down
Dr.11583.1.A1_at	Dr.75327	Zgc:162964	-	-	down	-	-
Dr.7273.1.A1_at	Dr.80345	zgc:66484	-	-	down	-	-
Dr.25285.1.S1_at	Dr.78528	zgc:73223	-	-	down	-	-
Dr.13512.1.S1_at	Dr.82983	zgc:73376	-	-	down	-	-

Dr.10016.1.A1_at	Dr.82081	zgc:77556	-	-	-	-	down
Dr.5934.1.A1_at	Dr.113528	zgc:85981	up	-	-	-	-
Dr.9746.8.S1_at	Dr.76432	zgc:86706	-	-	down	down	down
Dr.4911.1.A1_at	Dr.77249	zgc:92000	-	-	down	-	down
Dr.20008.2.A1_at	Dr.32396	zgc:92061	up	up	-	-	-
Dr.14685.1.S1_at	Dr.81719	zgc:92379	-	-	-	down	-
Dr.12321.1.A1_at			-	down	down	down	down
Dr.13438.1.A1_at	Dr.123250	Transcribed locus	-	-	-	-	down
Dr.18183.1.A1_at			up	-	-	-	-
Dr.18374.1.A1_at			up	-	-	-	-
Dr.21698.1.A1_at	Dr.78933	CDNA clone IMAGE:6034266	down	down	down	down	down
Dr.26534.1.A1_at	Dr.76999	Transcribed locus	down	down	down	down	down
Dr.7806.1.A1_at	Dr.124155	Transcribed locus	-	-	down	-	-
Dr.9875.1.A1_at	Dr.81956	Transcribed locus	-	down	down	down	down

(B) Cluster 2

Probe Set ID	Unigene ID	Gene Title	Cu	Ca	Na	CaCu	NaCu
Dr.24206.1.S1_at	Dr.72266	chaperonin containing TCP1, subunit 4 (delta)	-	-	-	-	up
Dr.13284.2.S1_at	Dr.108026	choline kinase alpha	down	-	-	-	-
Dr.11504.1.A1_at	Dr.80698	ecdysoneless homolog (Drosophila)	-	-	-	-	up
Dr.10717.1.S2_at	Dr.119569	estrogen receptor 1	down	down	down	-	up
Dr.7972.1.S1_at	Dr.77165	GTP binding protein 4	-	-	-	-	up
Dr.6932.3.S1_at	Dr.76671	high-mobility group box 3a	-	up	-	-	up
Dr.1149.1.A1_a_at	Dr.35374	heterogeneous nuclear ribonucleoprotein A0	-	-	-	-	up
Dr.19560.1.S2_at	Dr.115835	insulin induced gene 1	down	-	-	-	-
Dr.11707.2.A1_at	Dr.132454	spermine oxidase /// hypothetical LOC554383	-	-	-	up	-
Dr.236.1.S1_at	Dr.75518	similar to LOC494737 protein	-	-	down	-	-
Dr.11399.2.S1_at		hypothetical protein LOC791639	-	-	-	-	up
Dr.12594.1.S1_at	Dr.114931	X-box binding protein 1 /// hypothetical protein LOC793034	down	-	-	-	-
Dr.2850.1.S1_at	Dr.132178	methionine adenosyltransferase II, alpha	down	down	down	-	-

Dr.18414.1.S1_at	Dr.18414	programmed cell death 4a	-	-	-	-	up
Dr.6191.1.S1_at	Dr.78102	pim-1 oncogene	-	-	-	up	-
Dr.73.1.A1_at	Dr.73	sb:cb360	down	-	-	-	-
Dr.7467.1.S1_at	Dr.115188	serine incorporator 5	-	-	-	up	-
Dr.9272.1.A1_at	Dr.47567	si:dkey-30h14.2	down	-	down	-	-
Dr.5307.1.S1_at	Dr.5307	solute carrier family 20, member 1b	down	-	down	-	-
Dr.6847.1.S1_a_at	Dr.79948	solute carrier family 31 (copper transporters), member 1	down	-	-	-	up
Dr.11296.1.S1_at	Dr.106021	solute carrier family 43, member 1	down	down	down	-	-
Dr.24245.3.S1_a_at	Dr.76177	staphylococcal nuclease domain containing 1	-	-	up	-	up
Dr.3648.1.S1_at	Dr.35540	translocating chain-associating membrane protein 1	down	-	-	-	-
Dr.25607.1.S1_at	Dr.104447	wu:fi34d08	-	-	up	-	up
Dr.14725.1.A1_at	Dr.84733	zgc:112350	-	-	-	-	up
Dr.8914.1.S1_at	Dr.80042	zgc:113054	down	down	down	-	-
Dr.25243.1.S1_at	Dr.39091	zgc:113200	-	-	up	-	up
Dr.9003.1.A1_at		zgc:123010	-	-	down	-	-
Dr.774.1.S1_at	Dr.75933	zgc:136866	-	-	-	-	up
Dr.22015.1.A1_at	Dr.108327	zgc:158135	-	-	down	-	-
Dr.25593.2.A1_at	Dr.105866	Zgc:171426	-	-	-	-	up
Dr.13813.1.A1_at	Dr.83439	zgc:171663	-	-	-	up	-
Dr.9070.1.A1_at	Dr.9070	zgc:63770	-	-	-	-	up
Dr.23925.1.A1_at	Dr.80026	Transcribed locus	down	down	down	-	-
Dr.24938.1.S1_a_at			down	up	down	-	up
Dr.24938.1.S1_x_at			down	up	-	-	up

(C) Cluster 3

Probe Set ID	Unigene ID	Gene Title	Cu	Ca	Na	CaCu	NaCu
AFFX-Dr-acta1-5_x_at	Dr.75552	actin, alpha 1, skeletal muscle	-	-	up	-	up
Dr.14922.1.A1_at	Dr.81194	abhydrolase domain containing 4	-	-	up	-	-
AFFX-Dr-acta1-M_at	Dr.75552	actin, alpha 1, skeletal muscle	-	up	-	-	-
Dr.19542.1.A1_at	Dr.13893	autocrine motility factor receptor	-	-	up	-	-

Dr.5195.1.A1_at	Dr.5195	apolipoprotein L, 1	-	-	up	-	-
Dr.2022.1.A1_at	Dr.77297	arginase, type II	down	-	up	down	down
Dr.10283.1.A1_at	Dr.78270	BCL2/adenovirus E1B interacting protein 3-like	-	-	up	-	-
Dr.959.2.S1_at	Dr.75609	basic transcription factor 3	-	-	up	-	-
Dr.21005.1.S1_s_at	Dr.88584	complement component c3b /// complement component c3c	-	up	up	-	-
Dr.941.1.S1_at	Dr.32745	cell cycle associated protein 1	-	-	up	-	up
Dr.1357.1.S1_at	Dr.75181	chaperonin containing TCP1, subunit 8 (theta)	-	-	up	-	up
Dr.26344.3.S1_at	Dr.104797	cell division cycle 42	-	-	up	-	-
Dr.637.1.S1_at	Dr.75081	cadherin 2, neuronal	-	-	up	-	-
Dr.12605.1.S1_at	Dr.75267	cyclin-dependent kinase inhibitor 1b (p27, kip1)	-	-	up	-	-
Dr.535.2.A1_at		carboxyl ester lipase, like	up	up	up	-	up
Dr.7190.1.A1_at	Dr.80131	coiled-coil-helix-coiled-coil-helix domain containing 3	-	-	up	-	-
Dr.15261.1.A1_at	Dr.89300	cytoplasmic polyadenylation element binding protein 4	-	-	up	-	-
Dr.10433.1.S1_at	Dr.116325	cryptochrome 2a	-	-	up	-	-
Dr.25214.1.A1_at	Dr.87867	cytochrome P450, family 24, subfamily A, polypeptide 1, like	down	-	up	down	down
Dr.25208.2.S1_at	Dr.77160	cytochrome P450, family 3, subfamily A, polypeptide 65	-	up	up	-	-
Dr.664.2.S1_at	Dr.114326	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 /// similar to Ddx5 protein	-	-	up	-	-
Dr.20598.1.S1_at	Dr.20756	eukaryotic translation initiation factor 3, subunit D	-	-	up	-	up
Dr.1710.2.S1_at	Dr.33806	eukaryotic translation initiation factor 4E family member 3	-	up	up	-	-
Dr.10306.1.S1_at	Dr.121185	ets variant gene 6 (TEL oncogene)	-	up	up	-	-
Dr.7171.2.S1_a_at	Dr.33603	glutamic pyruvate transaminase (alanine aminotransferase) 2	-	up	up	-	-
Dr.24246.1.S1_at	Dr.24246	H1 histone family, member X	up	up	up	up	-
Dr.6382.1.A1_at	Dr.138736	hedgehog interacting protein	-	-	up	-	-
Dr.14662.1.S1_at	Dr.118179	HNF1 homeobox b	-	up	up	-	-
Dr.12882.1.A1_at	Dr.43052	heparan sulfate 6-O-sulfotransferase 1a	-	up	up	-	-
Dr.25548.1.S1_at	Dr.76602	heat shock protein 90kDa beta (grp94), member 1	-	-	up	-	-
Dr.7103.1.S1_at	Dr.7103	inhibitor of DNA binding 3	-	up	up	-	-
Dr.8587.1.A2_at	Dr.76315	insulin-like growth factor binding protein 1	-	up	up	-	-
Dr.12583.1.S1_at	Dr.12583	insulin-like growth factor binding protein 3	-	-	up	-	-
Dr.25874.1.A1_at	Dr.66408	im:7151086	-	-	up	-	-
Dr.374.1.S1_at	Dr.35325	interferon regulatory factor 2 binding protein 2	-	up	up	-	-
Dr.16331.2.S1_at	Dr.114298	Similar to Mitogen-activated protein kinase 3	-	-	up	-	-

Dr.7722.1.A1_at		similar to complement C3-H1	-	up	up	up	-
Dr.25379.1.S1_at	Dr.118237	hypothetical LOC567732	-	up	up	-	-
Dr.25331.1.S1_at	Dr.75455	hypothetical LOC567732	-	up	up	-	-
Dr.7111.1.S1_a_at	Dr.115516	similar to Probable RNA-dependent helicase p72	-	-	up	-	-
Dr.10226.1.S1_at	Dr.77293	similar to phosphoinositide-3-kinase, regulatory subunit, polypeptide 1	down	-	-	down	down
Dr.1815.1.A1_at	Dr.132528	similar to microfilament and actin filament cross-linker protein	-	up	up	-	-
Dr.16652.1.S1_at	Dr.75158	hypothetical LOC562726	-	-	up	-	-
Dr.10476.1.A1_at	Dr.75427	hypothetical LOC567461	down	-	-	-	-
Dr.1964.1.A1_at	Dr.132290	hypothetical LOC569591	up	up	up	-	-
Dr.96.1.A1_at	Dr.107751	similar to complement protein component C7-1	-	-	up	-	-
Dr.17464.1.A1_at	Dr.74715	hypothetical protein LOC571260	-	-	up	-	-
Dr.26268.1.A1_at	Dr.87685	similar to putative scavenger receptor MARCO	-	up	up	-	-
Dr.17059.1.S1_at	Dr.104967	similar to meningioma-expressed antigen 5	-	up	up	up	-
Dr.16658.1.S1_at	Dr.143695	hypothetical protein LOC792055	-	-	up	-	-
Dr.16095.1.S1_at	Dr.16095	insulin-like growth factor binding protein-1b	-	-	up	up	-
Dr.10442.1.A1_at	Dr.117624	makorin, ring finger protein, 1 /// similar to Makorin RING zinc-finger	-	-	up	-	-
Dr.15836.1.A1_at	Dr.77432	lyric-like	-	-	up	-	-
Dr.15836.2.A1_at	Dr.77432	lyric-like	-	-	up	-	-
Dr.608.1.A1_at	Dr.75475	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B	-	up	-	-	-
Dr.16754.1.S1_at	Dr.86078	mitogen-activated protein kinase kinase kinase 7 interacting protein 1	-	-	up	-	-
Dr.17570.1.S1_at	Dr.116082	MAP kinase-interacting serine/threonine kinase 2b	-	up	up	-	-
Dr.23065.1.S1_at	Dr.81717	muscle, skeletal, receptor tyrosine kinase	-	up	-	up	-
Dr.16048.1.S1_at	Dr.78260	myelocytomatosis oncogene b	down	-	down	-	-
Dr.12684.1.S1_at	Dr.7230	nuclear factor (erythroid-derived 2)-like 2	down	-	-	-	-
Dr.3986.1.S1_at	Dr.77152	nuclear receptor subfamily 2, group F, member 2	-	up	up	-	-
Dr.11921.1.S1_at	Dr.11921	nuclear receptor subfamily 5, group A, member 5	-	up	up	-	up
Dr.10500.1.A1_at	Dr.76399	oxysterol binding protein-like 9	-	-	up	-	-
Dr.12233.1.S1_at	Dr.24504	poly A binding protein, cytoplasmic 1 a	-	up	up	up	up
Dr.763.1.A1_at	Dr.7296	protein phosphatase 1, catalytic subunit, beta isoform, like	-	up	up	-	up
Dr.19303.1.A1_at	Dr.86354	protein phosphatase 1, regulatory (inhibitor) subunit 13 like	-	-	up	-	-
Dr.17113.1.A1_a_at	Dr.143803	phosphatase and tensin homolog A	-	-	up	-	-
Dr.14305.1.A1_at	Dr.82680	sal-like 1a (Drosophila)	-	up	up	-	-

Dr.26405.1.S1_at	Dr.74531	syndecan 4 like	-	-	up	-	-
Dr.10320.1.S1_at	Dr.78523	serum/glucocorticoid regulated kinase 1	down	-	-	-	down
Dr.7568.1.A1_at	Dr.78433	si:ch211-132p20.4	down	-	-	down	down
Dr.22971.1.A1_at	Dr.81479	si:ch211-235e18.3	down	-	-	down	down
Dr.17322.1.A1_at	Dr.17322	si:ch211-81a5.3	-	-	-	down	-
Dr.19979.1.S1_at	Dr.77248	si:dkey-261e22.2	-	-	up	-	up
Dr.24312.1.S1_at	Dr.82478	solute carrier family 16 (monocarboxylic acid transporters), member 1	-	-	up	-	-
Dr.20663.1.S1_at	Dr.76663	solute carrier family 25, member 33	down	-	-	down	down
Dr.12410.1.S1_at	Dr.32057	sprouty (Drosophila) homolog 4	-	up	-	-	-
Dr.4713.1.S1_at	Dr.75888	staufen, RNA binding protein, homolog 2 (Drosophila)	-	-	up	-	-
Dr.8233.1.S1_at	Dr.8233	T-box 2b	down	-	-	down	down
Dr.10250.1.A1_at	Dr.10250	L-threonine dehydrogenase	-	up	up	down	down
Dr.578.2.S1_a_at	Dr.32598	thyrotroph embryonic factor	-	up	-	-	-
Dr.12632.1.S1_at	Dr.78052	transcription factor binding to IGHM enhancer 3a	-	up	up	-	-
Dr.5123.2.S1_at	Dr.77214	tight junction protein 2b (zona occludens 2)	-	-	-	-	-
Dr.18844.1.S1_at	Dr.18844	T-cell leukemia, homeobox 1	-	-	up	-	-
Dr.4590.1.A1_at	Dr.79310	transducer of ERBB2, 1a	down	-	-	-	down
Dr.2710.1.S1_at	Dr.2710	Similar to target of myb1 (chicken)	-	up	up	-	-
Dr.4186.1.S1_at	Dr.4186	tumor protein D52-like 2	-	-	up	-	-
Dr.17866.1.S1_at		wu:fa12f04	-	-	up	-	-
Dr.17775.1.A1_at	Dr.76322	wu:fa99c11	-	-	up	up	-
Dr.4738.1.A1_at		wu:fb12g02	-	-	up	-	-
Dr.3479.1.A1_at		wu:fb37e01	up	up	up	-	-
Dr.2518.1.A1_at		wu:fb51f10	-	-	up	-	-
Dr.3649.1.A1_at		wu:fb76b09	-	-	up	-	-
Dr.11357.1.S1_at		wu:fc17e06	-	-	up	-	-
Dr.17714.1.S1_at		wu:fc55g02	-	up	up	-	-
Dr.5309.1.A1_at	Dr.76654	wu:fc66f06	-	-	up	-	-
Dr.22087.1.A1_at		wu:fd12f01	-	up	up	-	-
Dr.6952.1.A1_at		wu:fe25f07	-	up	-	-	-
Dr.24042.1.A1_at		wu:fj17e06	-	-	up	-	-
Dr.7230.1.A1_at		wu:fj67e03	-	up	up	-	-

Dr.9920.1.A1_at		wu:fk63e10	-	-	up	up	-
Dr.3139.1.S1_at	Dr.76498	zinc finger protein 36, C3H type-like 2	-	up	-	-	-
Dr.4213.1.S1_at	Dr.140544	zgc:111986	-	-	up	-	-
Dr.25598.1.A1_at	Dr.75936	zgc:114051	up	up	up	-	-
Dr.2204.1.A1_at	Dr.77881	zgc:136474	-	up	up	-	-
Dr.3936.1.A1_at	Dr.106159	zgc:162730	-	up	up	-	-
Dr.14046.1.S1_at	Dr.75179	zgc:55886	-	up	up	-	-
Dr.8025.1.S1_at	Dr.115955	zgc:56324	-	up	up	-	-
Dr.9112.1.S1_at	Dr.77889	zgc:63976	-	-	up	-	-
Dr.25776.1.S1_at	Dr.85899	zgc:63987	-	up	up	-	-
Dr.5325.1.A1_at	Dr.4035	zgc:65870	-	up	up	up	-
Dr.701.1.S1_at	Dr.114995	zgc:77155	-	up	up	-	up
Dr.3518.1.A1_at	Dr.140561	zgc:77551	-	-	up	-	-
Dr.15501.1.S1_at	Dr.15501	zgc:85866	-	up	-	-	-
Dr.10045.1.S1_at	Dr.76599	zgc:86598	-	-	up	-	-
Dr.11473.2.S1_at	Dr.132698	zgc:92578	-	-	up	-	up
Dr.1593.1.A1_at	Dr.77305	Transcribed locus	-	-	up	-	-
Dr.16319.1.A1_at	Dr.16319	Transcribed locus	up	up	up	up	-
Dr.16387.1.A1_at	Dr.123601	Transcribed locus	-	-	up	-	-
Dr.18657.1.S1_at	Dr.86964	Transcribed locus	-	up	up	-	-
Dr.19004.1.S1_at	Dr.133759	Transcribed locus	-	-	up	-	-
Dr.26107.1.A1_at			-	up	up	-	-
Dr.26218.1.A1_at	Dr.134514	Transcribed locus	-	-	-	up	-
Dr.26372.1.A1_at	Dr.122837	Transcribed locus	-	-	up	-	up
Dr.9217.1.A1_at	Dr.105658	Transcribed locus	-	-	up	up	-

(D) Cluster 4

Probe Set ID	Unigene ID	Gene Title	Cu	Ca	Na	CaCu	NaCu
Dr.3910.1.S1_at	Dr.107967	acyl-Coenzyme A dehydrogenase family, member 8	-	-	-	-	down
Dr.19974.1.S1_at	Dr.76282	Rho GDP dissociation inhibitor (GDI) gamma	up	-	-	-	-
Dr.9277.1.S1_at	Dr.67666	arrestin domain containing 3	up	-	up	up	-

Dr.7102.1.S1_at	Dr.78272	chromosome 20 open reading frame 149, like	up	up	up	up	up
Dr.5504.1.S1_at	Dr.76887	cystathionine-beta-synthase	-	-	-	down	down
Dr.20083.1.A1_at	Dr.21399	cyclin G2	-	-	-	-	down
Dr.10688.1.S1_at	Dr.105878	cyclin-dependent protein kinase 5	-	-	-	down	down
Dr.23612.1.A1_at	Dr.41241	cadherin EGF LAG seven-pass G-type receptor 1a	up	-	-	-	-
Dr.9.1.S1_at	Dr.75198	hypothetical LOC563969	up	up	up	-	-
Dr.3560.1.A1_at	Dr.76994	cystathionase (cystathionine gamma-lyase)	up	-	-	down	down
Dr.10070.1.A1_at	Dr.10070	fructose-1,6-bisphosphatase 1, like	-	-	up	-	-
Dr.25520.1.A1_at	Dr.84666	F-box protein 25	up	-	-	-	-
Dr.1544.1.A1_at	Dr.17618	glutamic-oxaloacetic transaminase 2a	-	-	-	-	down
Dr.17145.1.S1_at	Dr.85820	potassium channel tetramerisation domain containing 12.1	-	-	-	down	down
Dr.1368.4.A1_at	Dr.117367	hypothetical protein LOC100005355	up	-	up	-	-
Dr.16935.1.A1_a_at	Dr.16935	Hypothetical LOC558832	up	-	up	-	-
Dr.4661.1.A1_at	Dr.132403	hypothetical LOC559003	-	-	-	-	down
Dr.6172.1.A1_at	Dr.81026	hypothetical LOC567192	up	-	-	-	-
Dr.3974.1.A1_at	Dr.86160	similar to PAM	-	up	up	-	-
Dr.12107.1.A1_at	Dr.12107	N-myc downstream regulated gene 1 /// hypothetical protein LOC792085	up	-	-	-	-
Dr.24726.1.S1_at	Dr.77225	microtubule-associated protein 1 light chain 3 beta	up	-	up	-	-
Dr.23324.1.A1_at	Dr.81778	matrix metalloproteinase 14 (membrane-inserted) beta	-	-	up	-	-
Dr.9142.1.S1_at	Dr.77491	myosin, heavy polypeptide 10, non-muscle	-	-	up	-	-
Dr.2973.1.A1_at	Dr.2973	neutrophil cytosolic factor 1	up	-	up	-	-
Dr.23465.1.S1_at	Dr.76653	neuroepithelial cell transforming gene 1	up	up	-	-	-
Dr.3216.1.A1_at	Dr.105888	nucleoside phosphorylase	down	-	-	down	down
Dr.26132.1.S1_at	Dr.37928	phosphoenolpyruvate carboxykinase 1 (soluble)	down	up	down	down	down
Dr.1519.1.S1_at	Dr.116775	RAB6A, member RAS oncogene family	-	-	-	down	-
Dr.13041.1.A1_at	Dr.108840	retinol dehydrogenase 12, like	up	-	-	-	down
Dr.14097.1.S1_at	Dr.33203	Similar to Myelin basic protein	up	-	-	-	-
Dr.18513.2.S1_a_at	Dr.82774	saccharopine dehydrogenase b	-	-	up	-	-
Dr.26392.1.A1_at	Dr.74531	syndecan 4 like	-	-	up	-	-
Dr.11588.1.S1_at	Dr.117332	sideroflexin 2	-	-	-	-	down
Dr.9611.1.A1_at	Dr.107310	si:ch211-241e15.2	up	-	-	-	-
Dr.20551.1.A1_at	Dr.41221	si:dkey-222b8.2	-	-	-	-	down

Dr.2788.1.S1_at	Dr.76517	si:dkeyp-86b9.2	-	-	-	-	down
Dr.4948.1.A1_at	Dr.77323	solute carrier family 26, member 5	up	up	up	-	-
Dr.4351.1.A1_at	Dr.105401	striatin, calmodulin binding protein 3	-	-	-	-	down
Dr.578.1.A1_at	Dr.32598	thyrotroph embryonic factor	-	-	-	down	-
Dr.20029.1.A1_at	Dr.33137	tissue factor pathway inhibitor a	up	up	up	-	-
Dr.12134.2.A1_at	Dr.104473	wu:fb34a04	-	-	-	-	down
Dr.8747.1.A1_at		wu:fc16h10	up	up	up	-	-
Dr.1854.1.A1_at		wu:fc96c10	down	-	-	down	down
Dr.23743.1.S1_at	Dr.72340	wu:fj58g06	-	-	-	-	down
Dr.7226.1.S1_at		wu:fj65h10	-	-	-	down	-
Dr.9564.1.A1_at		wu:fj88f05	-	-	-	-	down
Dr.7535.1.A1_at		wu:fp56f09	-	-	-	-	down
Dr.11661.1.S1_at	Dr.114303	yippee-like 3	up	-	up	-	-
Dr.5605.4.S1_at	Dr.104496	zgc:123210	up	-	-	-	-
Dr.24907.1.A1_at	Dr.33774	zgc:136656	up	-	-	-	-
Dr.7099.2.A1_at	Dr.81135	zgc:153424	-	-	-	-	down
Dr.2433.2.A1_at	Dr.37700	zgc:153863	-	down	-	-	down
Dr.10914.1.A1_at	Dr.10914	zgc:154020	up	up	-	up	-
Dr.12072.1.A1_at	Dr.123501	zgc:154085	-	-	up	-	-
Dr.18410.1.S1_at	Dr.18410	zgc:56136	up	-	up	up	-
Dr.12459.1.A1_at	Dr.82369	zgc:63561	-	-	-	-	down
Dr.13966.1.S1_at	Dr.116215	zgc:63767	up	-	up	-	-
Dr.26476.1.S1_at	Dr.88906	zgc:64119	up	up	up	-	-
Dr.3873.1.A1_at	Dr.76245	zgc:73347	up	-	up	-	-
Dr.23613.1.A1_at	Dr.23613	zgc:77033	-	-	-	-	down
Dr.5385.1.A1_at	Dr.77527	zgc:77259	-	-	up	-	-
Dr.20525.1.A1_a_at	Dr.79390	zgc:77868	up	-	-	-	-
Dr.5399.1.S1_at	Dr.32015	zgc:91854	-	-	-	-	down
Dr.14577.1.A1_at	Dr.83602	zgc:92090	-	-	-	-	down
Dr.11062.1.A1_at	Dr.82842	zgc:92294	up	-	-	-	-
Dr.12194.1.A1_at	Dr.42256	Transcribed locus	up	-	-	-	-
Dr.12232.1.A1_at	Dr.83076	CDNA clone IMAGE:7250984	-	-	-	down	down

Dr.13160.1.A1_at	Dr.83987	Transcribed locus	up	-	-	-	-
Dr.15287.1.S1_at	Dr.143198	Transcribed locus, Lsm3 protein [Homo sapiens]	-	-	-	-	down
Dr.17.1.A1_at	Dr.17	Transcribed locus	up	-	up	-	-
Dr.18025.1.A1_at	Dr.18025	Transcribed locus, cAMP responsive element binding protein 1	-	-	up	-	-
Dr.18880.1.A1_at	Dr.84419	Transcribed locus	up	-	-	-	-
Dr.5569.1.S1_at	Dr.5569	Transcribed locus	up	up	up	-	-
Dr.7870.1.A1_at	Dr.7870	Transcribed locus	up	-	up	up	-
Dr.906.1.S1_at	Dr.82153	Transcribed locus	up	-	-	-	-

Table A.5: Unique (A) and shared (B) effects of Ca and Na in the presence of Cu compared to copper alone indicating the significant changes to gene expression in the liver of zebrafish. Na or Ca associated Cu exposure either enhanced or prevented the expression of genes compared to Cu alone, and the direction of regulation is indicated. Fold change is based upon change in expression from Ctrl values. The gene list was compiled based on a >2 difference in gene expression.

(A)

Probe Set ID	Fold Change Cu	Fold Change CaCu	Effect	Regulation	Gene Title
Dr.813.1.S1_at	-1.4	-3.8	Enhanced	Decrease	acetyl-CoA acetyltransferase 2
Dr.16095.1.S1_at	1.7	4.5	Enhanced	Increase	insulin-like growth factor binding protein-1b
Dr.23065.1.S1_at	1.3	5.0	Enhanced	Increase	muscle, skeletal, receptor tyrosine kinase
Dr.9920.1.A1_at	1.5	3.5	Enhanced	Increase	wu:fk63e10
Dr.5325.1.A1_at	1.6	3.8	Enhanced	Increase	zgc:65870
Dr.9277.1.S1_at	6.6	3.0	Prevented	Decrease	si:ch211-235e18.3
Dr.22971.1.A1_at	-5.0	-2.7	Prevented	Increase	arrestin domain containing 3
Dr.9611.1.A1_at	3.7	-1.3	Prevented	Increase	si:ch211-241e15.2
Dr.2871.1.A1_at	4.3	1.0	Prevented	Increase	similar to Tmc4 protein

Probe Set ID	Fold Change Cu	Fold Change NaCu	Effect	Regulation	Gene Title
Dr.10688.1.S1_at	-1.1	-3.4	Enhanced	Decrease	cyclin-dependent protein kinase 5
Dr.1603.1.A1_at	-2.8	-5.0	Enhanced	Decrease	Cytochrome P450, family 51
Dr.17283.1.A1_at	-2.4	-4.5	Enhanced	Decrease	hypothetical LOC558794
Dr.3216.1.A1_at	-2.5	-6.4	Enhanced	Decrease	nucleoside phosphorylase
Dr.9564.1.A1_at	-1.1	-3.3	Enhanced	Decrease	wu:fj88f05
Dr.9746.8.S1_at	1.0	-3.1	Enhanced	Decrease	zgc:86706
Dr.5399.1.S1_at	1.0	-3.1	Enhanced	Decrease	zgc:91854
Dr.9277.1.S1_at	6.6	2.0	Prevented	Increase	arrestin domain containing 3
Dr.2871.1.A1_at	4.3	-1.7	Prevented	Increase	similar to Tmc4 protein
Dr.2433.2.A1_at	1.3	-3.5	Prevented	Increase	zgc:153863

Dr.13966.1.S1_at	3.2	1.1	Prevented	Increase	zgc:63767
Dr.14577.1.A1_at	1.6	-7.6	Prevented	Increase	zgc:92090

(B)

Probe Set ID	Fold Change Cu	Fold Change CaCu	Fold Change NaCu	Effect	Regulation	Gene Title
Dr.21698.1.A1_at	-2.4	-12.1	-8.0	Enhanced	Decrease	CDNA clone IMAGE:6034266
Dr.12232.1.A1_at	-1.3	-4.7	-5.3	Enhanced	Decrease	CDNA clone IMAGE:7250984
Dr.1603.1.A1_x_at	-2.9	-5.3	-8.3	Enhanced	Decrease	Cytochrome P450, family 51
Dr.4267.1.A1_at	-2.4	-5.0	-4.8	Enhanced	Decrease	hypothetical LOC558794
Dr.6321.1.A1_at	-1.8	-7.9	-5.0	Enhanced	Decrease	hypothetical LOC559001
Dr.13321.1.S2_at	-1.4	-4.1	-3.5	Enhanced	Decrease	hypoxia induced gene 1
Dr.3698.1.A1_at	-2.0	-7.6	-21.3	Enhanced	Decrease	wu:fd02f07
Dr.7168.1.S1_at	-2.0	-5.6	-5.9	Enhanced	Decrease	wu:fd56d05
Dr.13284.1.A1_at	-4.2	1.2	-1.2	Prevented	Decrease	choline kinase alpha
Dr.13284.2.S1_at	-4.1	1.1	-1.3	Prevented	Decrease	choline kinase alpha
Dr.10717.1.S2_at	-9.0	1.8	2.4	Prevented	Decrease	estrogen receptor 1
Dr.10717.1.S1_at	-7.8	2.2	2.8	Prevented	Decrease	estrogen receptor 1
Dr.8000.1.S1_at	5.9	1.3	-1.3	Prevented	Decrease	insulin induced gene 1
Dr.6064.1.A1_at	14.6	3.2	1.5	Prevented	Decrease	myelocytomatosis oncogene b
Dr.19560.1.S2_at	-4.1	-1.8	-1.9	Prevented	Decrease	solute carrier family 43, member 1
Dr.16048.1.S1_at	-4.2	-1.3	-1.6	Prevented	Decrease	zgc:113054
Dr.2973.1.A1_at	5.2	1.7	-1.1	Prevented	Increase	glutathione peroxidase 1b
Dr.11296.1.S1_at	-4.3	1.2	1.3	Prevented	Increase	hypothetical protein LOC100000433
Dr.11310.1.S1_at	4.0	1.5	1.3	Prevented	Increase	neutrophil cytosolic factor 1
Dr.23582.1.A1_at	3.6	1.2	-1.0	Prevented	Increase	tubulin, alpha 1
Dr.1659.1.A1_at	1.5	-4.2	-3.8	Prevented	Increase	wu:fb13f03
Dr.8914.1.S1_at	-3.4	1.1	1.2	Prevented	Increase	wu:fc02a12
Dr.20008.2.A1_at	9.2	1.6	-1.2	Prevented	Increase	zgc:92061

Table A.6: Unique (A) and shared (B) effects of Ca and Na in the presence of Cu compared to the respective ion alone indicating the significant changes to gene expression in the liver of zebrafish. Na or Ca either enhanced or prevented the expression of genes compared to lone ion exposure, and the direction of regulation is indicated. The gene list was compiled based on a >2-fold difference in gene expression.

(A)

Probe Set ID	Fold Change Na	Fold Change NaCu	Effect	Regulation	Gene Title
Dr.5504.1.S1_at	-1.3	-3.4	Enhanced	Decrease	cystathionine-beta-synthase
Dr.3216.1.A1_at	-1.5	-6.4	Enhanced	Decrease	nucleoside phosphorylase
Dr.6847.1.S1_a_at	1.7	3.8	Enhanced	Decrease	zgc:153863
Dr.2433.2.A1_at	-1.2	-3.5	Enhanced	Increase	solute carrier family 31 (copper transporters), member 1
Dr.2022.1.A1_at	2.1	-4.8	Prevented	Decrease	Cytochrome P450, family 51
Dr.9277.1.S1_at	4.7	2.0	Prevented	Decrease	Cytochrome P450, family 51
Dr.535.2.A1_at	6.4	3.6	Prevented	Decrease	fatty acid binding protein 7, brain, a
DrAffx.2.58.A1_at	3.9	-1.1	Prevented	Decrease	solute carrier family 43, member 1
Dr.1603.1.A1_x_at	-13.5	-8.3	Prevented	Decrease	sterol-C4-methyl oxidase-like
Dr.1603.1.A1_at	-10.1	-5.0	Prevented	Decrease	Transcribed locus
Dr.15261.1.A1_at	3.1	1.1	Prevented	Decrease	zgc:73223
Dr.20850.1.S1_at	-5.3	-1.8	Prevented	Increase	arginase, type II
Dr.24246.1.S1_at	6.1	1.6	Prevented	Increase	arrestin domain containing 3
Dr.14662.1.S1_at	4.0	1.8	Prevented	Increase	carboxyl ester lipase, like
Dr.16652.2.A1_at	3.6	1.2	Prevented	Increase	cytochrome P450, family 24, subfamily A, polypeptide 1, like
Dr.25331.1.S1_at	4.1	1.4	Prevented	Increase	cytoplasmic polyadenylation element binding protein 4
Dr.8587.1.A2_at	4.3	-1.0	Prevented	Increase	H1 histone family, member X
Dr.16095.1.S1_at	3.4	-1.1	Prevented	Increase	HNF1 homeobox b
Dr.374.1.S1_at	3.3	1.1	Prevented	Increase	hypothetical LOC562726
Dr.7722.1.A1_at	5.6	2.0	Prevented	Increase	hypothetical LOC567732
Dr.96.1.A1_at	4.7	1.5	Prevented	Increase	insulin-like growth factor binding protein 1
Dr.2710.1.S1_at	3.5	1.3	Prevented	Increase	insulin-like growth factor binding protein-1b
Dr.4948.1.A1_at	4.1	1.3	Prevented	Increase	interferon regulatory factor 2 binding protein 2
Dr.11296.1.S1_at	-3.4	1.3	Prevented	Increase	similar to complement C3-H1

Dr.12110.1.S1_at	-4.5	-2.4	Prevented	Increase	similar to complement protein component C7-1
Dr.23925.1.A1_at	-4.5	-1.0	Prevented	Increase	Similar to target of myb1 (chicken)
Dr.18657.1.S1_at	3.3	-1.2	Prevented	Increase	solute carrier family 26, member 5
Dr.16319.1.A1_at	4.4	1.4	Prevented	Increase	Transcribed locus
Dr.9217.1.A1_at	5.5	1.3	Prevented	Increase	Transcribed locus
Dr.17775.1.A1_at	5.0	1.2	Prevented	Increase	Transcribed locus
Dr.3479.1.A1_at	4.3	1.4	Prevented	Increase	wu:fa99c11
Dr.22087.1.A1_at	3.7	-1.3	Prevented	Increase	wu:fb37e01
Dr.9920.1.A1_at	7.4	1.4	Prevented	Increase	wu:fd12f01
Dr.25598.1.A1_at	3.7	1.7	Prevented	Increase	wu:fk63e10
Dr.5325.1.A1_at	3.6	1.4	Prevented	Increase	zgc:114051
Dr.25285.1.S1_at	-4.6	-1.6	Prevented	Increase	zgc:65870
Dr.14577.1.A1_at	1.8	-7.6	Prevented	Increase	zgc:92090

Probe Set ID	Fold Change Ca	Fold Change CaCu	Effect	Regulation	Gene Title
Dr.21698.1.A1_at	-8.9	-12.1	Enhanced	Decrease	CDNA clone IMAGE:6034266
Dr.6321.1.A1_at	-2.6	-7.9	Enhanced	Decrease	hypothetical LOC559001
Dr.13321.1.S2_at	-1.6	-4.1	Enhanced	Decrease	hypoxia induced gene 1
Dr.7168.1.S1_at	-3.3	-5.6	Enhanced	Decrease	wu:fd56d05
Dr.9920.1.A1_at	1.2	3.5	Enhanced	Increase	wu:fk63e10
Dr.10717.1.S2_at	-3.9	1.8	Prevented	Decrease	estrogen receptor 1
Dr.16095.1.S1_at	-1.3	4.5	Prevented	Decrease	insulin-like growth factor binding protein-1b
Dr.3936.1.A1_at	3.9	1.3	Prevented	Increase	zgc:162730
Dr.26476.1.S1_at	3.8	1.6	Prevented	Increase	zgc:64119
Dr.20008.2.A1_at	5.4	1.6	Prevented	Increase	zgc:92061

(B)

Probe Set ID	Fold Change Na	Fold Change Ca	Fold Change NaCu	Fold Change CaCu	Effect	Regulation	Gene Title
Dr.12232.1.A1_at	-1.4	-1.5	-5.3	-4.7	Enhanced	Decrease	CDNA clone IMAGE:7250984
Dr.3698.1.A1_at	-17.4	-4.7	-21.3	-7.6	Enhanced	Decrease	wu:fd02f07
Dr.2051.1.S1_at	-17.2	-9.3	-5.3	-7.2	Prevented	Decrease	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
Dr.21005.1.S1_s_at	4.3	3.5	1.3	1.3	Prevented	Increase	complement component c3b /// complement component c3c
Dr.25208.1.A1_at	6.2	3.5	1.0	1.4	Prevented	Increase	cytochrome P450, family 3, subfamily A, polypeptide 65
Dr.25208.2.S1_at	6.8	4.1	1.3	1.4	Prevented	Increase	cytochrome P450, family 3, subfamily A, polypeptide 65
Dr.7171.2.S1_a_at	5.3	4.5	1.9	1.5	Prevented	Increase	glutamic pyruvate transaminase (alanine aminotransferase) 2
Dr.17570.1.S3_at	6.0	4.1	1.8	1.1	Prevented	Increase	MAP kinase-interacting serine/threonine kinase 2b
Dr.26268.1.A1_at	5.9	5.0	1.5	1.9	Prevented	Increase	similar to putative scavenger receptor MARCO