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Copper Toxicity and Accumulation: Physiology, Chemistry, and Molecular Biology

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UNIVERSITY OF MIAMI

COPPER TOXICITY AND ACCUMULATION ACROSS SALINITIES: PHYSIOLOGY, CHEMISTRY, AND MOLECULAR BIOLOGY

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Our knowledge of aquatic Cu toxicity has increased greatly over the past several years culminating with the incorporation of a model (the Biotic Ligand Model (BLM)) into the regulatory framework which allows for the site specific adjustment of water quality criteria based on water chemistry. However, our understanding of Cu toxicity in the aquatic environment is limited mostly to freshwater (FW). Because of this limited knowledge, this dissertation set out to examine the affect of salinity on Cu toxicity and accumulation across salinities from FW to sea water (SW).

First, this work examined tissue specific Cu accumulation in five salinities (FW, 5 ppt, 11 ppt, 20 ppt, 28 ppt) from waterborne Cu exposure at two [Cu] (30 and 150 μg Cu L^{-1}) in the euryhaline killifish, *Fundulus heteroclitus*. Branchial and hepatic accumulation followed a pattern that would be expected based on speciation and competition from cations. [Cu] were high in FW and decreased as salinity increased. However, in the intestine, [Cu] were highest at 5 ppt and were also elevated in the higher salinities. The elevation at the higher salinities was most likely due to drinking by the fish which increases as salinity increases above the isoosmotic point of the fish (~10 ppt) for osmoregulatory purposes and showed a trend toward increasing [Cu] with increasing salinity as would be expected.

Secondly, the mechanism of Cu toxicity in FW and SW was examined in killifish. The mechanism of Cu toxicity in the killifish in FW was the same as had been seen for other FW fish. Cu exposure caused a decrease in Na^+ / K^+ ATPase activity which led to a decrease in whole body [Na^+] which is the likely cause of death. In SW, surprisingly no ionoregulatory disturbances were observed. The only measured parameter that was changed in SW was net ammonia which showed a substantial decrease. Therefore, the mechanism of acute copper toxicity in FW and SW differed suggesting that physiology may need to be considered in future development of a BLM for SW.

Next, the effect of salinity on Cu accumulation from a naturally incorporated dietary source was examined in FW and SW in *Fundulus heteroclitus*. Cu accumulation was not seen to differ in the two salinities in spite of differences in gut fluid chemistry that would lead to an ~11 fold difference in free Cu ion between FW and SW. This indicated that Cu accumulation from a dietary source was not as the free ion but most likely as organically (amino acid) bound Cu. In addition to this, subcellular fractionation of the diet revealed that Cu available to the next trophic level did not change with increasing waterborne [Cu], increasing tissue [Cu], or time in oysters which has implications for Cu accumulation in the environment.

Finally, the molecular mechanisms behind effects and acclimation to waterborne and dietary Cu exposure in FW and SW were examined using suppression subtraction hybridization in the killifish intestine. Exposure to Cu induced a stress response which could be responsible for upregulation of genes involved in protein synthesis, proteolysis and ATP production. At the tissue level, two main responses were observed. First, genes necessary for muscle function were upregulated potentially in response to previously

observed decreased intestinal motility. Secondly, apoptotic genes were upregulated corresponding to increased rates of apoptosis in intestines during dietary exposures. At the cellular level, metal chelators and oxidative stress genes were upregulated in response to increased free Cu in the cell and the subsequent free metal induce free radical formation.

This work has advanced our understanding of Cu toxicology in saline environments and emphasized the importance of considering both chemistry and physiology in analyzing and interpreting Cu toxicology especially in saline environments. Hopefully, it will contribute to the future development of Cu water quality criteria.

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CHAPTER 1

INTRODUCTION

1.1 Copper and United States environmental regulation

The United States Environmental Protection Agency (EPA) is required to regulate [Cu] in aquatic ecosystems due to its toxicity. It accomplishes this through a variety of means in both fresh (FW) and sea water (SW) including the establishment of water quality criteria to regulate the allowable [Cu] in the environment (USEPA, 2002; USEPA 2003). In addition, to this more sensitive monitoring endpoints are always being sought in order to identify areas that are impacted by Cu contamination. Biomarkers, such as genes or proteins that change their level of expression in response to low doses of a particular contaminant, are highly prized as sublethal indicators of pollution (Fowle and Sexton, 1992; van der Oost et al. 2003). However, Cu is an interesting toxicant as it is not only toxic but also necessary for life (Uauy et al., 1998). Because of this, all organisms have developed systems to obtain necessary amount of Cu and to excrete excess Cu (Harris, 2000; Peña et al. 1999; Uauy et al., 1998).

In SW, criteria calculations are developed from waterborne Cu exposure data which use mortality as their endpoint (USEPA, 2003). From these studies, a distribution based on the genus mean acute toxicity values (the mean concentration at which 50% of the organisms within a genus die) is established. The fifth percentile of this distribution is calculated yielding a final acute value of 12.3 µg Cu L^{-1} which was lowered to 6.19 µg Cu L^{-1} in order to protect commercially important oysters which were more sensitive. This value was then divided by two to provide additional protection yielding the acute

criteria value of 3.1 μg Cu L^{-1}. This criteria value is used for all salinities greater than 11 ppt. At salinities from 1-10 ppt, either the FW or the SW criteria value is used depending on which is lower. The limitation of this approach is that it does not consider detailed variations in water chemistry over a wide range of salinities.

In FW, variations in water chemistry have been taken into account through the incorporation of the Biotic Ligand Model (BLM) (Paquin et al., 2002; USEPA, 2003, Hydroqual, 2005). The BLM is a mathematical model that can predict the concentrations at which 50% of the organisms die (LC 50) for Cu based on the water chemistry of the site. It has been implemented to reduce the need for water effect ratio testing which will reduce the need to use animals for developing site specific water quality criteria. Instead, using pH, dissolved organic cabon (DOC), percent humic acid, temperature, major cations, major anions, dissolved inorganic carbon (DIC), and sulfide measured in the site waters, it predicts the LC 50 for the appropriate test organism: fat head minnows (*Pimephales promelas*), *Ceriodaphnia dubia, Daphnia pulex,* or *Daphnia magna.*

The BLM estimations are based on calibration tests in which short term Cu binding to the biotic ligand was correlated to 96 hour LC 50 values (Hydroqual, 2005). It assumes that the free ion (Cu^{2+}) and a fraction of the $CuOH^{+}$ are available for binding to the biotic ligand. Speciation calculations are used to determine the amounts of copper available in these forms and take into account binding to other ligands (DOC, CO_3^{2-}, etc.). It also estimates the binding of other cations to the biotic ligand and therefore reduces the predicted amount of Cu bound to the biotic ligand due to this competition. In this manner, taking into account both speciation and competition, and obviously Cu

concentration, the model calculates the Cu burden on the biotic ligand and estimates an LC 50 based on the calibration tests. Thus in FW, the criteria can be adjusted based on water chemistry.

However, both the SW and FW criteria are based on waterborne Cu exposure and dietary Cu exposure is neglected (USEPA, 2003). This may lead to severe under-protection of organisms in aquatic systems. Researchers have recently demonstrated that at [Cu] below the water quality criteria dietary Cu exposure can have an effect on an organism's (Hook and Fisher, 2001; Bielmeyer et al., 2006) reproductive output in *Acartia tonsa* after feeding on an algal diet exposed to Cu for seven days. It found that the EC 20 (the concentration at which there was a 20% reduction in reproduction) was 1.5 µg Cu L^{-1} which is below both the acute (3.1 µg Cu L^{-1}) and chronic (1.9 µg Cu L^{-1}) criteria values (USEPA, 2003).

1.2 Copper and physiology

In addition to water chemistry and the influence of diet, physiology may play a prominent role in Cu toxicology. In FW fish, Cu has been demonstrated to decrease branchial Na^+ uptake by inhibiting the basolateral Na^+ / K^+ ATPase (Laurén and McDonald, 1987a; Laurén and McDonald, 1987b). This causes an ionoregulatory disturbance that leads to the death of the fish. In addition to the decrease in Na^+ uptake, Cl^- uptake, which exacerbates the ionoregulatory disturbance, and NH_3 excretion are inhibited (Laurén and McDonald, 1985; Wilson and Taylor, 1993; Grosell et al., 2002 Grosell et al., 2004a).

The mechanism of Cl^- inhibition has yet to be elucidated however it may be due to the inhibition of carbonic anhydrase (CA). CA catalyzes the hydration of CO_2 to form H^+ and HCO_3^- and has been demonstrated to be inhibited in the gills of crustaceans exposed to waterborne Cu (Vitale et al., 1999). The HCO_3^- generated through this catalysis is used for apical Cl^-/HCO_3^- exchange and any decrease in branchial $[HCO_3^-]$ will lead to a decrease in Cl^- uptake (Marshall and Grosell, 2005)..

The hypothesized CA inhibition will also affect two other processes: Na^+ uptake and NH_3 excretion. Na^+ uptake will be inhibited because of a reduction in the amount of H^+ available to exchange for Na^+ at the apical Na^+/H^+ exchanger or to export from the gill by the apical proton pump providing energy for Na^+ uptake via the Na^+ channel (Marshall and Grosell, 2005). The lack of H^+ extrusion will also effect NH_3 excretion because gill boundary layer acidification is necessary for NH_3 excretion. NH_3 passively moves across the cell membrane of the gill where in the acidified boundary layer it becomes NH_4^+. This maintains a high outwardly directed NH_3 transepithelial gradient by reducing the amount of NH_3 in the boundary layer and prevents NH_3 from diffusing back into the cell because NH_4^+ cannot passively diffuse across the cell membrane (Wilson et al., 1994; Wilkie, 2002).

In SW, Cu has been observed to consistently raise plasma $[NH_3]$ (Wilson and Taylor, 1993; Grosell et al., 2004a). The only link between Cu toxicity and NH_3 excretion in SW is at the basolateral Na^+/K^+ ATPase where a portion of the K^+ transported is displaced by NH_4^+ to enter the cell and be excreted at the apical membrane (Wilkie, 2002). This process may be inhibited by Cu inhibiting the Na^+/K^+ ATPase (Laurén and McDonald, 1987a; Laurén and McDonald, 1987b). However, this is only a

small portion of the ammonia excretion as the majority is believed to diffuse passively out of the fish as either NH_4^+ through the shallow paracellular junctions or NH_3 (Wilkie, 2002). Therefore, the link between Cu and the disruption of ammonia excretion is unknown at this time.

The second effect often noted in most waterborne Cu studies in SW is an increase in plasma Na^+ and Cl^- (Grosell et al., 2004a; Wilson and Taylor, 1993; Larsen et al., 1997; Stagg and Shuttleworth, 1982a). This can be caused in two ways. The first is through a decrease in the excretion of both Na^+ and Cl^- which occurs at the gill in SW fish. Na^+ excretion relies on the basolateral Na^+/K^+ ATPase which has been shown to be inhibited by Cu in FW (Laurén and McDonald, 1987a; Laurén and McDonald, 1987b). Cl^- excretion relies in part on the Na^+/K^+ ATPase which keeps the intracellular Na^+ concentration low allowing for the proper functioning of the $Na^+/K^+/2\ Cl^-$ transporter (NKCC) (Marshall and Grosell, 2005). NKCC brings Cl^- into the cell from the blood and it accumulates above its equilibrium point with external Cl^- allowing for its excretion through an apical Cl^- channel.

Secondly, an increase in internal Na^+ and Cl^- could be the results of Cu causing the branchial epithelium to become leakier. It has been demonstrated at high [Cu] in FW that the epithelium will become more leaky presumably because of Cu^{2+} displacing Ca^{2+} in the tight junctions which leads to a greater loss of ions from the animal (Laurén and McDonald, 1985). In SW, the same could happen and therefore lead to an increase in the ion content of the plasma.

1.3 Biomarkers

Biomarkers are measurements of pollutants or their consequences which indicate exposure, effect, and/or susceptibility of an organism to a pollutant ((Fowle and Sexton, 1992; van der Oost et al. 2003). Exposure biomarkers, which include measurements of the pollutant or a secondary metabolite of the pollutant in an organism's tissues, indicate that an organism has been at some time in the past exposed to the pollutant of interest. Physiological, biochemical, and/or behavioral measurements that are indicative of exposure to a pollutant or class of pollutants (such as a decrease in plasma [Na^+] which can be indicative of acute Cu exposure) are considered to be effect biomarkers and demonstrate both exposure and potential toxic effects. Susceptibility biomarkers, such as uptake rates, can determine whether a population is more sensitive to a pollutant based on environmental, demographic, and/or genetic factors.

In all cases good biomarkers are specific and stable (Fowle and Sexton, 1992; van der Oost et al. 2003). They are specific in that they indicate exposure, effect, and/or susceptibility to a single compound or a small group of related compounds. There also should be a clear demonstrable connection between the pollutant and the biomarker. They are also stable in that they should not change with changing environmental conditions. An alternative to this is that the interaction between the environmental variable of interest and the change of the biomarker is well established for the both the biomarker alone and the biomarker in the presence of the pollutant.

1.4 Scope of the research

This dissertation examines Cu toxicity from waterborne and dietary exposures with increasing salinity from FW to SW. It considers both the effect of the environmental variables indicated to be important by the BLM as well as the physiological effects of the Cu on the organism. The experiments presented in chapters 2 and 3 aimed to demonstrate whether Cu accumulation and toxicity are dependant solely on speciation which the research leading to the BLM suggests or whether physiology may also have an effect upon them. In addition to this, chapter 4 is one of the first studies to look at Cu accumulation from a naturally incorporated dietary source. Finally attempts are reported, in chapter 5 to identify potential changes in mRNA expression that can be utilized as specific biomarkers of Cu exposure and perhaps even indicate the route of the exposure.

CHAPTER 2

THE EFFECTS OF SALINITY ON COPPER ACCUMULATION IN THE COMMON KILLIFISH, *FUNDULUS HETEROCLITUS*

2.1 Summary

Laboratory and field studies have demonstrated that salinity influences the accumulation of copper. This study is the first to examine the effect of salinity on copper accumulation in a teleost fish across a comprehensive range of salinity from freshwater to seawater. This was done in an effort to identify potential target tissues and differences in chemical interactions across salinities that will aid in the development of a sea water biotic ligand model (BLM) for copper. Killifish, *Fundulus heteroclitus* were acclimated to five salinities (0, 5, 11, 22, and 28 ppt) and exposed to three copper concentrations (nominal 0, 30, and 150 $\mu g\ L^{-1}$) yielding 15 treatment groups. Fish from each group were sampled for tissue copper analysis at 0, 4, 12, and 30 days post-exposure. Whole body and liver accumulation was highest at the low salinities. The liver accounted for 57 to 86% of the whole body copper even though it accounts for < 4% of the body mass. Similarly, the gill accumulated more copper at lower salinities while the intestine generally accumulated more copper at higher salinities. Speciation calculations indicate that $CuCO_3$ is likely to account for much of the accumulation possibly with some contributions from $CuOH^+$ and $Cu(OH)_2$. The free ion, Cu^{2+}, does not appear to be associated with copper accumulation. However, the differences in physiology and in the concentrations of competing cations across salinities suggest that speciation alone can not

explain accumulation. The findings presented in this study may have implications for future development of a BLM for saline environments by identifying potential target tissues.

2.2 Background

Copper is an essential yet potentially toxic element to all organisms (Harris, 2000; Uauy et al., 1998). It is used as a co-factor in several enzymes and several proteins have evolved to tightly regulate the distribution of copper in the cell (Harris, 2000; Peña et al. 1999; Uauy et al., 1998). In humans, failure to maintain the proper level of copper has been implicated in two diseases: Wilson's disease (excess) and Menke's syndrome (deficiency). In aquatic systems, copper accumulation and mechanisms of copper toxicity have been studied mostly in fresh water (FW) (Grosell et al., 2002; Paquin et al., 2002). In seawater (SW) little is known (Grosell et al., 2004a; Grosell et al., 2004b; Stagg and Shuttleworth, 1982a; Stagg and Shuttleworth, 1982b; Wilson and Taylor, 1993) and even less is known about copper accumulation and toxicity at intermediate salinities.

During aqueous exposures of FW fish, it has been established that copper is readily taken up by the gill where it inhibits the action of Na^+-K^+ ATPase (Grosell et al., 2002). This inhibition causes an osmoregulatory disturbance, as Na^+ is lost to the dilute external environment. A similar loss of Cl^- has been observed in fish adding to the osmoregulatory disturbance (Grosell et al., 2002). In SW fish, several authors have proposed that an osmoregulatory disturbance is also responsible for toxicity during waterborne exposures (Grosell et al., 2004a; Stagg and Shuttleworth, 1982a; Stagg and

Shuttleworth, 1982b; Wilson and Taylor, 1993). However, in SW a net gain of Na^+ is observed because the gill is important for the excretion of excess Na^+ gained from the concentrated external environment and copper appears to disturb this process. Fish must drink in SW because they constantly lose water through diffusion to the concentrated external environment (Karnaky, 1998). In drinking SW, aqueous copper can accumulate in the intestine (Grosell et al., 2004b), as it does with aqueous silver exposures (Grosell et al., 1999; Grosell and Wood, 2001).

This study examined how salinity affects the accumulation of copper and related copper accumulation back to physiology, competitive cations, and speciation as the BLM predicts that these factors are important for short-term accumulation (Paquin et al., 2002). The gill, intestine, liver, gall bladder, kidney, muscle, and carcass were examined in an effort to identify potential target tissues for copper accumulation and toxicity as outlined below.

It was expected that gill copper accumulation would be high at low salinities and decrease with increasing salinity as a consequence of speciation and/or competition. In contrast, the intestine was expected to contain higher concentrations of copper in the high salinities due to the increased drinking rate (Grosell et al., 1999; Karnaky, 1998; Wilson and Taylor, 1993). The liver was examined due to its importance in maintaining copper homeostasis and was expected to accumulate copper regardless of salinity (Peña et al., 1999). The liver may, therefore, serve as a good indicator of whole animal copper uptake and accumulation (Peña et al., 1999). The gall bladder was examined to evaluate the importance of biliary excretion of excess copper (Grosell et al., 1998; Peña et al., 1999). The kidney has been proposed as a potential site of copper accumulation and copper

toxicity in SW but not in FW, and therefore the copper concentration was expected to increase with increasing salinity (Stagg and Shuttleworth, 1998a). Finally, the muscle and carcass were not expected to exhibit changes in copper concentrations as the carcass and muscle previously have been shown to not accumulate copper during aqueous copper exposure (Grosell et al., 2004b; Kamunde et al., 2003).

As mentioned earlier, inorganic speciation of the metal is an important factor influencing the toxicity of a metal. The free ion, Cu^{2+}, has been best correlated with toxicity (Andrew et al., 1977; Howarth and Sprague, 1978; Paquin et al., 2002) although $CuOH^{+}$ may also be toxic Andrew et al., 1977; Howarth and Sprague, 1978). We expected that the accumulation on a whole body basis would be best correlated with the free ion due to its importance in toxicity.

Finally, it has been observed that along a salinity gradient in polluted environments, mollusks, and other invertebrates accumulated more copper in their tissues as salinity decreased (Bryan and Hummerstone, 1971; Huggett et al., 1975; Phillips, 1977; Wright and Zamuda, 1987). Accumulation was positively associated with free ion activity and inversely related to salinity (Wtight and Zamuda, 1987). The present study attempted to further elucidate the reasons for this salinity effect, in this case using a fish model, while at the same time verifying the effect of the free Cu^{2+} ion across a wider range of salinities. As such, this study is the first to examine copper accumulation in fish across a comprehensive range of salinities from FW to SW.

For the present experiments, we chose to use *Fundulus heteroclitus*, the common killifish. Osmoregulation in this organism has been well studied and it tolerates wide salinity ranges (Wood and Marshall, 1994). In addition, the killifish is easy to obtain and adapts well to laboratory conditions.

2.3 Materials and methods

2.3.1 Experimental animals

Fundulus heteroclitus (1.5-11.2 g) were collected during June of 2003 north of St. Augustine (FL, USA) in the Atlantic Intracoastal Waterway near the Guana River by the Whitney Laboratory, University of Florida, St. Augustine (FL, USA) and shipped to Miami (FL, USA). After two weeks of acclimation to laboratory conditions, the killifish were acclimated to five different salinities (0, 5, 11, 22, 28 ppt) for a minimum of two weeks before experimentation. During this time, they were kept in aerated, filtered, recirculated water of the appropriate salinity and a natural photoperiod. The water used for holding and for exposure was Bear Cut (25° 43.9' N 80° 09.7' W) SW (28 ppt) that had been passed through a sand filter and diluted with dechlorinated Miami City tap water to reach the desired salinities. The physical and chemical characteristics of the waters used are listed in Table 2.1. Fish were fed a dense commercial fish feed from Aquatic Eco-Systems (Apopka, FL, USA) ad libitum every other day with any uneaten food being removed a few hours after feeding.

2.3.2 Experimental design of the copper exposure

Killifish were kept in 28 L tanks receiving flow through of 100 ml min^{-1} aerated water at a density of 17 to 18 fish per tank. Fish were kept in five salinities (0, 5, 11, 22, 28 ppt) at three copper concentrations (nominal 0, 30, and 150 μg L^{-1}; for measured concentrations see Table 2.2) throughout 30 days of exposure for a total of fifteen tanks and 265 fish. All flow through water was well mixed prior to entering tanks through the use of separate mixing chambers to set first the salinity and then the copper concentrations. The residence time in the mixing chambers was approximately 2 minutes for setting the salinity and approximately 1.5 minutes for the copper. Food was withheld for 72 hours prior to sampling. Due to an otherwise highly intense sampling schedule, tanks were randomly assigned to two groups. The first group's exposure started one day before the second group's exposure and thus allowed for slightly staggered sampling events.

2.3.4 Sampling

Water was sampled on 0, 2, 4, 8, 12, 16, 25, and 30 days of exposure. Water samples for both total and dissolved copper with the latter being defined as that which passed through a 45 μm syringe filter (Acrodisc syringe filters, Pall Life Sciences, Houston, TX, USA,) were acidified using concentrated nitric acid (Trace metal grade, Fisher Scientific, Pittsburgh, PA, USA, 1% volume/volume, v/v). Fish were sampled at four time points: Days 0, 4, 12, and 30. At each time point, five fish were taken from each tank with exception of treatments where mortality occurred. For these treatments *n*-numbers were adjusted to allow for sampling at all of the above mentioned time points

(See Table 2.3 for *n*-numbers). Euthanized fish (Tricaine methanesulfonate [MS-222]; 0.2 g L^{-1}) were dissected to obtain gill, liver, intestine, gall bladder, kidney, and a small muscle sample for analysis of copper content. These samples as well as the remaining carcass were weighed and digested in approximately five times their volume of 1N sulfuric acid (Trace metal grade, Fisher Scientific) at 80 °C for 24 hours.

Tissue somatic index for gill, liver, muscle, and intestine was determined by dissecting and weighing tissues from six fish: Three male and three female. The remaining portion of the fish (gall bladder, kidney, skin, nervous system, the remaining muscle, and bone) was called the carcass. The contribution of individual tissues to total body mass was calculated by dividing the tissue weight by the fish's body mass and was as follows: Carcass 59.80 ± 2.29 %, muscle 31.10 ± 1.90 %, liver 3.35 ± 0.50 %, gill 3.04 ± 0.19 %, intestine 2.71 ± 0.26 %, gall bladder 0.99 ± 0.14 %, and kidney 0.05 ± 0.01%.

2.3.5 Copper analysis

Copper concentrations in the fresh water (0 ppt) samples and tissue digests were determined by graphite furnace (Varian 220Z graphite furnace atomic absorbtion spectrophotometer, Mulgrave, Australia) using standard operating conditions and dilutions as necessary. However, a co-precipitation with iron was performed on the samples from the other salinities to eliminate the interference by sodium (Weisel et al., 1984). Briefly, an iron solution (iron (III) nitrate; Ultra Scientific, North Kingstown, RI, USA, 10,000 μg ml^{-1}, 1:50 v/v) was added to water samples. The pH was subsequently raised to 7.5 to 8.0 using trace metal grade ammonium hydroxide (Fisher Scientific) and

allowed to sit overnight. The next day, samples were centrifuged at 5° C and approximately 5000 rpm for ten minutes. Subsequently, the supernatant was removed and the precipitate was dissolved in 5% nitric acid (Trace metal grade, Fisher Scientific). The Cu concentrations in the resulting salt-free samples were then compared to a certified standard (Fisher Scientific). Known concentrations of Cu were added to samples for determination of percent recovery (generally 90%) and concentrations measured were adjusted accordingly.

2.3.6 pH, total organic carbon, dissolved organic carbon, cations, anions, and tirtratable alkalinity

The pH in the tanks was measured using a Radiometer Analytical PHM 201 portable pH meter fitted with a Radiometer Analytical pHC 3005 combined pH electrode (Lyon, France). Samples were taken on two occasions for determination of total organic carbon and dissolved organic carbon by high temperature catalytic oxidation using a Shimadzu total organic carbon –VCSH (V series, combustion catalytic oxidation / nondispersive infrared method, standalone, high sensitivity) total organic carbon analyzer (Kyoto, Japan) (Hansell and Carlson, 2001). Cations in water samples were determined using a Varian 220FS flame atomic absorbtion spectrophotometer while anions were determined using a Dionex DX-120 ion chromatograph (Sunnyvale, CA, USA), in both cases after appropriate dilution. Titratable alkalinity was determined by a double endpoint titration (Hills, 1973). In brief, samples were aerated with O_2 (Airgas, Radnor, PA, USA, Ultra pure grade) and the pH was allowed to stabilize after de-gassing of CO_2 (30 to 35 min). The pH was lowered to 3.8 using 0.02 N HCl in Gilmont micro burettes

(Barrington, IL, USA) and the volume used was recorded. After 15 minutes of continuous gassing with O_2 to drive off any CO_2, the pH was raised to the initial pH using 0.02 N NaOH also in micro burettes and the volume was recorded. The difference between the volume of acid and base required to reduce the pH to 3.800 and to return to starting pH, respectively, was equal to the titratable alkalinity present in the sample.

2.3.7 Speciation calculations

For the purpose of copper speciation calculations, linear regressions (r^2 = 0.954-0.999) were performed on measured concentrations of cations, anions, and titratable alkalinity to estimate the characteristics of waters at the actual exposure salinities as listed in Table 2.1. The data found in Table 2.1 were fed into a chemical speciation program based on natural waters for calculation of copper speciation (Millero and Pierrot, 1998).

2.3.8 Whole body copper and relative distribution

Whole body copper concentrations were determined by summing the contributions from various tissues and dividing by the body mass. The relative contribution of individual organs to whole body copper was calculated using the tissue somatic index as follows.

$$\text{Relative contribution} = \frac{\text{tissue somatic index x body mass x copper concentration in that tissue}}{\text{total amount of copper in the fish}}$$

2.3.9 Statistical analysis and data presentation

Data are presented as mean ± standard error of the mean with *n*-numbers presented in Table 2.3. Numerical data were log transformed prior to analysis because the variances generally increased with increasing means. Copper distribution data (expressed as percent of total) were arcsine transformed prior to analysis (Sokal and Rohlf, 1995). Where appropriate, one-way analysis of variance (ANOVA) was performed to evaluate potential differences from control and differences among salinities. Where data did not fit the assumptions of the ANOVA (i.e., the data were not normally distributed or did not have equal variances even after the log transformation), a Kruskal-Wallis analysis of variance on ranks was performed (Sokal and Rohlf, 1995). Due to differences among controls over time and across salinity, all comparisons were made to a simultaneous control and all *p*-values were subjected to a Bonferroni multi-comparison correction for the ANOVAs and a Dunn's multi-comparison correction for the Kruskal-Wallis analyses for each comparison independently. Correlation analyses were performed using either a Pearson product moment correlation for data that fit the assumptions of the test (i.e., the data are not distributed as a bivariate normal distribution) or a Spearman rank order correlation for data that did not fit the assumptions of the Pearson correlation. A p-value ≤ 0.05 was considered significantly different throughout for both the ANOVA and correlation analyses. All statistical analysis was performed using Sigma Stat 3.0 (Chicago, IL, USA).

2.4 Results

2.4.1 Water chemistry

Measured copper concentrations were not significantly different between the salinities with dissolved concentrations being slightly lower than total copper concentrations (Table 2.2). However, the dissolved concentrations of copper tended to differ among treatments by up to 21% in the high copper exposure and by 47% at the low exposure concentration. The pH (8.00 to 8.39), total organic carbon (0.1618 ± 0.0015 mM carbon), dissolved organic carbon (0.1607 ± 0.0010 mM carbon), and temperature (25º C) did not vary significantly over time or among treatments.

2.4.2 Copper uptake and accumulation

There were significant increases in whole body copper in FW and at 5 ppt (Table 2.4; gill, liver, and intestine are presented in Figs. 2.1-2.3) while whole body copper concentrations remained similar to control levels at the higher salinities. The gill, intestine, and liver all exhibited significant copper accumulation in some but not all treatments and these changes showed dose, time, and salinity dependence. However, in the gall bladder, kidney, muscle, and carcass, the few significant changes observed did not exhibit dose, time, or salinity dependence (Table 2.5).

In the gill, elevated copper concentrations were observed in all salinities except SW at both concentrations of copper tested (Fig. 2.1). Fish in both the low and high copper treatments in FW showed significantly elevated branchial levels of copper by day 4 and remained high throughout the exposure at the low copper concentration (Fig. 2.1). At the high copper concentration, all remaining fish died before day 12. In 5 ppt, at

the low copper concentration, gill copper was elevated at day 4 and 12 and subsequently returned to control levels by day 30. In contrast, at the high copper concentration, gill copper increased over time and was significantly elevated at day 30. At 11 ppt, the gill copper concentration was elevated in both copper concentrations by day 4. It returned to control levels regardless of the copper concentration by day 12 and became elevated by day 30 only in the high copper exposure. At 20 ppt, the gill copper concentrations were significantly elevated on day 30 at both copper concentrations.

In the intestine, copper concentrations were observed to be elevated in all salinities except SW (Fig. 2.2). The highest concentration of copper was seen in the intestine at the high copper concentration on day 30 at 5 ppt. In FW in the low copper exposure, the intestinal copper levels started significantly lower than control levels at day 4 and became significantly elevated at day 12 returning to control levels by day 30. At 11 ppt, copper was significantly elevated at the low copper concentration on day 4 and 12 and at the high copper concentration at day 12 and 30. In 20 ppt, the intestinal copper levels were significantly elevated only at the high copper concentration at day 30.

Hepatic copper was elevated in FW, 5 ppt, and 11 ppt (Fig. 2.3) but not at the higher salinities. The only significant increase in hepatic copper at the low copper concentration was observed in FW on day 30, while hepatic copper concentrations were significantly elevated at the high copper concentration on day 4 at 5 ppt and day 30 at 11 ppt.

2.4.3 Distribution of whole body copper

The liver accounted for 57 to 86% of the copper found in body in fish not exposed to elevated aqueous copper (Table 2.5). The carcass (6.84 – 28.14%), muscle (3.21 – 12.3%), intestine (1.34 – 8.06%), and gill (0.57 – 2.30%) followed the liver, in order of decreasing contribution to whole body concentration of copper. The gall bladder and the kidney combined accounted for less than 0.1% of the copper burden in the fish with no change in response to treatment and therefore were included in the carcass (skin, nervous system, gonads, bone, gall bladder, and kidney).

The relative distribution of copper in the fish generally showed modest changes in response to copper exposure in all tissues except the intestine. Both the gill and the liver exhibited increases in their contributions while the muscle exhibited decreased contributions. The carcass showed both increased and decreased contributions to the body burden. In FW, the only difference was observed on day 4 at the high copper concentration when the gill contributed significantly more to the body burden. At 5 ppt, on day 4 the carcass contributed significantly less at both copper concentrations. At the high copper concentration, the muscle contributed significantly less and the liver contributed significantly more to the whole body copper. At 11 ppt on day 4, both the gill and the carcass made significantly larger contributions to the whole body copper levels at the high copper concentration, while on day 30, the liver contributed significantly more and the carcass significantly less at both copper concentrations. At 20 ppt, only on day 4 at the low copper concentration was a difference observed when the carcass made a significantly larger contribution. In SW on day 12, the muscle contributed significantly less to the body burden at both copper concentrations.

2.4.5 Speciation

Speciation calculations revealed that $CuCO_3$ is the dominant form of inorganic copper at all salinities (Table 2.6). The abundance of $CuCO_3$ and $CuHCO_3^+$ decreased with increasing salinity, but $CuHCO_3^+$ comprised little of the total of copper present (≤ 0.15%). Both $Cu(CO_3)_2^{2-}$ and $CuSO_4$ increased with increasing salinity. However, while $Cu(CO_3)^{2-}$ contributed significantly to the total copper present in the higher salinities, $CuSO_4$ stayed relatively low (≤ 1%). In contrast, Cu^{2+}, $CuOH^+$, and $Cu(OH)_2$ all increased in abundance from FW to 5 ppt. The free ion, Cu^{2+}, increased slightly (0.04%) at 11 ppt before decreasing in abundance with increasing salinity, while both $CuOH^+$ and $Cu(OH)_2$ decreased as salinity increases from 5 ppt to SW. Overall, Cu^{2+}, $CuOH^+$, and $Cu(OH)_2$ contributed less than 14.6% combined at their peak in abundance.

2.4.6 Correlation analysis

The gill, intestine, and liver were chosen for further analysis due to the significant amounts of copper accumulation that occurred in these tissues. In addition, the whole body was chosen for analysis because it provided a sum of the individual tissue accumulation trends. Dissolved copper, the free ion, $CuOH^+$, $Cu(OH)_2$, $CuCO_3$, and $Cu(CO_3)_2^{2-}$ were chosen for analysis because Cu^{2+}, $CuOH^+$, and $Cu(OH)_2$ have been shown to be important for toxicity and $CuCO_3$ and $Cu(CO_3)_2^{2-}$ are high in concentration relative to the other species of copper present. Dissolved copper concentrations were analyzed to verify that the accumulation is not well correlated with the amount of copper in the water. Both $CuHCO_3^+$ and $CuSO_4$ were excluded from the analysis due to their

low contributions. The influence of salinity and time were also analyzed. Salinity and time appeared to be important for accumulation. Cations, anions, and titratable alkalinity were not considered separately because they all directly co-vary with salinity.

Tissue copper concentrations showed positive correlations with time, $CuCO_3$, $Cu(CO_3)_2^{2-}$, $CuOH^+$, and $Cu(OH)_2$ in the gill, liver, and whole body (Table 2.7). Tissue copper concentrations also showed significant correlations to both $CuCO_3$ and $Cu(CO_3)_2^{2-}$ in the intestine. Tissue copper concentrations were negatively correlated to salinity in the intestine, gill, and whole body. When tissue copper concentrations were significantly correlated to the free ion, they always exhibited a negative correlation. Tissue copper concentrations were at times significantly correlated with dissolved copper concentrations in the four compartments examined but were both negatively and positively correlated with dissolved copper and thus showed no clear trend.

2.5 Discussion

2.5.1 Target tissues

As one of the goals of this study, we investigated many tissues to identify potential target tissues for toxicity. The gill followed the hypothesis of being important at low salinities, as the copper concentrations in the gill decreased with increasing salinity. The intestine did show a trend towards increasing accumulation with increasing salinity above the isoosmotic point as expected, and exhibited very high amounts of accumulation at 5 ppt. These results suggest that the intestine may be an important target for copper toxicity in saline environments. The liver also showed high accumulation of copper which warrants further study as a potential site of toxicity especially during

chronic exposure as had already been suggested by previous work (Peña et al., 1999). These tissues will all be discussed in detail below (see relevant sections of the discussion: gill, intestine, and liver).

Our study does not confirm the hypothesis that the kidney is an important site of accumulation in high salinities (Stagg and Shuttleworth, 1982a) and therefore it is not likely to be a target tissue, as we see no trends towards elevated copper with either salinity or copper concentration. The gall bladder also did not respond as expected (Grosell et al., 1998; Peña et al., 1999) and no elevations of the copper concentrations were observed in the bile. The lack of copper accumulation in the bile suggests that either the killifish does not excrete copper via the bile like rainbow trout (Grosell et al., 1998) which would be interesting physiologically, or that the copper burden in the fish never reached a high enough concentration to initiate copper excretion. The muscle and carcass did not show any trends toward increased accumulation with either salinity or time which is in agreement with previously published results (Grosell et al., 2004b; Kamunde et al., 2002). This lack of copper accumulation indicates that these two compartments are unlikely to be important sites of copper toxicity.

2.5.2 The gill

The BLM identifies the gill as an important target for metal toxicity and accumulation in FW (Paquin et al., 2002). Because Cu is able to substitute for Na^+ in several uptake pathways (Grosell et al., 2002; Grosell and Wood, 2002), it was expected that Cu would accumulate in the gill at salinities below the isoosmotic point (~10 ppt; euryhaline teleost = 297 mOsmol kg^{-1} , SW = 32 ppt = 1050 mOsmol kg^{-1} (Karnaky,

1998); mOsmol represents the amount of substance that dissociates in solution to form one millimole of osmotically active particles). At these lower salinities the gill takes up Na^+ to compensate for the loss of Na^+ to the dilute environment (Karnaky, 1998). Our study demonstrated that the gill is an important site of copper uptake at low salinities. The highest accumulation in the gill occurred in FW while no accumulation occurs in SW and there is a strong negative correlation between salinity and branchial copper concentrations. Accumulation was continuous over time at the low copper concentration in FW and also at 5 ppt in the high copper concentration. Interestingly, in FW, the maximal concentration in the gill was reached at day 4 in the high copper concentration and it was equal to the concentration at day 30 in the low exposure concentration.

The decrease in copper accumulation seen with increasing salinity is most likely due to competition by competing cations at the gill and a change in the physiology of the gill at high salinities. In salinities above the isoosmotic point (~10ppt and higher), the gill excretes ions. Therefore, since copper is thought to be taken up on the same pathways as Na^+ it is likely that copper uptake is decreased at 5 ppt by the large increases in Na^+ and increasing competition for Na^+ uptake pathways. At the higher salinities the reduction of accumulation in the gill is most likely due to the fact that Na^+ is not being taken up at the gill.

However, above the isoosmotic point, we observed branchial copper accumulation at 11 and 20 ppt but the concentrations accumulated were lower than those observed at the low salinities. A possible explanation for this accumulation trend is sodium insensitive uptake pathways (Grosell and Wood, 2002). Fish have been shown to take up copper in fresh water by a path which is inhibited by increasing sodium and by

one that is not inhibited by sodium (the sodium insensitive pathway). As salinity increases above the isoosmotic point the pathways that can be inhibited by sodium would be shut down leaving only the sodium independent pathways for uptake of copper. This sodium independent pathway would likely be dependant on the concentration of copper in the water and especially the concentration of the free ion. Both the dissolved copper concentration in the high copper exposure (Table 2.2; 5 ppt to SW) and the percentage of the free ion decreased as salinity increased in this experiment which could account for the decreased accumulation observed. However, we saw no significant accumulation in SW.

With one exception, we observed that the fish exhibited homeostatic control of copper levels in the gill above the isoosmotic point. Although the branchial copper concentrations in fish exposed to the high copper concentration were often elevated relative to their simultaneous unexposed controls, the concentrations measured reached a plateau and stabilized instead of increasing constantly over time. The stabilization of copper concentrations in the gill is indicative of homeostatic control albeit at a different set point. The control of copper levels in the gill was also exhibited at the low copper concentration at 5 ppt. This homeostatic control of copper concentrations followed two patterns. In one pattern, the concentration of branchial copper increased early in the exposure and returned to control levels by day 30. In the second pattern, copper concentrations increased in the gill and were maintained at this higher, second steady-state concentration throughout the remainder of the exposure. This demonstrated that the fish were able to cope with the increased levels of copper uptake to maintain appropriate concentrations of copper in the tissues and possibly to avoid toxicity while maintaining proper cellular function (Harris, 2000; Peña et al. 1999; Uauy et al., 1998). The only

exception to this was that the concentration in the gill at 20 ppt and the low exposure concentration increased above control levels on day 30 and this level was higher than any other concentration observed in the gill at 20 ppt.

The observed patterns of copper accumulation can be explained by changes in uptake, excretion, and internalization. Where branchial copper accumulation occurs during exposure to elevated levels of aqueous copper, uptake into the gill exceeds the gill's ability to excrete the copper either by moving it back into the environment or moving it into the body. Where no branchial accumulation is observed, the uptake is, of course, matched by the excretion / internalization. This is also true when the gill during exposure to elevated copper establishes a second steady state at a constant copper concentration above a measured control concentration and maintains this elevated copper concentration. When the branchial copper levels initially rise and then return to control levels during exposure, alterations in uptake into, or elimination from, the gill must have occurred. These adjustments could include both reduced uptake rates and increased elimination. During the reduction of branchial copper levels, the rate of excretion / internalization must be higher than the uptake rate in the gill. We cannot determine from these experiments whether the branchial uptake is decreased and/or if excretion / internalization is increased. The mechanisms involved in maintaining branchial copper homeostasis offer an exciting area for future research.

2.5.3 The intestine

The intestine in salinities above the isoosmotic point is also a potential target for copper accumulation because fish in these salinities drink to compensate for water loss to the concentrated external environment (Grosell et al., 1999; Grosell and Wood, 2001; Karnaky, 1998). The drinking was illustrated by a trend for an increase in intestinal copper concentrations with salinity above the isoosmotic point. We observed increased copper concentrations in the intestines of fish at 11 and 20 ppt, but intestinal copper concentrations were not significantly elevated in SW probably due to the limited *n*-numbers on day 30 (see Table 2.3). In FW, we observed no apparent trend towards an increase in intestinal copper. However, from the correlation analyses (Table 2.7), we observed that the intestine was poorly correlated with the water chemistry parameters investigated including salinity. This is probably because the intestine exerts strong control over the intestinal fluid chemistry (Wilson et al., 2002).

In contrast to our expectations, we observed the highest concentrations of copper in the intestine at 5 ppt and the high exposure concentration. These concentrations were about four times higher than the next highest concentration observed and about eight times higher than the control levels of copper. It is unlikely that this result was due to a sampling error or contamination. First, the fish showed a trend towards increased copper in the intestine on day 12 at this salinity (~4.5 fold increase over control). Secondly, even though all tissues from the fish were sampled at the same time, no other tissues show contamination. Finally, the relatively small standard error (~18 %) associated with the measurements suggest that contamination was not an issue. The alternative explanation that the higher exposure concentration (~21% higher than the lowest

exposure concentration) coupled with the low salinity caused the increased intestinal accumulation is also unlikely. This explanation is unlikely because a 21% increase in copper concentration is unlikely to cause four times as much accumulation in the intestine at 5 ppt as in the intestines of fish exposed to copper at the other salinities.

At this salinity, the fish were still below their isoosmotic point and therefore expected to be drinking little or no water. However, the high levels of copper in the intestine might indicate that the fish exhibited a high drinking rate at this salinity. Another, possible explanation is that the copper found in the intestine is accumulated from within the fish. Finally, if drinking occurs at 5 ppt, the chemistry in the intestine could be unique and thereby account for the high copper levels found in the intestine at this salinity. At present, the excessive intestinal copper accumulation at 5 ppt is unexplained but is likely due to an interesting combination of chemistry and physiology at this intermediate salinity.

2.5.4 The liver

The liver contains more copper than any other tissue and dominates the whole body copper levels (57-86% of whole body copper). Again, the highest accumulation occurred at the lowest salinity and decreased as the salinity increased. Because the liver is the main organ involved in copper homeostasis, it dictates the trends for whole body copper accumulation. The intestine and the gill were the major sites of uptake for final accumulation in the liver and whole body. From the present data it is difficult to separate

intestinal and branchial contributions to copper accumulation in the liver and whole body. Further studies are necessary to address the contribution of uptake by the intestine and gill to liver and whole body accumulation

Currently, copper is thought to impair osmoregulation and ionoregulation at the gill and that this is why copper is toxic. This impairment is also thought to occur at the intestine in sea water as this organ is important for water absorbtion driven by NaCl transport. Accumulation in the gill and the intestine occur relatively rapidly and it is likely that the intestine and gill would be important for acute toxicity. However, the liver exhibits substantial copper accumulation and might be important for chronic toxicity.

2.5.5 Accumulation, speciation, and competition

From the correlation analysis (Table 2.7), it was apparent that tissue copper concentrations were poorly correlated with both the free ion and dissolved copper concentrations. Tissue copper concentrations exhibited both significant positive and negative correlations to dissolved copper concentrations while they were significantly negatively correlated to the free ion at several time points. As such, the free ion and dissolved copper concentrations alone are likely to be poor predictors of copper accumulation of fish in saline environments. Tissue copper concentrations exhibited positive correlations with salinity, $CuOH^{+}$, $Cu(OH)_2$, and $CuCO_3$ in all compartments except the intestine. The intestinal copper concentrations showed significant correlations to many of the factors tested at one time point only which suggests that the intestinal fluid chemistry and water chemistry were poorly correlated.

Taken together $CuCO_3$ appeared to be the most important form of copper for accumulation while $CuOH^+$ and $Cu(OH)_2$ also contributed in the gill, liver, and whole body. The uncharged $CuCO_3$ was probably accumulated via a passive diffusion process and is perhaps less likely to bind to any target in the cell. Thus, $CuCO_3$, although apparently available for accumulation, may not be available for exerting toxicity. However, the mechanism of uptake, cellular distribution, and potential toxic action of $CuCO_3$ is not known at this time. Both $CuHCO_3^+$ and $CuSO_4$ were not considered very important because of the low concentration of the two forms present at all salinities.

However, this interpretation is complicated due to the co-variance of $CuCO_3$ and salinity and due to the physiological changes in the tissues responsible for uptake and excretion that occur as salinity increases. The strong correlation observed between salinity and accumulation in the gill is likely due to competition for uptake by cations at the gill at salinities below the isoosmotic point. However, the gill physiology changes from FW to SW from taking up ions to excreting ions respectively and it is likely that the competition from cations is no longer as important at the gill in SW.

Tissue copper concentrations and time are significantly correlated in all compartments examined. Tissue copper concentrations appeared to be correlated with salinity, $CuOH^+$, and $Cu(OH)_2$ generally before day 12. Because these correlations appeared early in the exposure, these factors may have a greater influence on accumulation during short-term accumulation. Both $CuOH^+$ and $Cu(OH)_2$ may be taken up and internalized more readily than other forms of copper. In the liver and whole body, tissue copper concentrations are correlated with $CuCO_3$ suggesting that longer term

accumulation may be dependant on the amount of $CuCO_3$ available. However, more detailed work needs to be done to determine which factors (speciation, physiology, or competition) are important for accumulation at intermediate and high salinities.

2.5.6 Conclusion

The most important inorganic form for accumulation (but not necessarily for toxicity) at all salinities is likely to be $CuCO_3$ with $CuOH^+$ and $CuOH_2$ also appearing to contribute. Overall, accumulation is highest at the lowest salinities and decreases with increasing salinity. As could be expected, the gill accumulates more copper at the low salinities while the intestine appears to accumulate more at high salinities. However, we observed high copper accumulation in the intestine at 5 ppt that does not follow this trend. It thus appears that more than one target tissue (both the gill and the intestine) must be considered for future development of a BLM for saline environments. In spite of the significant correlations between copper accumulation and both copper speciation and salinity, it is unlikely that chemistry alone explains our results. The low accumulation at high salinities and the unusually high accumulation at 5 ppt indicate that other factors, such as physiology, probably play important roles in copper accumulation across salinities.

Table 2.1 Chemical characteristics of waters used in the exposures

Salinity (ppt)	Na^+ (mmol)	K^+ (mmol)	Mg^{2+} (mmol)	Ca^{2+} (mmol)	SO_4^{2-} (mmol)	Cl^- (mmol)	Tiratable Alkalinity (mmol)
0.0 ± 0.0	0.93	0.08	0.14	0.54	0.12	0.95	0.86
4.7 ± 0.1	63.39	1.45	7.76	2.03	3.17	65.33	1.06
10.7 ± 0.1	138.35	3.09	16.89	3.81	6.84	142.59	1.29
19.9 ± 0.2	250.78	5.55	30.59	6.48	12.34	258.48	1.65
27.5 ± 0.2	350.72	7.73	42.77	8.86	17.23	361.49	1.96

Table 2.2 Measured copper concentrations in the test waters. Total and dissolved copper concentration in control water showed no difference and are presented as one.

Salinity (ppt)	Control Total [Cu] (µg L^{-1})	Low Total [Cu] (µg L^{-1})	Low Dissolved [Cu] (µg L^{-1})	High Total [Cu] (µg L^{-1})	High Dissolved [Cu] (µg L^{-1})
0	0.37 ± 0.22	22.58 ± 0.74	20.55 ± 0.88	146.35 ± 2.57	140.60 ± 2.94
5	0.84 ± 0.10	18.45 ± 0.85	17.94 ± 0.85	179.85 ± 15.51	178.91 ± 15.82
11	1.10 ± 0.08	31.81 ± 0.73	30.22 ± 1.15	174.12 ± 30.68	168.90 ± 31.77
20	0.63 ± 0.09	33.54 ± 0.82	33.87 ± 0.68	169.19 ± 26.07	163.05 ± 24.85
28	0.83 ± 0.18	29.66 ± 4.12	29.38 ± 3.84	147.12 ± 18.37	142.93 ± 16.78

Table 3. *n*- numbers for mean values reported in tables 4 and 5 and figures 1-3.

Salinity	Day 4			Day 10			Day 30		
	Control	Low	High	Control	Low	High	Control	Low	High
0	5	5	5	5	4	0	3	3	0
5	5	5	5	3	5	5	1	4	3
11	5	5	5	5	5	5	2	3	6
20	5	5	5	5	5	5	5	5	5
28	5	5	5	5	5	3	6	6	1

Table 2.4. Copper concentration (µg g^{-1}) in tissues of killifish exposed to nominal 30 and 150 µg Cu L^{-1} for up to 30 days at five salinities; * indicates a significant difference ($p \leq 0.05$) from the simultaneous control. See table 2.2 for measured copper concentrations and table 2.3 for *n* -numbers

	Control			Low Cu			High Cu		
	4 d	12 d	30 d	4 d	12 d	30 d	4 d	12 d	30 d
Salinity of 0 ppt									
Whole Body	3.88 ± 0.55	2.36 ± 0.55	4.65 ± 2.25	2.94 ± 0.56	5.43 ± 1.17	7.38 ± 2.70 *	3.28 ± 0.84		
Gall Bladder	2.30 ± 0.58	2.79 ± 0.45	3.20 ± 1.35	1.39 ± 0.15	3.29 ± 1.23	3.43 ± 1.40	4.81 ± 1.77		
Kidney	19.67 ± 8.86	11.73 ± 1.67	21.47 ± 4.31	20.00 ± 3.95	24.59 ± 8.67	11.85 ± 1.74	39.06 ± 13.83		
Muscle	0.45 ± 0.04	0.26 ± 0.02	0.30 ± 0.04	0.42 ± 0.05	0.29 ± 0.02	0.58 ± 0.31	0.41 ± 0.04		
Carcass	0.38 ± 0.04	0.42 ± 0.03	0.41 ± 0.05	0.35 ± 0.02	0.41 ± 0.04	0.49 ± 0.07	0.61 ± 0.14		
Salinity of 5 ppt									
Whole Body	2.18 ± 0.41	6.02 ± 3.31	5.14	3.40 ± 0.64	4.70 ± 1.79	5.86 ± 1.49	7.15 ± 1.87 *	6.57 ± 2.05	13.51 ± 2.52
Gall Bladder	2.19 ± 0.84	0.84 ± 0.30	1.71	2.99 ± 0.47	3.48 ± 1.58	11.20 ± 8.26	2.74 ± 0.44	1.56 ± 0.53	4.32 ± 2.10
Kidney	45.22 ± 11.86	23.57 ± 8.82	8.95	42.33 ± 9.79	59.66 ± 49.38	19.98 ± 6.24	37.28 ± 9.72	24.61 ± 2.88	12.95 ± 2.62
Muscle	0.57 ± 0.10	0.38 ± 0.08	0.36	0.52 ± 0.04	0.41 ± 0.08	0.32 ± 0.02	0.56 ± 0.05	0.37 ± 0.04	0.56 ± 0.30
Carcass	0.39 ± 0.05	0.30 ± 0.01	0.36	0.37 ± 0.02	0.55 ± 0.05	0.57 ± 0.14	0.38 ± 0.03	0.75 ± 0.12 *	0.70 ± 0.18
Salinity of 11 ppt									
Whole Body	5.50 ± 1.41	3.07 ± 0.54	1.47 ± 0.43	2.92 ± 1.06	2.94 ± 0.60	4.74 ± 2.32	2.83 ± 0.99	3.42 ± 0.46	5.40 ± 1.10
Gall Bladder	2.95 ± 0.32	1.21 ± 0.38	2.65 ± 0.77	3.24 ± 0.71	2.30 ± 0.41	3.65 ± 1.94	2.54 ± 0.19	1.37 ± 0.32	4.35 ± 1.34
Kidney	19.56 ± 3.76	28.17 ± 10.40	35.77 ± 4.16	18.53 ± 3.64	9.84 ± 2.59	10.64 ± 2.98	45.39 ± 18.68	35.39 ± 11.09	23.72 ± 12.74
Muscle	0.53 ± 0.07	0.32 ± 0.04	0.26 ± 0.04	0.63 ± 0.11	0.44 ± 0.10	0.51 ± 0.15	0.36 ± 0.02	0.77 ± 0.33	0.86 ± 0.42
Carcass	0.41 ± 0.04	0.45 ± 0.02	0.41 ± 0.04	0.36 ± 0.03	0.42 ± 0.01	0.32 ± 0.02	0.40 ± 0.03	0.45 ± 0.03	0.47 ± 0.04
Salinity of 22 ppt									
Whole Body	2.94 ± 1.10	2.60 ± 0.61	3.04 ± 0.67	3.26 ± 0.90	2.18 ± 0.49	5.43 ± 2.05	2.52 ± 0.55	4.06 ± 1.10	5.69 ± 2.26
Gall Bladder	1.76 ± 0.17	2.75 ± 0.49	2.69 ± 0.71	2.16 ± 0.80	0.73 ± 0.14 *	2.14 ± 0.91	1.84 ± 0.06	3.54 ± 0.59	2.31 ± 0.65
Kidney	16.61 ± 4.32	10.11 ± 3.03	22.53 ± 4.39	26.53 ± 7.88	10.71 ± 2.49	13.96 ± 3.07	35.09 ± 13.02	25.48 ± 14.28	10.86 ± 2.10
Muscle	0.45 ± 0.03	0.42 ± 0.09	0.22 ± 0.01	0.53 ± 0.07	0.40 ± 0.04	0.50 ± 0.05 *	0.51 ± 0.07	0.47 ± 0.10	0.40 ± 0.07 *
Carcass	0.41 ± 0.05	0.56 ± 0.02	0.40 ± 0.03	1.05 ± 0.34 *	0.48 ± 0.06	0.43 ± 0.03	0.39 ± 0.03	0.65 ± 0.04	0.45 ± 0.04
Salinity of 28 ppt									
Whole Body	2.25 ± 0.47	2.21 ± 0.91	3.36 ± 0.65	3.67 ± 0.90	2.95 ± 0.40	4.08 ± 0.98	2.51 ± 0.72	5.02 ± 2.38	6.86
Gall Bladder	3.33 ± 1.52	0.89 ± 0.22	3.79 ± 0.77	2.25 ± 0.26	3.62 ± 0.82 *	4.21 ± 0.90	2.37 ± 0.74	1.83 ± 0.80	3.88
Kidney	15.44 ± 2.60	65.76 ± 15.45	11.18 ± 2.82	15.99 ± 2.48	18.24 ± 6.22 *	29.62 ± 12.07	15.58 ± 2.25	13.08 ± 2.07 *	10.22
Muscle	0.47 ± 0.05	0.58 ± 0.24	0.35 ± 0.07	0.40 ± 0.08	0.35 ± 0.03	0.23 ± 0.04	0.40 ± 0.04	0.36 ± 0.02	0.72
Carcass	0.38 ± 0.02	0.52 ± 0.03	0.37 ± 0.01	0.63 ± 0.10	0.46 ± 0.05	0.48 ± 0.04	0.37 ± 0.04	0.59 ± 0.07	0.43

Table 2.5. Relative distribution (%) of copper in killifish exposed to nominal 30 and 150 µg Cu L^{-1} for up to 30 days at five salinities; * indicates a significant difference ($p \leq 0.05$) from the simultaneous control. See table 2.3 for *n* -numbers.

	Control			Low Cu			High Cu		
	4 d	12 d	30 d	4 d	12 d	30 d	4 d	12 d	30 d
Salinity of 0 ppt									
Carcass	11.97 ± 4.45	19.67 ± 6.78	11.89 ± 1.57	12.74 ± 2.85	7.77 ± 1.68	6.04 ± 0.26	17.77 ± 3.58		
Gill	1.54 ± 0.63	1.64 ± 0.44	1.22 ± 0.45	3.96 ± 0.65	1.90 ± 0.39	2.65 ± 1.23	5.98 ± 1.11 *		
Intestine	8.06 ± 2.70	4.44 ± 1.30	3.14 ± 0.77	6.24 ± 2.16	3.33 ± 0.81	2.28 ± 0.19	5.71 ± 0.89		
Liver	72.66 ± 8.95	67.91 ± 11.08	79.85 ± 2.88	69.18 ± 8.21	84.48 ± 3.01	84.89 ± 4.41	65.03 ± 5.56		
Muscle	5.77 ± 1.31	6.34 ± 2.73	3.90 ± 0.57	7.89 ± 2.90	2.52 ± 0.49	4.15 ± 2.81	5.50 ± 0.82		
Salinity of 5 ppt									
Carcass	16.05 ± 2.39	6.84 ± 1.70	8.79	9.61 ± 0.79 *	14.82 ± 3.09	11.03 ± 3.72	5.19 ± 0.88 *	10.27 ± 2.37	6.75 ± 3.51
Gill	1.41 ± 0.31	0.57 ± 0.12	0.68	2.36 ± 0.96	2.56 ± 0.63	0.61 ± 0.09	0.84 ± 0.22	1.23 ± 0.35	1.04 ± 0.15
Intestine	4.98 ± 1.30	2.28 ± 0.56	1.34	2.67 ± 0.44	4.11 ± 0.87	2.14 ± 0.72	2.44 ± 0.62	7.85 ± 5.42	11.85 ± 5.42
Liver	65.25 ± 6.53	86.40 ± 2.45	84.96	79.40 ± 1.76	73.25 ± 5.76	83.47 ± 4.18	88.19 ± 2.07 *	77.78 ± 6.97	78.61 ± 8.98
Muscle	12.30 ± 4.37	3.91 ± 0.69	4.24	5.96 ± 0.75	5.26 ± 1.85	2.75 ± 0.55	3.34 ± 0.64 *	2.87 ± 0.76	1.74 ± 0.46
Salinity of 11 ppt									
Carcass	7.43 ± 1.60	12.54 ± 2.11	28.14 ± 0.53	13.67 ± 2.54	11.76 ± 1.30	9.67 ± 4.70 *	20.24 ± 4.30 *	10.12 ± 0.97	8.12 ± 1.44 *
Gill	0.57 ± 0.16	1.01 ± 0.17	1.89 ± 0.06	1.90 ± 0.28	0.96 ± 0.06	0.81 ± 0.28	3.13 ± 0.68 *	1.02 ± 0.03	1.11 ± 0.24
Intestine	1.52 ± 0.40	1.31 ± 0.32	5.13 ± 0.49	7.14 ± 3.12	3.56 ± 0.97	2.35 ± 0.85	7.22 ± 1.98	4.17 ± 0.87	3.26 ± 0.55
Liver	85.83 ± 3.23	81.11 ± 2.87	56.93 ± 1.16	65.45 ± 9.95	77.77 ± 2.50	82.18 ± 6.38 *	61.34 ± 8.35	76.59 ± 3.24	82.25 ± 2.44 *
Muscle	4.67 ± 1.25	4.03 ± 0.57	7.91 ± 1.26	11.84 ± 4.65	5.95 ± 1.48	4.99 ± 0.78	8.06 ± 1.70	8.11 ± 3.65	5.26 ± 1.55
Salinity of 20 ppt									
Carcass	14.29 ± 3.32	18.35 ± 2.10	12.39 ± 1.92	26.82 ± 4.18 *	20.07 ± 4.38	8.16 ± 1.15	13.05 ± 1.41	15.22 ± 2.57	7.71 ± 0.74
Gill	1.19 ± 0.35	1.62 ± 0.38	1.04 ± 0.22	1.84 ± 0.35	2.17 ± 0.60	2.12 ± 0.84	1.57 ± 0.45	1.60 ± 0.25	1.10 ± 0.17
Intestine	3.26 ± 0.85	4.27 ± 0.93	2.67 ± 0.82	4.39 ± 1.06	5.67 ± 0.87	2.71 ± 0.50	6.00 ± 1.26	7.89 ± 2.99	5.80 ± 2.00
Liver	73.76 ± 6.03	68.68 ± 2.58	80.68 ± 3.29	59.41 ± 4.56	63.92 ± 6.97	82.55 ± 1.99	71.58 ± 2.89	70.65 ± 5.39	82.28 ± 3.33
Muscle	7.49 ± 1.67	7.08 ± 1.56	3.21 ± 0.55	7.54 ± 0.91	8.18 ± 1.75	4.47 ± 0.39	7.81 ± 1.10	4.65 ± 0.79	3.10 ± 0.77
Salinity of 28 ppt									
Carcass	16.10 ± 1.93	25.45 ± 5.16	10.38 ± 1.55	17.29 ± 5.03	13.18 ± 2.19	10.89 ± 0.91	17.28 ± 3.76	11.84 ± 3.04	8.41
Gill	2.09 ± 0.36	2.30 ± 0.60	0.97 ± 0.18	1.07 ± 0.22	0.97 ± 0.15	0.98 ± 0.16	2.36 ± 0.66	0.75 ± 0.14	1.19
Intestine	4.52 ± 1.44	4.56 ± 1.02	2.99 ± 0.80	3.83 ± 1.27	2.80 ± 0.61	2.68 ± 0.72	5.30 ± 1.59	5.77 ± 2.67	6.55
Liver	68.60 ± 3.60	58.11 ± 5.99	81.49 ± 2.86	73.05 ± 5.56	78.58 ± 2.61	83.05 ± 1.79	65.85 ± 8.63	78.10 ± 6.49	77.55
Muscle	8.70 ± 0.63	9.57 ± 1.74	4.18 ± 0.84	4.76 ± 0.87	4.46 ± 0.63 *	2.40 ± 0.60	9.21 ± 2.74	3.55 ± 0.77 *	6.29

Table 2.6 Inorganic copper speciation expressed as percent total copper present in water with the composition described in Table 2.1 as determined by an inorganic chemical speciation model (Millero and Pierrot, 1998)

Salinity (ppt)	Cu^{2+}	$CuOH^{+}$	$Cu(OH)_2$	$CuHCO_3^{+}$	$CuCO_3$	$Cu(CO_3)_2^{2-}$	$CuSO_4$
0	3.30%	4.45%	3.68%	0.15%	85.33%	3.04%	0.06%
5	4.96%	5.20%	4.44%	0.12%	77.37%	7.30%	0.61%
11	5.00%	4.88%	3.99%	0.11%	74.01%	11.24%	0.76%
20	4.76%	4.40%	3.30%	0.10%	70.67%	15.94%	0.82%
28	4.52%	4.03%	2.78%	0.09%	68.49%	19.09%	1.00%

Table 7. Correlation analysis of copper concentration in the gill, intestine, liver, and whole body with form of Cu, salinity, and time for all individuals exposed to high and low waterborne [Cu]. Values presented are r values; * ($p \leq 0.05$) denotes significant correlation between compartment copper concentration and the parameter listed.

High [Cu]

	Gill			Intestine			Liver			Whole body		
	Day 4	Day 12	Day 30	Day 4	Day 12	Day 30	Day 4	Day 12	Day 30	Day 4	Day 12	Day 30
Cu^{2+}	-0.22	0.23	-0.12	-0.17	-0.17	-0.26	0.07	0.11	0.20	-0.01	0.08	0.25
$CuOH^{+}$	0.39	0.32	0.40	0.24	-0.07	0.33	0.41 *	0.27	0.46	0.45 *	0.35	0.56 *
$Cu(OH)_2$	0.39	0.32	0.40	0.30	-0.07	0.33	0.41 *	0.27	0.46	0.45 *	0.35	0.56 *
$CuCO_3$	0.75 *	0.34	0.40	0.36	-0.07	0.33	0.31	0.27	0.46	0.43 *	0.35	0.56 *
$Cu(CO_3)_2^{2-}$	-0.75 *	-0.33	-0.40	-0.38	0.07	-0.33	-0.31	-0.27	-0.46	-0.43 *	-0.35	-0.56 *
Salinity	-0.75 *	-0.33	-0.40	-0.39	0.07	-0.33	-0.31	-0.27	-0.46	-0.43 *	-0.35	-0.56 *
Dissolved copper	-0.24	0.46	0.40	0.14	-0.07	0.33	0.35	0.27	0.46	0.27	0.35	0.56 *
Time	0.61 *			0.05			0.36 *			0.39 *		

Low [Cu]

	Gill			Intestine			Liver			Whole body		
	Day 4	Day 12	Day 30	Day 4	Day 12	Day 30	Day 4	Day 12	Day 30	Day 4	Day 12	Day 30
Cu^{2+}	-0.19	-0.25	-0.62 *	-0.13	-0.25	-0.44 *	0.06	-0.44 *	-0.31	0.11	-0.43 *	-0.34
$CuOH^{+}$	0.44 *	0.44 *	-0.30	0.08	0.26	-0.16	0.05	0.05	0.18	-0.01	0.09	0.19
$Cu(OH)_2$	0.44 *	0.44 *	-0.30	0.08	0.33	-0.09	0.03	0.15	0.25	-0.04	0.18	0.26
$CuCO_3$	0.77 *	0.71 *	0.07	0.32	0.47 *	0.28	-0.04	0.49 *	0.44 *	-0.13	0.50 *	0.48 *
$Cu(CO_3)_2^{2-}$	-0.77 *	-0.71 *	-0.07	-0.32	-0.47 *	-0.19	0.02	-0.44 *	-0.42	0.12	-0.46 *	-0.45 *
Salinity	-0.77 *	-0.71 *	-0.07	-0.32	-0.47 *	-0.15	0.03	-0.39	-0.40	0.12	-0.41 *	-0.43
Dissolved copper	-0.35	-0.58 *	0.14	-0.01	-0.37	-0.07	-0.20	-0.45 *	-0.34	-0.06	-0.48 *	-0.37
Time	0.35 *			0.26 *			0.29 *			0.27 *		

Fig. 2.1 Copper concentration (μg g^{-1} wet wt) in the gill of killifish exposed to nominal 30 μg L^{-1} (open bar) and 150 μg L^{-1} (solid bar) copper for 30 days of waterborne exposure at five salinities; * indicates a significant difference ($p \leq 0.05$) from the simultaneous control (hatched bar). See Table 2.2 for measured copper concentrations and Table 2.3 for *n*-numbers.

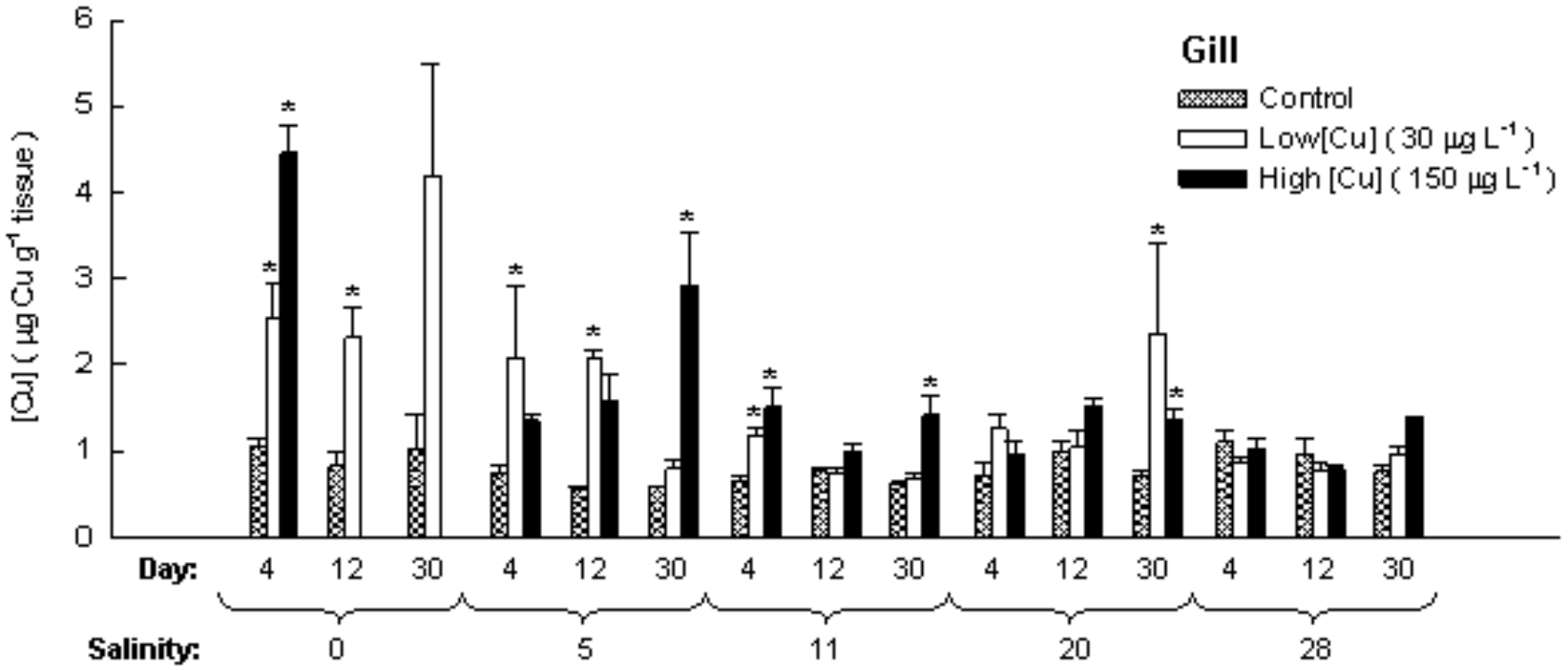


Fig. 2.2 Copper concentration (μg g^{-1} wet wt) in the intestine of killifish exposed to nominal 30 μg L^{-1} (open bar) and 150 μg L^{-1} (solid bar) copper for 30 days of waterborne exposure at five salinities; * indicates a significant difference ($p \leq 0.05$) from the simultaneous control (hatched bar). See Table 2.2 for measured copper concentrations and Table 2.3 for *n*-numbers. (Please note the break in the y-axis)

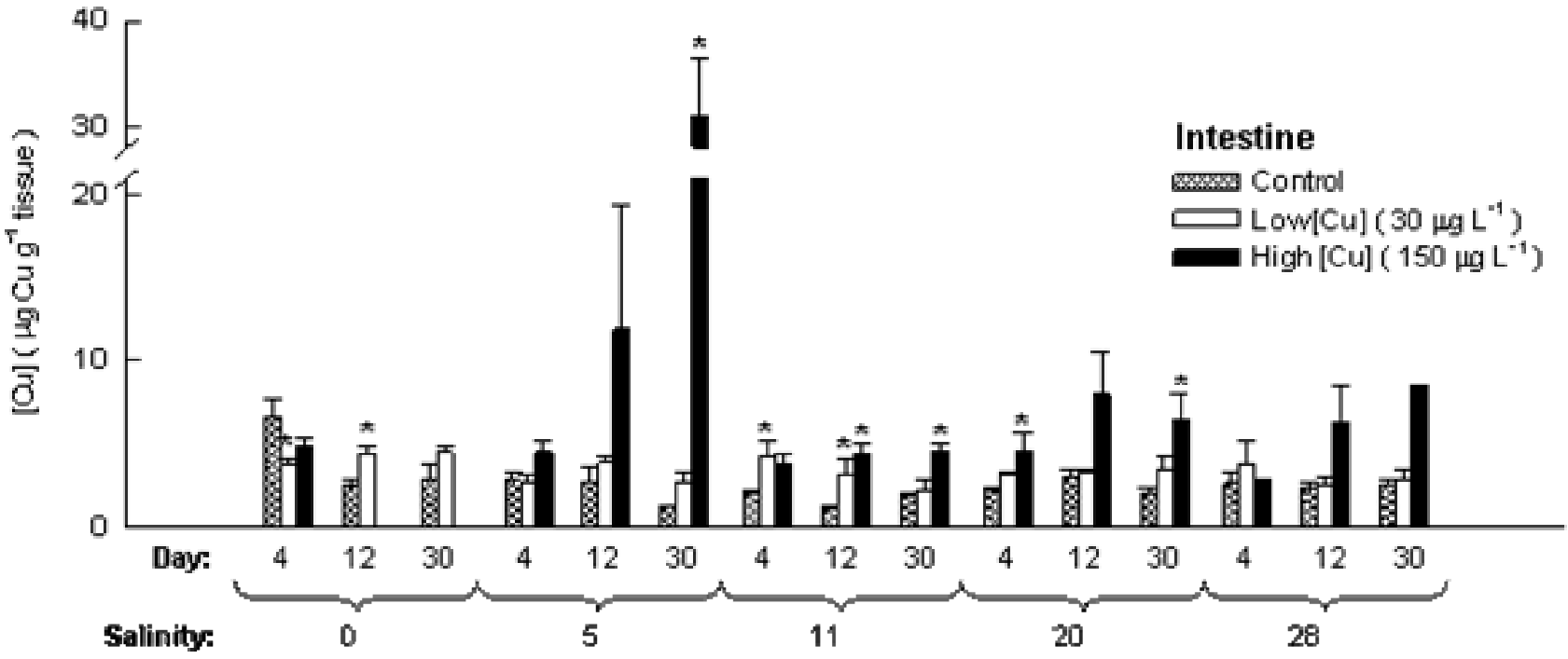
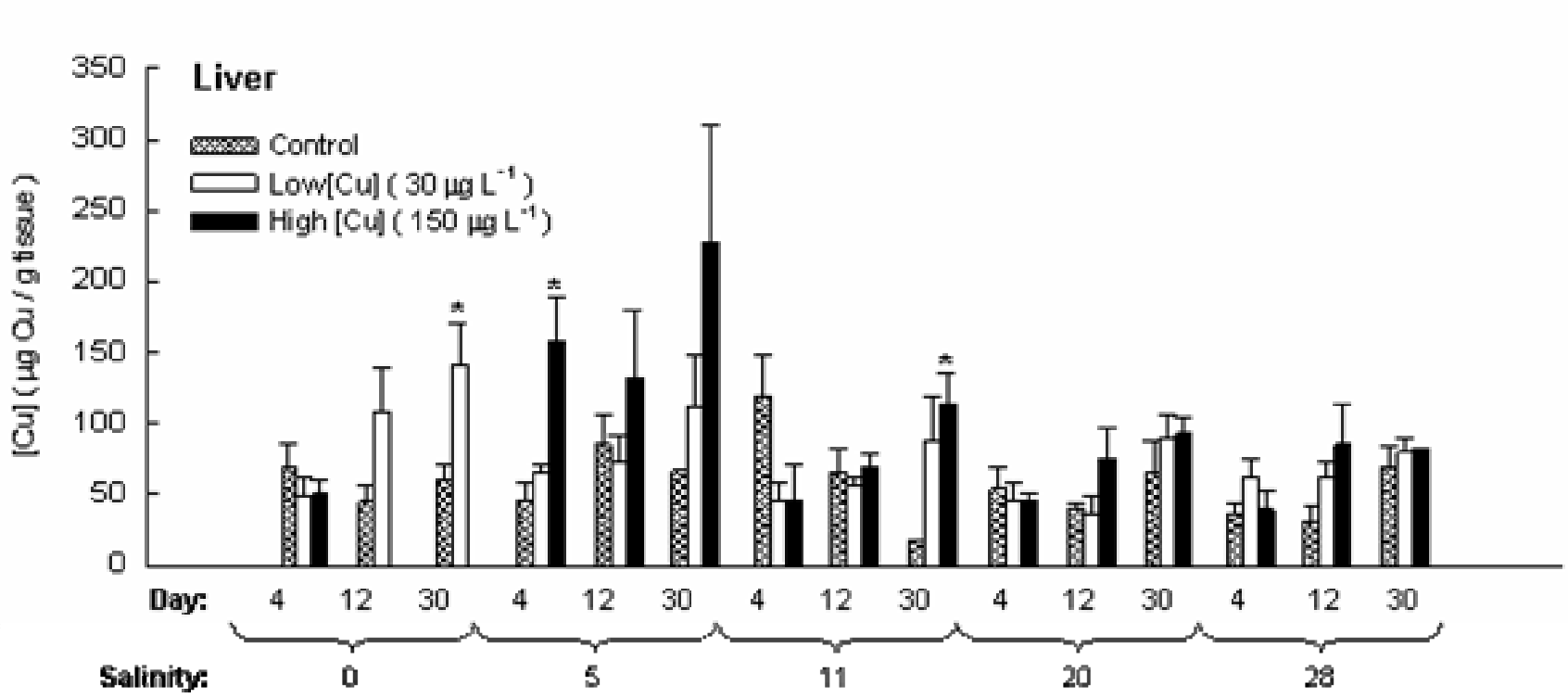


Fig. 2.3 Copper concentration (μg g^{-1} wet wt) in the liver of killifish exposed to nominal 30 μg L^{-1} (open bar) and 150 μg L^{-1} (solid bar) copper for 30 days of waterborne exposure at five salinities; * indicates a significant difference ($p \leq 0.05$) from the simultaneous control (hatched bar). See Table 2.2 for measured copper concentrations and Table 2.3 for *n*-numbers.



CHAPTER 3

COPPER TOXICITY ACROSS SALINITIES FROM FRESHWATER TO SEAWATER IN THE EURYHALINE FISH *FUNDULUS HETEROCLITUS*: IS COPPER AN IONOREGULATORY TOXICANT IN HIGH SALINITIES?

3.1 Summary

Two waterborne Cu exposures were performed to investigate if Cu is an ionoregulatory toxicant at all salinities in the killifish, *Fundulus heteroclitus*. A thirty day flow through exposure in 0 (FW), 5, 11, 22, and 28 (SW) ppt and three [Cu]'s (nominal 0, 30, and 150 μg Cu L^{-1}) revealed no apparent Cu induced mortality at the intermediate salinities and high mortality in FW and SW. Fish were sampled at 4, 12, and 30 days after the start of the exposure and both Na^+/K^+ adenosine triphosphatase (Na^+/K^+ ATPase) and carbonic anhydrase (CA) activity in the gill and intestine as well as whole body [Na^+], and [Cl^-] were measured. At the high [Cu] a reduction of whole body [Na^+] after four days of exposure in FW was the only physiological parameter influenced. A second static 24 hour Cu exposure was performed in FW, 5 ppt, 13 ppt, and 29 ppt (SW) and two [Cu]'s (nominal 0 and 110 μg Cu L^{-1}). In addition to the parameters listed above, ammonia flux was measured at all salinities and Na^+ flux was measured in FW fish. Cu affected ionoregulation in FW where decreased Na^+ uptake associated with inhibition of Na^+/K^+ ATPase led to decreased whole body [Na^+] after 24 hours. The only affected parameter in SW was net ammonia excretion suggesting that Cu is not an ionoregulatory toxicant in SW at the concentrations employed. We propose that physiology rather than chemistry explain much of the variation in Cu toxicity seen across salinities.

3.2 Background

Salinity has a profound effect on a fish's osmoregulatory and ionoregulatory physiology (Marshall and Grosell, 2005; Loretz, 1995). In freshwater (FW), fish deal with a net gain of water and a net loss of ions to their dilute external environment which causes them to excrete copious amounts of dilute urine to eliminate the excess water and to compensate for the loss of ions by uptake at the gill. In seawater (SW), fish lose water to their concentrated external environment and experience a net gain of ions. Consequently, SW fish must drink in order to compensate for water loss and must take up ions across the gastro-intestinal tract to facilitate the absorption of water and the gill is responsible for excreting the resulting Na^+ and Cl^- gain (Marshall and Grosell, 2005; Loretz, 1995; Larsen et al., 2002

Cu has been demonstrated to elevate plasma ammonia concentrations through an unknown mechanism and to disrupt ionoregulation by inhibiting Na^+ K^+ adenosine triphosphatase (Na^+/K^+ ATPase) in the gill during waterborne Cu exposures in FW (Laurén and McDonald, 1987a; Laurén and McDonald, 1987b). The inhibition of the basolateral Na^+/K^+ ATPase causes a reduction in Na^+ uptake that leads to a decrease in whole body and plasma [Na^+]. This reduction can be exacerbated by a concomitant increase in gill permeability, resulting in elevated diffusive loss (Laurén and McDonald, 1985) which could also be responsible for the reduction of whole body and plasma [Cl^-] observed during FW waterborne Cu exposures. A second potential explanation for Cl^- loss during Cu exposure is inhibition of Cl^-/HCO_3^- exchange at the gill resulting from an inhibition of carbonic anhydrase (CA) (Vitale et al., 1999) which would limit the amount of HCO_3^- available for exchange (Perry et al., 2003).

In SW, Cu in some cases behaves like an ionoregulatory toxicant (Grosell et al., 2004a; Wilson and Taylor, 1993; Larsen et al., 1997; Stagg and Shuttleworth, 1982a) and in others exhibits no ionoregulatory effect (Grosell et al., 2003). An increase in whole body and plasma [Na^+] has been observed during waterborne Cu exposures in SW (Grosell et al., 2004a; Larsen et al., 1997; Stagg and Shuttleworth, 1982a; Wilson and Taylor, 1993) which could be caused either by an inhibition of water transport or an inhibition of Na^+ excretion. Both of these scenarios could be explained by an inhibition of Na^+/K^+ ATPase which is responsible for the Na^+ uptake that facilitates water transport in the intestine as well as the Na^+ excretion at the gill. However, no inhibition of Na^+/K^+ ATPase has been observed so far during waterborne Cu exposures in SW in either the intestine or the gill perhaps suggesting that the ionoregulatory disturbance is more likely caused by a change in gill permeability (Stagg and Shuttleworth, 1982b; Grosell at al., 2004b). The only consistent effect that has been demonstrated in response to Cu exposure in seawater fish is an increase in plasma ammonia (Grosell et al., 2004a; Wilson and Taylor, 1993).

The present study was designed to investigate mechanistic Cu toxicity across a range of salinities from FW to SW, testing the hypothesis that the mechanism of Cu toxicity does not change with salinity. We chose to eliminate species-specific effects among the different salinities through the use of a euryhaline fish, *Fundulus heteroclitus*. We employed endpoints that have classically been measured in FW toxicity studies: gill Na^+/K^+ ATPase activity, whole body [Na^+], whole body [Cl^-], ammonia flux, and Na^+ flux. Because of the physiological differences between a FW and SW acclimated fish and the importance of the intestine in SW, Na^+/K^+ ATPase activity was also measured in

the intestine. Finally, CA activity, which has been demonstrated to be inhibited during waterborne Cu exposures in crustaceans (Vitale et al., 1999) and in fish during silver exposure (Morgan et al., 2004) potentially inhibiting Cl^- uptake at the gill and intestine (Perry et al. 2003; Marshall and Grosell, 2005), was measured in both the gill and intestine.

We demonstrate that Cu is more toxic in FW and SW than at intermediate salinities and the mechanism of Cu toxicity appears to be different in FW and SW. This difference in potential mechanism of Cu toxicity across salinities and differences between fish species suggest that physiology rather than Cu geochemistry plays a crucial role in determining how sensitive an organism is to Cu and how salinity alters Cu toxicity.

3.3 Materials and methods

3.3.1 Experimental animals

Fundulus heteroclitus (1.5 to 11.2 g) were collected north of St. Augustine, FL in the Atlantic Intracoastal Waterway near the Guana River by the Whitney Lab, University of Florida and shipped to Miami. They were acclimated to the lab and their test salinity for at least two weeks prior to the Cu exposure as outlined previously (Blanchard and Grosell, 2005).

3.3.2 Cu exposures and sampling

Two separate copper exposures were performed. The initial exposure was a thirty-day flow through copper exposure explained briefly below and in detail in Blanchard and Grosell (2005). A second follow-up 24 hour static exposure was

performed after the initial 30 day exposure because no effects were observed in most of the physiological and biochemical parameters. These experiments were performed to determine whether the lack of changes in these parameters was due to differences between the killifish and other fish examined to date or because the fish were not sampled on a tight enough time scale in the initial thirty day exposure. The brief exposure time was chosen to catch any transient, early effects of Cu exposure. Also because no ionoregulatory effects were observed in SW yet mortality was high, these experiments allowed us to examine the ammonia excretion as a potential reason for the observed mortality in SW.

The first was a thirty-day flow through exposure at five different salinities (FW, 5 ppt, 11 ppt, 20 ppt, and 28 ppt (SW)) and three Cu concentrations (Table 3.1 a). Fish were kept in 24 liter tanks at a flow rate of 100 mL min^{-1} and fed daily except prior to sampling when food was withheld for 48 hours. Detailed water chemistry including inorganic speciation calculations for the test water is also reported in Blanchard and Grosell (2005) and there were no significant differences among the salinities in dissolved organic carbon concentrations (0.1607 ± 0.0015 mM) or pH (8.00-8.39). Inorganic speciation calculations using the measured water chemistry of the test waters indicate that the free ion percentage is 3.30% in FW, 4.96 % in 5 ppt, 5.00% in 11 ppt, 4.76 % in 20 ppt, and 4.52% in SW (Blanchard and Grosell 2005).

The second Cu exposure was a 24 hour static Cu exposure at two Cu concentrations (Table 3.1 b) and four salinities: FW, 5, 13, and 29 ppt (SW). Cu concentrations are reported as the mean of the initial and the final water samples because there was little change over the 24 hour period. The salinities were selected based on

their physiological importance: FW and SW are the ionoregulatory extremes, high copper accumulation was observed at 5 ppt (Blanchard and Grosell 2005), and 13 ppt is near the isoosmotic point. There were ten fish per treatment during the static exposures which were used in the ammonia and Na^+ flux experiments described below.

All fish were euthanized (Tricaine methanesulfonate, MS-222; 0.2 g L^{-1}) and gills and intestines were dissected out and flash frozen in liquid nitrogen for analysis of CA activity and Na^+/K^+ ATPase activity.

3.3.3 Whole body Na^+ and Cl^- Concentration

Whole body [Na^+] and [Cl^-] were measured using a Varian 220FS flame AAS (Mulgrave, Australia) for Na^+ and a Dionex DX-120 ion chromatograph (Sunnyvale, CA, USA) for Cl^- after heat assisted (70ºC) digestion in 1 N sulfuric acid (Trace metal grade, Fisher Scientific) overnight.

3.3.4 Ammonia and Na^+ flux

Since measurements of ionoregulatory endpoints did not indicate physiological disturbances in SW fish despite Cu induced mortality, ammonia excretion was considered as a potential cause of Cu toxicity in SW. After twenty hours of exposure, fish in the static Cu exposure were transferred to small individual flux chambers (100 mL exposure water per chamber) and allowed to acclimate for 45 minutes. The Na^+ flux measurements were made in FW only, where ^{22}Na (Amersham, Buckinghamshire, UK) was added to each chamber (~1 μCi / chamber) fifteen minutes prior to taking the first water sample. For both ammonia and Na^+ flux measurements, water samples were taken at 1, 2, 3, and 4

hours post transfer. The ^{22}Na activity was measured on a Perkin-Elmer Packard Cobra II Auto Gamma_counter (Wellesley, MA, USA) in water samples and total Na^+ concentration was determined by flame AAS (Varian 220FS flame AAS, Mulgrave, Australia).

The ammonia flux measurements were carried out simultaneously with the Na^+ flux in FW where two water samples were taken instead of one at each time point. At the other three salinities the procedure was the same as for the Na^+ flux. Ammonia concentrations in the water were measured using the indophenol blue spectrophotometric method as described in Pai et al. (2001) and measured on a Molecular Devices Spectramax Plus 384 microplate reader (Sunnyvale, CA, USA).

3.3.5 CA and Na^+/K^+ ATPase activity

CA activity was measured at 4ºC employing the ΔpH method as described in Tufts et al. (1999) using a water jacketed reaction chamber of 2 mL and a Radiometer Analytical pHc 4000 combined pH electrode (Lyon, France) coupled to a Radiometer Analytical PHM 220 lab pH meter (Lyon, France). The pH meter was interfaced with a PC allowing for high resolution data collection. The Na^+/K^+ ATPase activity was

determined in gill and intestinal homogenates as described by McCormick (1993) and both CA and Na^+/K^+ ATPase activity were normalized to total protein content of the sample determined by the Bradford assay (Bradford,1976).

3.3.6 Cu analysis

Cu was analyzed by graphite furnace atomic absorption (Varian 220Z, Mulgrave, Australia). During the thirty day exposure, Cu in salinities other than FW was coprecipitated with iron to remove matrix interference from Na^+ (Weisel et al., 1984). In the 24 hour exposure, Cu was extracted using a solvent extraction procedure described in Kinrade and Van Loon (1974) with subsequent back extraction as described by Danielsson et al. (1978) for all samples except FW. For both analyses, standards made from commercially available certified solutions (SPEX CertiPrep, Metuchen, NJ) in the appropriate medium were subjected to the coprecipitation and solvent extraction procedure to assess extraction efficiency. Measured values were corrected according to extraction efficiency which in all cases was > 90 %. Total Cu concentrations are given because little difference (<5%) was observed between total and dissolved Cu concentrations (Blanchard and Grosell, 2005).

3.3.7 Calculations and Statistical analysis

Na^+ uptake was calculated from the disappearance of ^{22}Na from the exposure chamber, the length of time of the flux, the weight of the fish, the specific activity for that flux period as described in detail previously (Grosell et al. 2000). Net Na^+ and ammonia

flux were determined by the change of the total [Na^+] and total [ammonia] in the exposure chamber over the flux period and all values are based on the two flux periods combined.

Data are presented as means ± SEM. Where appropriate, one-way analysis of variance (ANOVA) was performed to evaluate potential differences from control and differences among salinities. Where data did not fit the assumptions of the ANOVA (i.e. the data were not normally distributed or did not have equal variances), a Kruskal-Wallis analysis of variance on ranks was performed (Sokal and Rohlf, 1995). Due to differences among controls over time and across salinity, all comparisons were made to a simultaneous control and all *P*-values were subjected to a Bonferroni multi-comparison correction for the ANOVA's and a Dunn's multi-comparison correction for the Kruskal-Wallis analysis for each comparison independently. The Mann Whitney U-test (for data that were not normally distributed or have equal variance) or *t*-tests were used to determine differences from control for the flux data and the 24-hour enzyme activity data. A P-value < 0.05 was considered significantly different throughout. All statistical analysis was performed using Sigma Stat 3.0 (Chicago, IL, USA). Where statistical tests indicated no differences data were pooled from the two experiments.

3.4 Results

3.4.1 Mortality

During the thirty day exposure mortality was highest in FW (50% mortality by day 7)and SW (50% mortality by day12)(Figure 3.1). There was some mortality after day 20 at 5 ppt which could be due to a more chronic effect of Cu or due to social

interaction as indicated by mortality in the control and low Cu treatments. Control animals fared worse than animals exposed to the low Cu treatment which could be a result of prevention of infection by the low levels of Cu or through alteration of social behaviour (Scott and Sloman, 2004).

3.4.2 Whole body [Na^+] and [Cl^-]

Whole body [Na^+] and [Cl^-] responded as expected (Laurén and McDonald, 1987a; Laurén and McDonald, 1985) in the FW exposure whereas they demonstrated no response in SW (Figure 3.2) or at intermediate salinities. Consequently, Table 3.2 presents data from the intermediate salinities and SW as single means for all values except FW which is the mean of all control values. It is interesting to note that the control [Na^+] is significantly higher in SW fish than at the other salinities and that a similar trend exists in the Cl^- data.

Whole body [Na^+] was significantly lower than the corresponding control value at day 4 at both 146 ± 2.6 µg Cu L^{-1} and 23 ± 0.7 µg Cu L^{-1} treatments in FW. The fish in low Cu recovered their Na^+ by day 30 whereas in the 146 ± 2.6 µg Cu L^{-1} treatment all fish died by day 12. The [Cl^-] was reduced (significantly at day 12 and 30) at all time points measured in 23 ± 0.7 µg Cu L^{-1}, but no change was observed in 146 ± 2.6 µg Cu L^{-1}.

3.4.3 Enzyme activity

The only change observed in enzyme activity was the expected (Laurén and McDonald, 1987b) decrease in Na^+/K^+ ATPase activity in the gill at 24 hours in the FW Cu exposure (Figure 3.3). Both CA and Na^+/K^+ ATPase activity showed no Cu induced change at any of the other time points, salinities, or tissues and as such are presented as single means for each salinity in Table 3.3. A trend towards salinity dependence of branchial CA activity was observed in the gill, which displayed the highest CA activities in FW and SW and the lowest activity at near iso-osmotic salinities. Furthermore, Na^+/K^+ ATPase activity was found to be higher in the intestine than in the gill whereas the opposite was true for CA.

3.4.4 Flux data

The fish exposed to 120 ± 0.2 μg Cu L^{-1} in FW for 24 hours demonstrated a significant decrease in net Na^+ uptake as expected (Figure 3.4 a) (Laurén and McDonald, 1987a; Laurén and McDonald, 1987b). This decrease in net flux was caused by a decrease in influx which is likely attributable to the decreased Na^+/K^+ ATPase activity in the gill (Figure 3.3). There was no change in net ammonia flux in the FW exposed fish (Figure 3.4 b), but, in contrast, there was a significant reduction in net ammonia flux in SW.

3.5 Discussion

3.5.1 Water chemistry and mortality

Detailed water chemistry and modeling of Cu speciation on the test waters is presented elsewhere (Blanchard & Grosell, 2005). Due to relatively high pH in the freshwater employed and similar concentration of dissolved organic carbon across salinities only slight differences in the fraction of Cu existing as the free Cu^{2+} ion across salinities occurred.

Considerable control mortality occurred at some salinities presumable due to social (aggressive) interactions among individuals. However, despite this variable control mortality, Cu exposures at the extreme salinities (FW and 28 ppt) resulted in apparent Cu-induced mortality. This pattern of mortality compares well with observed physiological disturbances discussed in the following.

3.5.2 Mechanism of Cu toxicity

These experiments tested the hypothesis that Cu would act via the same general mechanism regardless of salinity. According to this hypothesis, fish below their isoosmotic point (~12 ppt) would suffer from a decrease in plasma Na^+ (Laurén and McDonald, 1987a; Laurén and McDonald, 1987b; Laurén and McDonald, 1985). This decrease in plasma Na^+ would be caused by diffusive loss of Na^+ to the environment and an inhibition of Na^+/K^+ ATPase by Cu in the gill causing a decrease in compensatory Na^+ uptake. Above the isoosmotic point, an increase in plasma Na^+ would be expected from inhibition of Na^+/K^+ ATPase in the gill by Cu (Marshall and Grosell, 2005; Loretz, 1995) and at near isoosmotic salinities limited effects would be expected.

The overall expected pattern across salinities of an ionoregulatory toxicant would be high toxicity in FW and in SW and the least toxicity would be at ~12 ppt where the external and internal Na and Cl concentrations are appromimately equal.. Thus the organism at 12 ppt would have no diffusive loss to the environment and an inhibiton of compensatory sodium uptake via Na^+/K^+ ATPase would have little to no effect on the organism. This is fully consistent with the pattern we observed where no Cu related acute mortality was seen in fish at intermediate salinities in contrast to both SW and FW where high mortality occurred (Figure 3.1).

The physiological mechanism of Cu toxicity at intermediate salinities can not be addressed because no apparent toxicity was observed and none of the measured physiological or biochemical parameters changed. In FW, the expected mechanism of toxicity was observed: A decrease in whole body Na^+ concentrations occurred (Figure 3.2), caused by inhibition of Na^+ uptake (Figure 3.4) which presumably was associated with the decrease in Na^+/K^+ ATPase activity (Figure 3.3). This phenomenon likely contributed to mortality. The decrease in whole body Na^+ concentrations, Na^+ uptake, and Na^+/K^+ ATPase activity have been demonstrated previously (Laurén and McDonald, 1987a; Laurén and McDonald, 1987b) and it is interesting to note that in spite of full recovery of Na^+/K^+ ATPase by day four (data not shown) mortality continues (Figure 3.1a). However, at the lower Cu concentration tested (23 ± 0.7 µg Cu L^{-1}) the decrease in whole body [Na^+] is the same as at the high concentration (146 ± 2.6 µg Cu L^{-1}) (Figure 3.2a) yet no mortality occurs (Figure 3.1c). This could be because the fish that survived four days at 146 ± 2.6 µg Cu L^{-1} are better at maintaining their whole body Na^+ levels or that the perturbation of Na^+ homeostasis is only part of the story in FW.

In addition to the gill, the intestine is also a likely target for Cu toxicity in seawater as outlined in the introduction. The intestine is responsible for taking up water to compensate for the diffusive loss of water to the concentrated external environment (Marshall and Grosell, 2005; Loretz, 1995). In order to accomplish this, the intestine absorbs Na^+ and Cl^- which are then excreted at the gill. In the intestine, the Na^+/K^+ ATPase is responsible for both Na^+ and Cl^- transport (Marshall and Grosell, 2005; Grosell and Genz, 2006), and Cu-induced inhibition of Na^+ / K^+ ATPase would result in reduced intestinal water uptake. The resulting dehydration would be evident from elevated $[Na^+]$ and $[Cl^-]$ in plasma and whole body.

However, in contrast to our hypothesis, there was no effect on Na^+ homeostasis in SW. We observed no inhibition of Na^+/K^+ ATPase activity in the gill (Figure 3.3) or the intestine and no increase in whole body Na^+ concentrations (Figure 3.2). The only parameter measured that indicated any effect is net total ammonia flux (Figure 3.4 b) which could contribute to mortality through disturbing acid base balance or allowing the accumulation of toxic levels of ammonia. This decrease in net ammonia excretion is consistent with earlier observations in both FW (Laurén and McDonald, 1985) and SW fish exposed to Cu (Grosell et al., 2004a, Wilson and Taylor, 1993; Grosell et al., 2003) and likely a result of impaired excretion rather than decrease in production (Wilson and Taylor, 1993). In FW, CA is known to be involved in ammonia transport as inhibition of CA leads to a decrease in ammonia excretion (Wright et al., 1989). However, the results obtained here suggest that even though Cu inhibits CA in crustaceans (Vitale, 1999) it has no effect on CA in *Fundulus heteroclitus*.

The present results contradict one of the major findings reported in most of the literature existing on the physiological responses of fish to Cu exposure in SW, demonstrating ionoregulatory disturbances (Grosell et al., 2004a; Wilson and Taylor, 1993; Larsen et al., 1997; Stagg and Shuttleworth, 1982a). However, most of these studies observe an increase in plasma [Na^+] within 4 days of exposure to Cu and use very high Cu concentrations (> 400 μg Cu L^{-1}). It appears that the likely cause of ionoregulatory disturbances at these high Cu concentrations is increased gill permeability (Grosell et al., 2004a; Wilson and Taylor, 1993; Larsen et al., 1997) because no decrease in Na^+/K^+ ATPase activity was observed by Grosell et al. (2003), Grosell et al. (2004b), or Stagg and Shuttleworth (1982b).

A single study (Stagg and Shuttleworth 1982a) used Cu concentrations similar to those employed in this study (170 μg of Cu L^{-1}), and observed no effect on Na^+ until day 35 and on Cl^- until day 21 suggesting a more chronic response. In agreement, Grosell et al. (2003) used similar Cu concentrations (110 μg of Cu L^{-1}) and observed no ionoregulatory effect after seven days of exposure. In the present study, only one fish survived in the high Cu treatment in SW until day 30 and therefore little can be said about any chronic effect on ionoregulation. However, the lack of mortality observed in previous studies, in spite of ionoregulatory effects (Grosell et al., 2004a; Wilson and Taylor, 1993; Larsen et al., 1997; Stagg and Shuttleworth, 1982a), and the agreement between a decrease of net ammonia flux observed here and an increase in plasma ammonia observed in other studies (Grosell et al., 2004a, Wilson and Taylor, 1993; Grosell et al., 2003) suggest that the ionoregulatory effects of Cu are less detrimental than the effect on net ammonia flux in SW.

3.5.3 The BLM and SW

The BLM considers water chemistry to estimate toxicity by calculating the speciation of Cu; only the free ion, Cu^{2+}, and $CuOH^{+}$ are considered to be toxic in the BLM analysis (USEPA, 2003; Paquin et al., 2002). When the Cu concentration is kept constant, these two species increase slightly from FW to SW (Blanchard and Grosell, 2005) while the toxicity decreases. The BLM also includes competition between Cu^{2+} and $CuOH^{+}$ and cations in the water for gill binding sites (USEPA, 2003; Paquin et al., 2002) which should greatly reduce copper toxicity in SW. When increasing the salinity from FW to SW the concentrations of the cations increase by two orders of magnitude which theoretically should eliminate or greatly reduce any possible toxic interaction between Cu and its biotic ligand. Thus, one would expect, if the BLM principles applied to SW, that Cu toxicity would decrease as salinity increased and that the highest salinity would be the least toxic if everything else was held constant.

In this experiment, we observed mortality in FW and SW. According to the measured Cu concentrations (Table 3.1) and chemical speciation calculations (Table 3.4), the free ion concentration would be higher in SW than FW. Dissolved organic carbon (DOC) was the same across all treatments in this experiment (Blanchard and Grosell, 2005) and thus would have only a minor influence on the copper toxicity. Therefore, the toxicity of copper should be governed by cation competition according to the BLM. However, the mortality does not follow this pattern. The higher free Cu ion concentration can not explain the high mortality in SW because at lower salinities, where

there are fewer competing cations, there was an even higher amount of free Cu ion yet no mortality. This suggests that Cu toxicity in saline environments is governed by physiology rather than copper geochemistry.

An alternate explanation for this, is that the assumptions regarding the relative toxicity of the different species of Cu in saline environments are incorrect. The relative amounts of Cu^{2+} and $CuOH^{+}$ remain fairly constant across salinities (Blanchard and Grosell, 2005) and therefore competing cations should govern the toxicity which is not what we observed. In contrast, $Cu(CO_3)_2^{2-}$ which is generally considered non toxic (USEPA, 2003; Paquin et al., 2002), increases about six fold from FW to SW. Because of the discrepancies between the observations and theoretical predictions, the potential toxicity of $Cu(CO_3)_2^{2-}$ should at least be reevaluated.

The BLM also utilizes a correlation between toxicity and short term gill binding to predict toxicity. Although we do not have short term gill binding data, we do have 96-hour gill and intestinal Cu accumulation data (Blanchard and Grosell, 2005). These data suggest that there is little to no Cu accumulation in SW in the killifish over the first four days and even out to thirty days in these tissues which is in marked contrast to FW where Cu rapidly accumulates in the gill even at low Cu concentrations. In studies on the toadfish using high Cu concentrations, Grosell et al (2004b) observed Cu accumulation in the intestine and the gill yet there was no mortality. These observations suggest that there is unlikely to be a correlation between short term gill or intestinal binding and subsequent toxicity.

3.5.4 Cu and environmental regulation

Our data indicate that Cu is least toxic at intermediate salinities. For regulatory purposes, the EPA suggests the use of the SW water quality criterion for salinities above 10 ppt and the lower of the FW and SW water quality criterion for salinities from 1 to 10 ppt (USEPA, 2002). This idea is supported by the data for salinities from 5 ppt to 28 ppt because toxicity is only observed at 28 ppt, however, two issues should be considered. The first is that from FW to 5 ppt, speciation models predict increases in what are thought to be the most toxic forms of Cu, Cu^{2+} and $CuOH^{+}$ and Cu accumulation appears to be very high at 5 ppt (Blanchard and Grosell, 2005) despite increased cation concentrations and thus competition. These two observations suggest that salinities between 1 ppt and 5 ppt ought to be more thoroughly investigated. The second is that the salinity of oceanic SW is 35 ppt in contrast to the highest salinity employed in the present study (28 ppt). Therefore, the trend of increasing Cu toxicity with increasing salinity ought to be evaluated at least to 35 ppt, which may potentially verify the EPA's recommendations for application of water quality criteria.

The present observations complicate the choice of endpoints for assessing Cu toxicity across salinities. In FW at the ninety six hour time point, fish exposed to 146 µg of Cu L^{-1} had the same whole body $[Na^{+}]$ as those exposed to 23 µg of Cu L^{-1} (Figure 3.2). The fish exposed to the low concentration had whole body Na^{+} levels similar to controls by day thirty but those in the high concentration died within 11 days of exposure. Whole body Cl^{-} concentrations showed no disturbance in the high concentration but displayed a significant drop in whole body Cl^{-} concentrations at the low concentration with no recovery and no mortality. A change is Na^{+}/K^{+} ATPase activity was only evident

in the first twenty hours of exposure (Figure 3.3) but not at later time points despite continued exposure. These observations make it difficult to select an appropriate parameter in the search for sensitive non-lethal endpoints but also when the best time to assess a given endpoint is.

3.5.5 Conclusions

The mechanism of Cu toxicity in SW remains unclear, but acute water borne exposure appears to disrupt nitrogen metabolism (Grosell et al., 2004a, Wilson and Taylor, 1993; Grosell et al., 2003; Figure 3.4 b). This mechanism has to date been considered a secondary effect of Cu toxicity and how Cu affects nitrogen metabolism is unknown (Laurén and McDonald, 1985). The apparently different mechanisms in FW and SW may explain why some of the assumptions (competing cations, a relationship between accumulation and toxicity, and the relative toxicity of the different forms of Cu (USEPA, 2003; Paquin et al., 2002)) of the BLM do not seem to apply to SW. A BLM for SW may require more detailed understanding and modeling of the organism's physiology because physiology rather than just chemistry seems to be dominating the effects of Cu in saline environments. Secondly, traditional test methods may need to be altered because potentially more sensitive endpoints (such as Na^+/K^+ ATPase activity or whole body [Na^+]) respond at different times during exposures. Thus, a 48 or 96 hour endpoint may not capture the acute response. Finally, our data suggests that the EPA's current recommendations for the application of the Cu water quality criteria may be overprotective at intermediate salinities where no Cu induced mortality or physiological disturbance was observed.

Table 3.1 Cu concentrations of the exposure waters. Values presented are means ± SEM. for the thirty day copper exposure, $n = 8$ except for the 0 ppt high Cu concentration where $n = 5$. The values presented are the mean ± SEM of the initial and final Cu concentrations for the 24 hour static exposure.

Exposure	Salinity (ppt)	Control Total [Cu] (μg L^{-1})	Low Total [Cu] (μg L^{-1})	High Total [Cu] (μg L^{-1})
Thirty Day Flow -through	0	0.37 ± 0.22	22.58 ± 0.74	146.35 ± 2.57
	5	0.84 ± 0.10	18.45 ± 0.85	179.85 ± 15.51
	11	1.10 ± 0.08	31.81 ± 0.73	174.12 ± 30.68
	20	0.63 ± 0.09	33.54 ± 0.82	169.19 ± 26.07
	28	0.83 ± 0.18	29.66 ± 4.12	147.12 ± 18.37
Twenty Four Hour Static	0	0.50 ± 0.16		119.82 ± 0.18
	5	1.17 ± 0.18		131.03 ± 3.87
	13	1.52 ± 0.12		179.55 ± 6.10
	29	1.35 ± 0.25		113.76 ± 2.77

Table 3.2 Whole body [Cl^-] and [Na^+] presented as means ± SEM. "a" indicates a significant ($P < 0.05$) from 0, 5, and 15 ppt. "b" indicates a significant difference (ANOVA, $P < 0.05$) from 0, 5, 11, and 15 ppt. Data from both exposures and all treatment groups were pooled after determining statistically that they were not different for all salinities except in FW where only the control values from both exposures were pooled.

Whole Body Chloride				Whole Body Sodium			
Salinity (ppt)	[Cl^-] (mM)		*n*	Salinity (ppt)	[Na^+] (nM)		*n*
0	36.23 ± 2.19		13	0	47.97 ± 1.36		20
5	45.38 ± 1.06		50	5	47.70 ± 0.76		50
11	51.52 ± 0.85	a	41	11	48.28 ± 0.45		41
15	39.98 ± 1.36		14	15	44.21 ± 1.95		14
22	52.57 ± 1.43	a	45	22	49.07 ± 0.51		45
28	52.79 ± 1.20	a	54	28	52.20 ± 0.73	b	54

Table 3.3 CA activity and Na^+/K^+ ATPase activity in the gill and intestine. Values presented are means ± SEM. "a" indicates a significant difference (ANOVA, $P < 0.05$) between 0 and 5 ppt. "b" indicates that 15 ppt is significantly different (ANOVA, $P < 0.05$) from 5, 22, and 28 ppt. "c" indicates a significant difference (ANOVA, $P < 0.05$) between 0 and 15 ppt. Data from both exposures and all treatment groups were pooled after determining statistically that they were not different for all salinities except for the Na^+/K^+ ATPase activity in FW and SW which pool only the thirty day exposure. The SW and FW 24 hour exposure Na^+/K^+ ATPase activity data are presented in Figure 3.3.

Tissue	Salinity (ppt)	Carbonic Anhydrase Activity (µmol H^+ µg $protein^{-1}$ min^{-1})				*n*	Na^+ / K^+ ATPase Activity (µmol ADP µg $protein^{-1}$ min^{-1})				*n*
Gill	0	0.794	±	0.081		25	0.813	±	0.111		28
	5	0.766	±	0.068		24	0.619	±	0.060		30
	11	0.683	±	0.081		10	0.829	±	0.118		19
	15	0.746	±	0.043		14	0.545	±	0.114		14
	22	0.766	±	0.101		16	1.058	±	0.185		21
	28	0.932	±	0.086		23	1.034	±	0.106		35
Intestine	0	0.094	±	0.009	a	35	2.196	±	0.311		23
	5	0.070	±	0.003		35	1.282	±	0.230		19
	11	0.080	±	0.001		21	2.266	±	0.391		6
	15	0.112	±	0.016	b	14	0.709	±	0.148	c	14
	22	0.073	±	0.001		21	1.778	±	0.301		6
	28	0.078	±	0.002		35	1.466	±	0.164		20

Table 3.4 Calculated inorganic speciation expressed as the percentage of total copper in the exposure waters for the thirty day copper exposure. (Millero and Pierrot, 1998)

Salinity (ppt)	Cu^{2+}	$CuOH^{+}$	$Cu(OH)_2$	$CuHCO_3^{+}$	$CuCO_3$	$Cu(CO_3)_2^{2-}$	$CuSO_4$
0	3.30%	4.45%	3.68%	0.15%	85.33%	3.04%	0.06%
5	4.96%	5.20%	4.44%	0.12%	77.37%	7.30%	0.61%
11	5.00%	4.88%	3.99%	0.11%	74.01%	11.24%	0.76%
20	4.76%	4.40%	3.30%	0.10%	70.67%	15.94%	0.82%
28	4.52%	4.03%	2.78%	0.09%	68.49%	19.09%	1.00%

Figure 3.1 Percent survival of *Fundulus heteroclitus* exposed to nominal 0 (a), 30 (b), 150 (c) µg Cu L^{-1} for 30 days in a flow through exposure. Panel (d) graphs the mortality as a function of salinity on day 13 illustrating the salinity dependence (Control = hatched bars; exposed to 150 µg Cu L^{-1} = clear bar). Please note that there was no mortality in the control fish at 28 ppt and the exposed fish at 5 ppt.

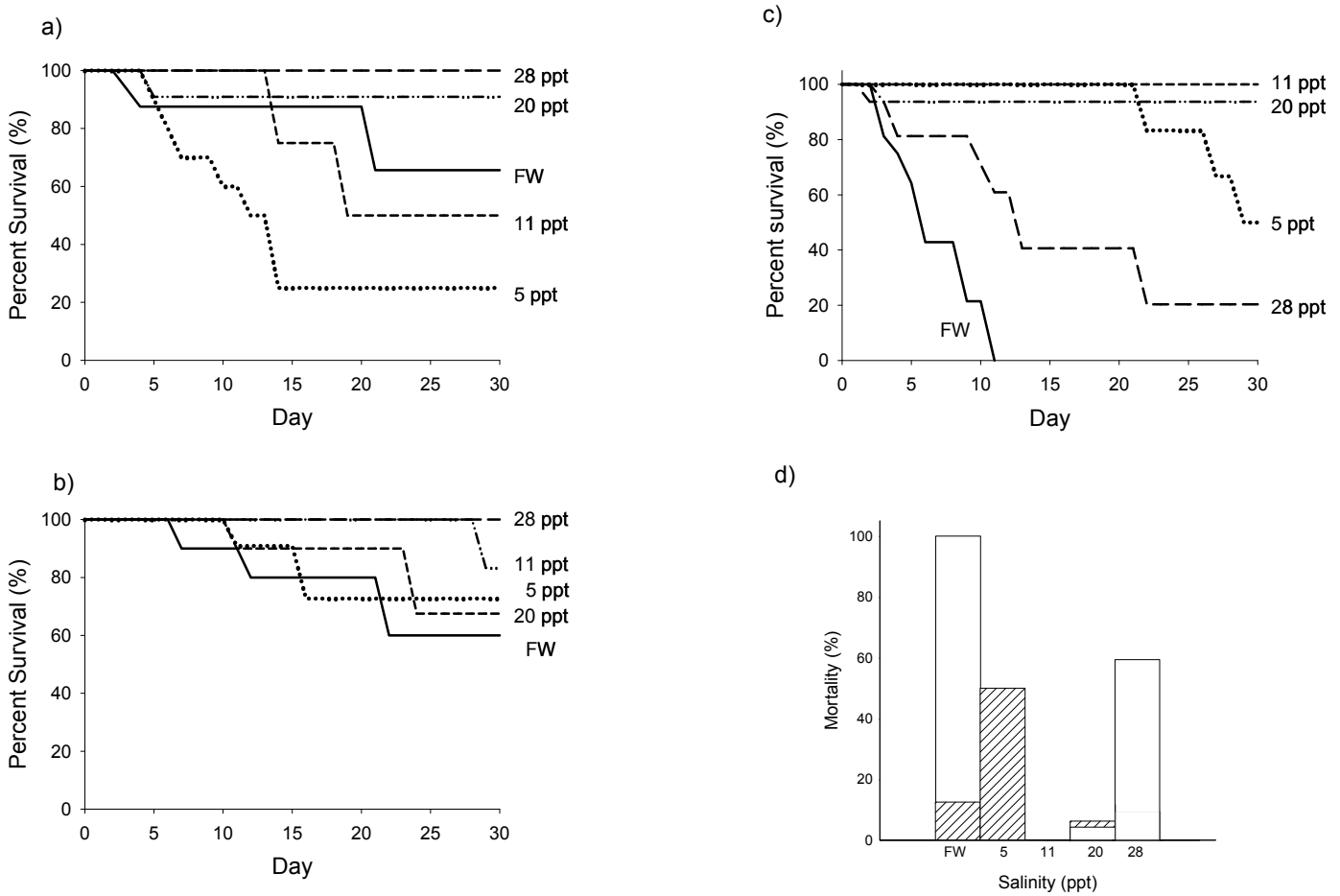


Figure 3.2 Whole body [Na^+] (a) and [Cl^-] (b) in fish exposed to Cu. Day 1 data were obtained in a twenty four hour static exposure to Cu at 120 ± 0.2 µg Cu L^{-1} in FW (control *n* = 7; exposed *n* = 8) and 114 ± 3 µg Cu L^{-1} in SW (control n=7; exposed n=7). Data for day 4 and greater were obtained from a thirty day flow through exposure at 146 ± 3 µg Cu L^{-1} (FW + High Cu; day 4 n=5) and 23 ± 0.7 µg Cu L^{-1} (FW + Low Cu; day 4 n = 5, day 12 n = 4, day 30 n = 3) in FW (control day 4 n= 5, day 12 n= 4, day 30 n = 3) and 147 ± 18 µg Cu L^{-1} (day 4 n = 5, day 12 n = 3, day 30 n= 1) in SW (day 4 n = 5, day 12 n = 5, day 30 n = 6). The low Cu concentration in FW was included to demonstrate the recovery of Na^+ and the altered pattern in Cl^-. "*" indicates significant difference (P<0.05) from simultaneous control and all data are presented as mean ± SEM. Please note that in panel (a) the FW + high is coincident with the FW + low Cu data point and in panel (b) the FW + high Cu is coincident with the FW **(b)** Con data point.

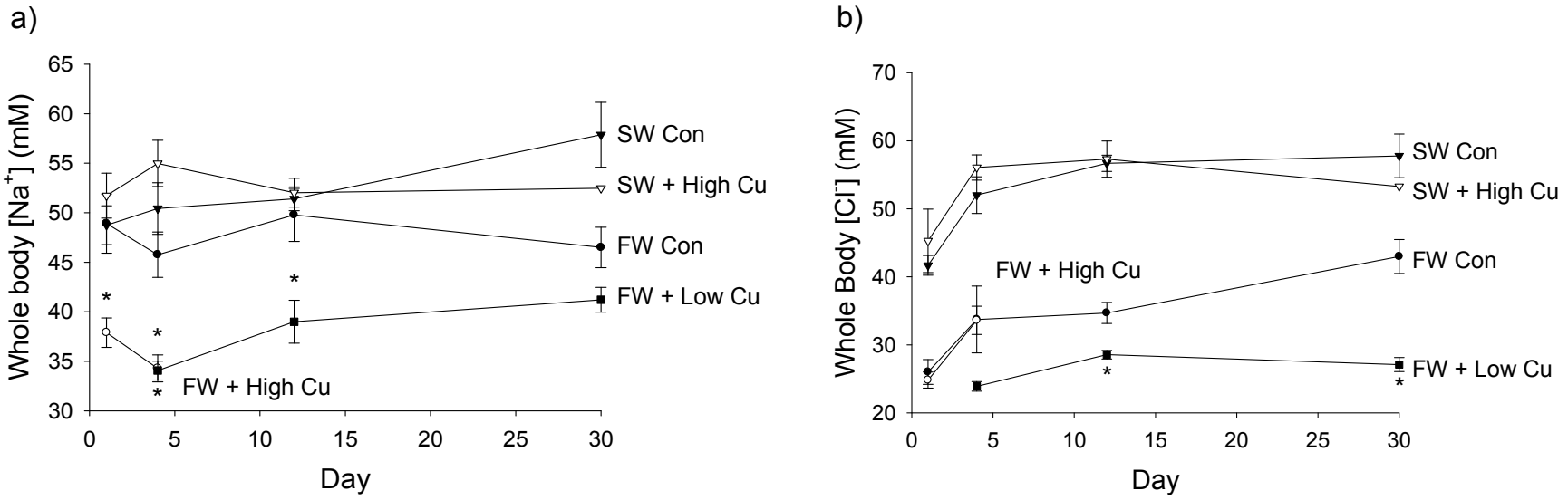


Figure 3.3 Na^+/K^+ ATPase activity in the gill of fish exposed for 24 hours to 120 ± 0.2 µg Cu L^{-1} in FW (control *n* = 7; exposed *n* = 8) and 114 ± 3 µg Cu L^{-1} in SW (control n=7; exposed n=7). "*" indicates significant difference ($P<0.05$) from simultaneous control and data is presented as mean ± SEM.

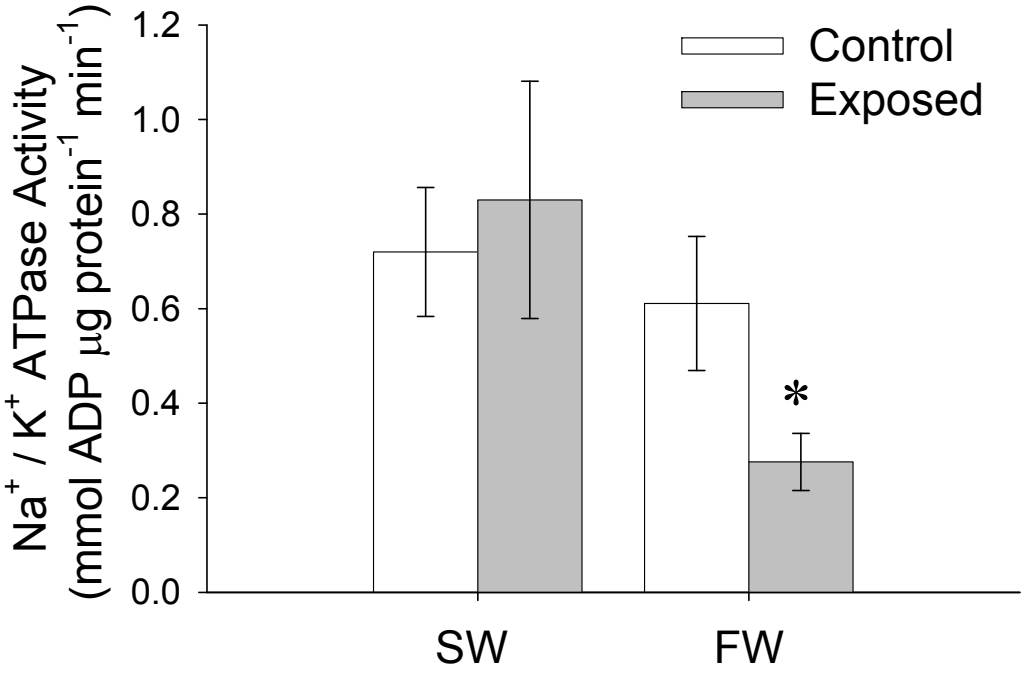
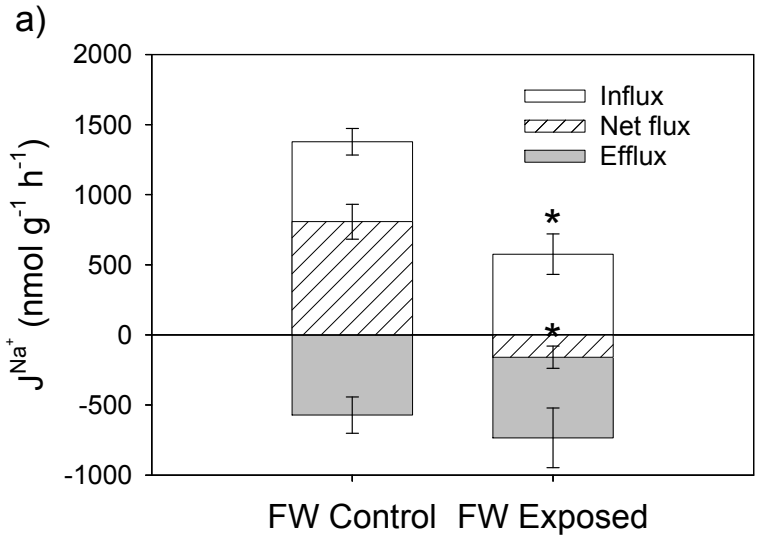
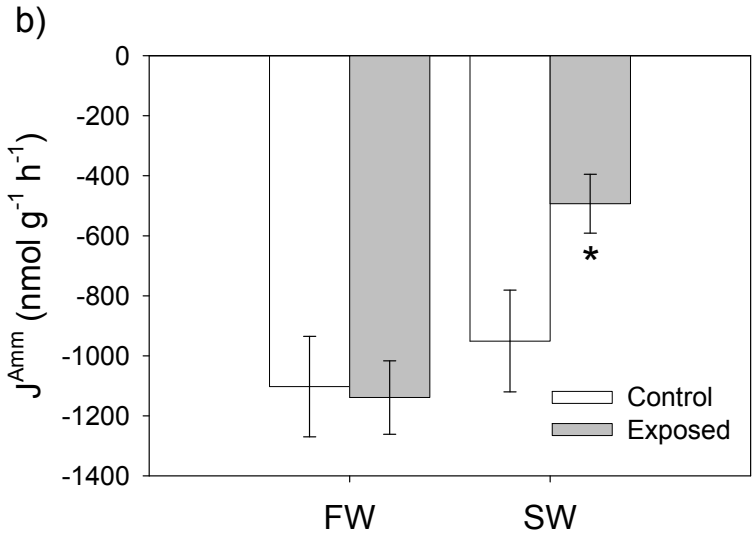


Figure 3.4 (a) Na^+ flux data for fish exposed to 120 ± 0.2 µg Cu L^{-1} in FW (control *n* = 7; exposed *n* = 8) for twenty four hours. (b) Ammonia flux data for at 120 ± 0.2 µg Cu L^{-1} in FW (control *n* = 7; exposed *n* = 8) and 114 ± 3 µg Cu L^{-1} in SW (control n=7; exposed n=7) for twenty four hours. "*" indicates significant difference ($P<0.05$) from simultaneous control and data is presented as mean ± SEM.





CHAPTER 4

SUBCELLULAR FRACTIONATION OF CU EXPOSED OYSTERS, *CRASSOSTREA VIRGINICA,* AND CU ACCUMULATION FROM A BIOLOGICALLY INCORPORATED CU RICH OYSTER DIET IN *FUNDULUS HETEROCLITUS* IN FRESH AND SEA WATER.

4.1 Summary

In order to examine the effect of salinity on Cu accumulation from a naturally incorporated diet, oysters were exposed in sea water for 96 days to four waterborne [Cu]: 2.9 ± 0.7 (control), 4.3 ± 0.6, 5.4 ± 0.5, and 10.7 ± 1.0 μg L^{-1}. After 96 days, the control whole body [Cu] increased from 2.1 ± 0.5 to 9.1 ± 1.1 μg g^{-1} w.w. and the highest [Cu] was 163.4 ± 27.1 μg g^{-1} w.w. in the oysters. Despite large differences in tissue [Cu], there was no effect on the fraction of trophically available metal in the oyster suggesting that trophic transfer will correlate well with tissue [Cu]. The control and highest [Cu] oysters became diet for killifish in fresh and seawater for 40 days. The two diets contained 84.7 ± 5.1 and 850.5 ± 8.8 μg Cu g^{-1} d.w. Fish were fed a combined diet of oyster and a pellet supplement (20.5 ± 1.0 μg Cu g^{-1} d.w.) both at 5% body mass day^{-1}. In killifish, Cu increased ~7% in gills and 100% in intestines after 6 weeks of exposure to the high Cu diet. No other tissues accumulated Cu above control levels. An 11-fold difference free Cu^{2+} concentrations was predicted in intestinal fluid between fresh and sea water, but there was no corresponding effect of salinity on intestinal Cu accumulation suggesting that Cu is not accumulated as the free ion.

4.2 Background

Many factors are thought to affect the accumulation and toxicity of metals from dietary sources. Spiking a diet with metal salts has been demonstrated to be less toxic than a diet in which the metal has been incorporated naturally (Hook and Fisher, 2001; Bielmyer et al., 2006; DeSchamphalaere et al., 2007). One reason for this is that natural incorporation may allow the metal to bind to complexes in the organism including amino acids that make the metal more available during digestion (Wapnir, 1998; Glover and Wood, 2008). This may account for the high toxicity observed in experiments using naturally incorporated diets.

Natural incorporation may also lead a change in the internal distribution of the metal in the organism that is being utilized as the diet. Subcellular fractionation techniques, such as those developed and utilized by Wallace and co-workers (2003), allow for the elucidation of what portions of the organism may be most available during digestion. They and others (Seebaugh et al., 2005; Rainbow et al., 2006) have found that there is a positive correlation between trophic transfer of a metal and the amount of metal found in the enzymes, organelles, and heat stable proteins which include the metallothionein-like proteins. In addition to these three trophically available fractions, the subcellular fractionation technique of Wallace et al. (2003) separates out two other pools of metal: metal rich granules and cellular debris. Both of these pools show poor correlations with trophic transfer and are less available to the consumer organism (Wallace et al., 2003; Seebaugh et al., 2005; Rainbow et al., 2006).

Another factor that may influence the uptake of metal from a dietary source is the gut fluid chemistry itself. This chemistry is different in unfed fish depending on the salinity to which the fish had been acclimated (Wilson, 1999; Marshall and Grosell, 2005; Grosell 2006). However, a recent study using the European flounder reported similar intestinal fluid chemistry from fresh (FW) and sea water (SW) acclimated fish up to twelve hours after feeding (Taylor et al., 2007). Whether these observations from European flounder apply generally across species or if ambient salinity influences gut fluid chemistry post feeding in other fish remains unknown at this time.

The gut fluid chemistry is especially important for Cu uptake in the intestine. If ambient salinity influences gut fluid chemistry post feeding, then the intestinal fluid in SW will contain high concentrations of Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, and HCO_3^- relative to FW (Wilson, 1999; Marshall and Grosell, 2005; Grosell, 2006). In addition, if the fish utilizes acidic digestion then the pH in FW is likely to be lower due to the high buffering capacity of the SW intestinal fluid and the low buffering in most FW. These differences in the gut fluid's physicochemical properties would cause a large difference in the speciation of Cu especially because of the high [HCO_3^-] and pH in marine fish intestinal fluids.

This study set out to examine several of the factors that influence Cu uptake from a naturally incorporated dietary source. The first part of the experiment exposed oysters (*Crassostrea virginica*) to low waterborne [Cu] (<11µg L^{-1}) for 96 days. This organism is generally considered a Cu hyper-accumulator with unusually high Cu body burdens even at low levels of ambient Cu (Zamuda and Sunda, 1982) with little if any toxic effect. The oysters were subcellularly fractioned in order to assess whether the distribution of Cu in

the oyster changed with time or ambient [Cu]. We expected to see changes in the distribution with both time and ambient [Cu] as has been reported for other metals accumulated by invertebrates (Seebaugh and Wallace, 2004; Seebaugh et al., 2005).

The second aim of the present study was to examine the influence of gut fluid chemistry and salinity on Cu accumulation from a dietary source in killifish. To pursue this goal, we prepared a naturally incorporated Cu rich diet from the eastern oyster (*Crassostrea virginica*). These oysters were subsequently used as a diet for *Fundulus heteroclitus* which is fully euryhaline and can tolerate salinities from FW to SW (Wood and Marshall, 1994). Furthermore, Cu accumulation and its effects in this species have already been examined for waterborne exposures across a wide range of salinities (Blanchard and Grosell, 2005; Blanchard and Grosell, 2006). Fish were exposed to a high Cu diet in both FW and SW and the accumulation of Cu was measured for 40 days in gill, gonad, intestine, liver, and the remaining carcass. We expected to see Cu accumulation in both the intestine and the liver which would be higher in the FW fish due to intestinal physicochemical environment which would be more favorable to Cu uptake. The intestine was an expected site of accumulation because it is the direct site for dietary uptake while the liver is the main homeostatic organ in organisms.

4.3 Materials and Methods

4.3.1 Experimental animals

Oysters, *Crassostrea virginica,* were obtained from a commercial supplier (Apalachicola Bay, FL, USA) and were acclimated to aerated unfiltered Bear Cut SW (25º 43.9’ N 80º 09.7’ W, Miami, FL, USA) for 1 week prior to the exposure in 750 L

tanks under flow through conditions at a rate of 750 ml min^{-1} or 1080 L day^{-1} and a natural photoperiod at a salinity of 36.0 ± 0.7 ppt (mean ± Standard error of the mean (SEM)). The physical chemical characteristics of SW are listed in table 4.1 and the [Cu] in table 4.2.a.

Fundulus heteroclitus were obtained from Aquatic Research Organisms and were collected in Hampton, New Hampshire, USA. These fish were fed the dry pellet food described below while acclimated to the laboratory for a month in SW and then acclimated to either FW (Miami dechlorinated tap water) or Bear Cut SW at a salinity of 34.8 ± 0.2 ppt (mean ± SEM) (see Table 4.1 for details of water chemistry) for two weeks prior to the exposure under a 16:8 Light:Dark cycle. Fish were kept under flow through conditions at the appropriate salinity in 24 L tanks with the males and females separated. The tanks containing males held twenty one fish per tank while there were thirty five fish in each tank containing females. The difference in the number of males and females was to obtain proper breeding ratios for the planned breeding experiments at the end of the dietary exposure.

4.3.2 Oyster Cu exposure and sampling

Oysters were exposed to 4 concentrations of Cu under flow through conditions in circular outdoor tanks (~750 L) fed a continuous flow of unfiltered Bear Cut seawater at a 750 mL min^{-1}: 0 (Control), 4, 5, 11 µg L^{-1} (nominal, see Table 4.2.a for measured concentrations). The Cu was supplied via a peristaltic pump for 96 days. Water samples were collected periodically for determination of exposure [Cu] and the oysters were sampled on days 0, 60, and 96 of exposure. Five oysters were sampled at each time point

for subcellular fractionation using the method of Wallace et al. (2003) and five for whole body Cu analysis. At 96 days, the remaining oysters were sacrificed and kept at -20º C until used for the dietary exposure.

4.3.3 Diet preparation and dietary exposure

Two diets were prepared from the oysters that were exposed to 11 μg L^{-1} for 96 days and from the controls (only two diets were prepared due to a lack of substantial change in the subcellular fractionation of the oysters). The [Cu] in these diets is listed in Table 4.3. The oyster diet was prepared by homogenization in a blender followed by "pelleting" in an agarose matrix (see below) for a final composition of 50% oyster, 46% water, and 4% agarose (Sigma-Aldrich, St. Louis, MO, USA) by weight which gave a final total water content of 87.6 ± 0.8% (Mean ± SEM). The diets were prepared by homogenizing oysters adding half of the water to facilitate the homogenization. An agarose gel was prepared at the proper concentration to obtain 4% agarose in the diet with the remaining water and allowed to cool to ~37º C. This was added to the homogenized oysters, thoroughly mixed, and stored at 4ºC. Fresh diet was prepared approximately every 7 days.

This diet was fed to killifish for 40 days at a rate of 5% body weight per day in the morning and was supplemented with a dry pellet food which was composed of 50% protein, 3% fiber, 16% fat, 0.35% Na, 2.2% Ca, and 1.3% phosphorous (Aquatic Eco-systems, Apopka, FL, USA; see table 4.3 for [Cu]) at the same rate in the evening. The killifish were kept in either FW or SW and the males (4.1 ± 0.1 g) and females (4.5 ± 0.1 g) were kept separated under flow through conditions. The light cycle was 16:8 light:dark

and the temperature was 26.0 ± 0.2º C. Fish were sampled at day 4, 10, and 40 post exposure for gill, liver, intestine, gonad, and the rest of the carcass. Prior to sampling fish were starved for 24 hours and were killed with an overdose of MS-222.

4.3.4 Gut fluid sampling

Ten fish (FW 6.9 ± 0.4 g, SW 6.3 ± 0.7 g) were acclimated to both FW and SW for at least 2 weeks prior to the experiment and were fed a single meal consisting of dry pellet food and sampled 8 hours later. The fish were killed with an overdose of MS-222 prior to sampling and the entire contents of the gut were removed. The gut contents were measured for pH, ionic composition, and total CO_2 by the methods listed below.

4.3.5 Analyses

All [Cu] were measured by graphite furnace atomic absorption spectroscopy (Varian Model 220 Z, Mulgrave, Australia) under manufacturer recommended conditions. Tissue samples were digested in 1 N trace metal grade nitric acid (Fisher Scientific, Pittsburg, PA, USA) overnight at 80º C and all samples were appropriately diluted prior to measurement. National Institute of Standards and Technology (USA) standard reference material 2976 (mussel tissue) was processed using the same tissue digestion procedure. We measured the tissue at 3.74 ± 0.04 mg Cu kg^{-1} which falls within the uncertainty reported and is 93% of the reported value for Cu in the tissue (4.02 ± 0.33 mg Cu kg^{-1}). All values presented are total Cu measurements as there was no difference between total and dissolved values. For the SW water samples, the Cu was removed from the matrix and resuspended in 1 % trace metal grade nitric acid before dilution using a

solvent extraction (Kinrade and Van Loon, 1974) and back extraction (Danielsson et al., 1978). All values were corrected based on extraction efficiency which was >90% in all cases.

Other cations were measured using flame atomic absorption spectroscopy (Varian Model 220 FS) under standard operating conditions after appropriate dilution and anions were measured on a Dionex DX 120 ion chromatograph (Sunnyvale, CA, USA). Total CO_2 was measured using a Corning 965 total CO_2 analyzer (Corning, NY, USA). In the exposure tanks, pH was measured using a Radiometer analytical PHM 201 portable pH meter fitted with a pHC 3005 combined pH electrode (Lyon, France) while the gut fluid pH was measured using a Radiometer analytical PHM 220 lab pH meter with an Accumet micro combination electrode (Fisher Scientific). Salinity was measured using a refractometer.

4.3.6 Statistical analysis

Sigmastat software (3.0) was used for statistical analyses. The oyster data were analyzed by a two way ANOVA using the Holm-Sidak pairwise comparison or an ANOVA on ranks with a Dunn's test for paired comparisons for data that were not normally distributed or have equal variances. The dietary data were analyzed using a four way ANOVA on ranks because of the lack of homoscadicity, followed by a least significant difference test for the paired comparisons. The gut fluid comparisons were two-tailed t-tests. All values presented are means ± the standard error of the mean and a *p*- value of <0.5 is accepted as significant throughout.

4.4 Results

4.4.1 Oyster Exposure

4.4.1.1 Exposure Concentrations

The exposure [Cu] remained fairly constant over time as reflected by the small error associated with the measured [Cu] (Table 4.2.a). However, the control water was fairly high in Cu (2.9 ± 0.7 µg Cu L^{-1}) and exceeded the current EPA chronic guideline of 1.9 µg L^{-1} (USEPA, 2003) in our oyster exposure tanks throughout the exposure period.

4.4.1.2 Cu accumulation in oysters

The oysters, including controls, accumulated a significant amount of Cu in all of the exposure concentrations (Figure 4.1). This accumulation increased with both time and exposure concentration. The oysters had an initial [Cu] of 2.1 ± 0.5 µg Cu g^{-1} w.w. at day 0 in the control water and significantly increased to 9.7 ± 1.1 µg Cu g^{-1} w.w. The whole body concentrations also increased in 4, 5, and 11 µg Cu L^{-1} from the initial concentration (2.1 ± 0.5 µg Cu g^{-1} w.w.) to 27.1 ± 4.9, 30.2 ± 4.6, and 163.4 ± 27.1 µg Cu g^{-1} w.w. respectively. It is interesting to note that the oysters at 5 µg Cu L^{-1} did not significantly increase their whole body Cu from day 60 to Day 96 and that the oysters at 4 µg Cu L^{-1} did not significantly accumulate Cu until after day 60 and yet still accumulated a similar amount of Cu compared to the oysters at 5 µg Cu L^{-1}.

4.4.1.3 Subcellular distribution

Cu had a significant effect on the distribution of Cu in the oysters in the cellular debris, the metallothionein-like fraction, and the enzymes (results from two-way ANOVA, $p < 0.05$). There was a significant effect of time and the interaction between time and Cu on the organelle fraction. There was no effect of either time or Cu on the metal rich granules. There are few paired comparison differences between the fractions within time or between times and most of these differences are from the higher exposure concentrations (Table 4.4). In particular, oysters exposed to 11 μg Cu L^{-1} had a significantly greater proportion of Cu in the metallothionein-like fraction than in the simultaneous control while the proportion of Cu in the enzymes was reduced.

However, in spite of significant differences in the organelle, enzyme, and metallothionein-like protein fraction, which make up the trophically available metal, there were no differences in the proportion of trophically available metal in the oysters (Table 4.4). The trophically available metal fraction was also unaffected by Cu exposure, time, or their interaction (results from two-way ANOVA, $p > 0.05$). The metal sensitive fraction, which consists of the organelle and the enzyme fractions, was affected by Cu, time, and their interaction while the biologically detoxified metal was affected only by the interaction of time and Cu. The paired comparisons, suggest that, as the exposure concentration and therefore [Cu] in the organism increases, the proportion of Cu in the metal sensitive fraction may decrease while the proportion detoxified may increase.

4.4.2 Dietary Cu exposure

4.4.2.1 Whole body ions

There was no effect of dietary Cu exposure on either whole animal cations (Na^{+}, Ca^{2+}, Mg^{2+}, and K^{+}) or Cl^{-} (data not shown).

4.4.2.2 Tissue [Cu] in killifish

Cu did not accumulate in the gonad (2.44 ± 0.47 µg Cu g^{-1}, n = 120), liver (34.09 ± 2.25 µg Cu g^{-1}, n = 120), or carcass (0.41 ± 0.01 µg Cu g^{-1}, n = 120) during the dietary exposure. Dietary [Cu] had a significant effect ($p<0.05$, results from the four way ANOVA) on [Cu] in the gill although there were few paired comparison differences (Figure 4.2.a and 4.2.b). There was also significant accumulation in the intestine which showed different patterns in males and females (Figure 4.3.a and 4.3.b). In females, there was a difference between the FW and SW fish whereas in males there was no difference between the two salinities. In males, accumulation increased over time. In females the [Cu] in FW was elevated at day 4 and returned to control levels by day 10 (Fig 3 a). At day 40, the females fed the high Cu diet had significantly lower [Cu] in their intestine then their corresponding control. In SW, the [Cu] were elevated at all days and the concentration was similar, however they were only significantly elevated relative to their control at day 4 and 40.

4.4.2.3 Gut fluid Chemistry

The pH, [Cl^-], and [K^+] of the gut fluids were not different in the two salinities (Table 4.5). However, the total CO_2, [SO_4^{2-}], [Ca^{2+}], and [Mg^{2+}] were significantly higher in the SW acclimated fish's gut fluid than the FW acclimated fish's gut fluid, while the [Na^+] was higher in FW fish gut fluid.

4.5 Discussion

4.5.1 Oysters

The oysters accumulated Cu even at low ambient Cu concentrations (2 fold increase in tissue concentration in the control oysters at 2.9 ± 0.7 µg Cu L^{-1}), and internal concentrations increased with increasing ambient Cu. The accumulation rate was higher in the control, 4, and 11 µg Cu L^{-1} groups in the final thirty days than during the first sixty which could potentially be explained by an increase in temperature over this period as the experiment was started in May and ended in August.

The subcellular fractionation of the oysters changed over 90 days, however there are no clear trends with time or concentration. It appears that at high whole body [Cu] the organism may detoxify a proportionally larger fraction of Cu in metallothionein-like protein, while the fraction detoxifying as metal rich granules fraction remains unchanged (Table 4.4). However, this increase in the proportion of Cu in the metallothionein-like protein fraction has no effect on the trophically available metal due to a simultaneous decrease in the proportion of Cu in the enzymatic fraction. This response is consistent with an organism trying to protect itself from the deleterious effects of a high Cu body burden. At higher exposure concentrations or longer time periods, the proportional

increase in the metallothionein-like protein fraction and decrease in the enzyme fraction may not offset each other directly leading to a potential increase in the trophically available metal fraction as proposed by Wallace et al (2003). However, at [Cu] below 11 μg L^{-1}, total [Cu] and the trophically available metal fraction are well correlated in these experiments. Therefore, the [Cu] in the trophically available fraction will be no better at predicting toxicity or accumulation than whole body [Cu] in this organism at low ambient [Cu].

4.5.2 Fundulus heteroclitus accumulation

In this study, Cu accumulated in the intestine and to some extent the gill. The gill has been noted to be a site of accumulation for dietary Cu in several studies (Miller et al., 1993; Kamunde et al., 2001; Kamunde et al., 2002 a). Branchial Cu accumulation could have come from the water but the [Cu] in the control and high Cu diet tanks did not differ (Table 4.2.b). An alternate explanation for branchial Cu accumulation during dietary exposure is that Cu is accumulated from the diet via intestinal absorption and subsequent transfer via the blood to the gills. In support of this proposal is a previous study demonstrating Cu accumulation in the gill from the blood side (Grosell et al. 2001).

The intestine is also a site in which dietary Cu accumulates commonly, however there are two distinct patterns of accumulation. In some cases, the intestine rapidly accumulates Cu and then the Cu remains at the same level throughout the exposure (Berntssen et al., 1999; Kamunde et al., 2001; Kamunde et al., 2002 a; Kamunde et al., 2002 b; Kamunde and Wood, 2003). At other times, the Cu concentrations increase

throughout the exposure period (Berntssen et al., 1999; Kamunde et al., 2001; Kamunde et al., 2002 a; Kjoss et al., 2005) as seen in the male intestinal tissue from the present study.

The liver is also a site in which many studies have seen accumulation of dietary Cu (Miller et al., 1993; Berntssen et al., 1999; Kamunde et al., 2001; Kamunde et al., 2002 a; Kamunde et al., 2002 b; Kamunde and Wood, 2003; Kjoss et al., 2005), but, as seen in this study, there are also instances in which this does not occur (Handy, 1992). Therefore, the tissue specific patterns of accumulation of dietary Cu observed here with a naturally incorporated Cu diet are not dissimilar to those seen for diets spiked with Cu salts. Although the patterns are similar, there appear to be no relationships between Cu dose, exposure duration, or salinity in regards to the amount of Cu accumulation. A review of the existing literature revealed that over a wide range of exposure times and concentrations no trends in accumulation exist between studies for the intestinal, hepatic, or branchial [Cu] (Figures 4.4.a, 4.4.b, and 4.4.c). The studies used in this analysis utilized different species, life stages, and exposure regimes which could account for some of the observed variability. But overall, the accumulation of Cu does not appear to be a good indicator of dietary Cu exposure and diets spiked with Cu salts appear to behave similarly to the naturally incorporated Cu diet used in the present study in that accumulation appears to be highly variable and occurs in the same tissues.

From the patterns of accumulation seen here it appears that Cu from dietary sources is dealt with differently by fish when compared to waterborne Cu (Blanchard and Grosell, 2005). During waterborne exposure Cu accumulates in the liver in both FW and SW, the gill in FW, and in the intestine in SW (Blanchard and Grosell, 2005) while liver

accumulation is not always seen in dietary Cu exposures (Handy, 1992). The reason for the difference in liver Cu accumulation could be that the form of Cu taken up in waterborne versus dietary exposures is different. During waterborne exposures, free ionic Cu is the form which is most likely to be taken up (Paquin et al., 2002), whereas in dietary studies the Cu could likely be taken up complexed to amino acids (Wapnir, 1998; Glover and Wood 2008). Also because fish can receive a large portion of their nutritive Cu from their diet (Kamunde et al., 2002 a), there is the potential that the cellular pathways used to transport this Cu are different from the pathways utilized by the gill perhaps leading to different internal dispositions of the Cu in the various tissues. Regardless of what leads to the differences in the pattern of accumulation, there are differences in the physiological effects of the Cu suggesting that there truly is a difference between Cu obtained through the diet and from the water. This is exemplified in a study by Milller et al. (1993) which demonstrates a difference increased resistance to waterborne Cu exposure after pre-exposure to waterborne Cu but not after pre-exposure to dietary Cu. This occurred even though gill Cu accumulation was attributable to the dietary Cu exposure.

4.5.3 Intestinal Cu Accumulation and salinity

In male fish, Cu accumulation in the intestine did not differ between the two salinities. In females, there were differences by day 40, but the amount of Cu in the intestine was much lower than in males at this point probably because female fish ate less

after day 10. Food intake was not quantified, but the amount of food that had to be siphoned from the tanks containing females increased after day 10. Therefore, the changes are probably due to differential feeding rather than salinity.

Differential feeding could also account for the greater amount of [Cu] found in the female control fish in FW relative to the female fish fed the high Cu diet. The control oyster diet still contained a relatively high [Cu] and if the control fish were eating some of the oyster diet while the fish exposed to the high Cu diet were not then we would expect them to have a higher [Cu] in their intestines. However, the food intake was not quantified and this cannot be verified.

The similarity between the two salinities however was not because of similarities in the gut fluid chemistry (Table 4.4). Unlike the Taylor et al. (2007) study, we found differences in several of the intestinal parameters measured. The SW fish had higher $[Mg^{2+}]$, $[Ca^{2+}]$, and $[SO_4^{2-}]$ than the FW fish because SW taken in with the food has much higher concentrations of these ions than FW (see Table 4.1) and these ions are not actively taken up in the gut (Marshall and Grosell, 2005). The $[HCO_3^-]$ was also significantly higher in the SW intestine because of active HCO_3^- excretion by the gut in SW which is thought to play a role in SW osmoregulation (Wilson 1999, Marshall and Grosell, 2005). Surprisingly, this had no significant effect on intestinal fluid pH suggesting the presence of non-HCO_3^- buffering in the intestinal lumen.

The $[Cl^-]$ were not different in spite of a ~200 fold difference in the ambient Cl^- which suggests high rates of Cl^- uptake in the SW intestine to aid water absorption and facilitate HCO_3^- excretion through Cl^-/HCO_3^- exchange (Marshall and Grosell, 2005). The $[Na^+]$ was higher in FW than in SW, which is caused by the active absorption of Na^+

by the SW intestine to aid water uptake (Marshall and Grosell, 2005). In FW, the gill is the site of Na^+ uptake to maintain homeostasis (Patrick et al., 1997; Marshall and Grosell, 2005) and the intestine plays a much smaller role. The difference between the [Na^+] and [Cl^-] occurs because killifish do not actively take up Cl^- at the gill (Patrick et al., 1997) and in FW the intestinal Cl^- uptake rate must be fairly high in order ionoregulate (Scott et al. 2006). Thus, some of the Cl^- from the food is taken up in FW leaving no difference between the two salinities.

Since the fish used in the present study were fed the same diet regardless of exposure salinity, the organic matrix of the intestinal fluids can reasonably be assumed to be the same regardless of Salinity. However, the differences in inorganic gut fluid chemistry between FW and SW discussed above have a profound effect on the speciation of Cu. Intestinal as well as internal Cu accumulation would therefore be expected to be different if inorganic speciation and competition controlled accumulation. Inorganic speciation calculations predict an ~11 fold difference in the free ion concentrations based on the gut fluid composition (Table 4.6) (Millero and Pierrot, 1998). Therefore, it is unlikely that Cu is being taken up solely as the free ion. Amino acid Cu complexes have been demonstrated to be taken up at different rates than both the amino acid alone and free Cu^{2+} (Wapnir, 1998; Glover and Wood 2008). These and similar complexes are likely to account for the Cu uptake observed due to the lack of pattern attributable to either inorganic speciation or competition.

It is also important to note that even without feeding, SW fish drink the ambient water and during waterborne exposures in SW will be exposed to waterborne Cu. The values presented in Table 4.4 are generally similar to the gut fluid of unfed fish (Marshall

and Grosell, 2005). The exceptions to this are the pH which in an unfed fish is higher (>8.0) and the HCO_3^- which is also generally higher. Table 4.6 (Millero and Pierrot, 1998) shows that Cu speciation in ambient SW and the intestinal fluid differ substantially with an ~35 fold difference in the free Cu^{2+} ion concentration between these two and the differences in gut fluid composition between unfed and fed fish should increase this difference further. It is likely that intestinal Cu accumulation during waterborne Cu exposures will be better correlated with Cu speciation in the gut fluid rather than the ambient SW.

4.5.5 Conclusions

The proportion of Cu in the trophically available metal fraction of oysters exposed to low levels of waterborne Cu for a prolonged period did not change Because of this, total [Cu] and the trophically available metal fraction will correlate equally well with assimilation efficiency and thus trophic transfer. However, as may be expected, the proportion of Cu in the biologically detoxified metal fraction may increase while it decreases in the metal sensitive fraction. The existing data suggest that there are no apparent differences between naturally incorporated and spiked diets and that dietary copper dose, duration, and salinity are unrelated to Cu accumulation. It appears that Cu assimilated from the diet is treated differently from waterborne Cu based on patterns of accumulation and acclimatory responses and therefore may have different toxic modes of action. Finally, the uptake of dietary Cu from a natural diet is not governed by inorganic speciation and is therefore unaffected by salinity.

Table 4.1 Physical and chemical characteristics of exposure waters.

	FW	SW
Salinity (ppt)	0.16	35
pH	8.29	8.16
Na^+ (mM)	2.0	438.1
K^+ (mM)	0.2	9.6
Mg^{2+} (mM)	0.3	53.4
Ca^{2+} (mM)	1.2	10.9
SO_4^{2-} (mM)	0.3	21.5
Cl^- (mM)	2.1	451.6
TA (mM)	1.9	2.2

Table 4.2.a Measured total copper concentrations to which oysters were exposed (mean ± SEM; $n = 15$). **Table 4.2.b** Measured total waterborne copper concentrations during the dietary copper exposure of killifish (mean ± SEM; $n = 8$).

Table 2. a. Nominal	Measured [Cu] ($\mu g\ L^{-1}$)
Control	2.9 ± 0.7
4 $\mu g\ L^{-1}$	4.3 ± 0.6
5 $\mu g\ L^{-1}$	5.4 ± 0.5
11 $\mu g\ L^{-1}$	10.7 ± 1.0

Table 2. b.	Male Control ($\mu g\ L^{-1}$)	Male Diet ($\mu g\ L^{-1}$)	Female Control ($\mu g\ L^{-1}$)	Female Diet ($\mu g\ L^{-1}$)
FW	3.5 ± 1.7	3.8 ± 1.2	6.7 ± 5.3	6.9 ± 2.6
SW	8.3 ± 1.6	10.9 ± 1.5	8.0 ± 1.5	6.9 ± 1.3

Table 4.3 Measured [Cu] (μg Cu g^{-1} dry weight) in the prepared oyster diets and the commercial pellet food. (n = 4)

Diet	[Cu]
Oyster Control	84.7 ± 5.1
Oyster High Cu	850.5 ± 8.8
Pellet	20.5 ± 1.0

Table 4.4 Subcellular fractionation (mean % ± SEM) of oysters exposed for 63 and 96 days to the indicated waterborne [Cu]. "a" indicates a significant difference between the two times. "b" indicates a significant difference from the control within time, "c" indicates a significant difference from 4 µg Cu L^{-1} within time, and "d" indicates a significant difference from 5 µg Cu L^{-1} within time. (n = 5)

		Metallothionein-like Proteins (%)		Cellular Debris (%)		Metal Rich Granules (%)		Enzymes (%)		Organelles (%)	
Day 63	Control	14.9 ± 4.4		19.8 ± 3.1		41.2 ± 2.3		15.4 ± 4.4		8.7 ± 2.4	
	4 µg Cu L^{-1}	24.4 ± 2.2		24.9 ± 3.3		38.6 ± 3.1		6.7 ± 1.2		5.4 ± 0.6	
	5 µg Cu L^{-1}	24.6 ± 5.2		19.9 ± 1.9		46.8 ± 6.4		3.1 ± 0.7	a,b,c	5.5 ± 1.1	
	11 µg Cu L^{-1}	27.3 ± 3.3	a,b	20.4 ± 3.3		36.2 ± 7.7		4.1 ± 0.9	b,c	12.0 ± 1.7	c,d
Day 96	Control	23.0 ± 10.3		20.1 ± 4.0		43.2 ± 6.7		9.4 ± 1.9		4.3 ± 0.8	
	4 µg Cu L^{-1}	23.3 ± 4.0		31.3 ± 3.7	b	35.5 ± 5.2		5.6 ± 1.2	b	4.4 ± 0.3	
	5 µg Cu L^{-1}	29.0 ± 3.5		27.8 ± 2.7		31.1 ± 2.5		6.3 ± 1.4		5.8 ± 0.4	
	11 µg Cu L^{-1}	36.7 ± 1.5	b,c	15.8 ± 3.0	c,d	39.1 ± 2.8		3.7 ± 0.7	b,d	4.7 ± 0.2	

		Biologically Detoxified Metal (%)		Metal Sensitive Fraction (%)		Trophically Available Metal (%)
Day 63	Control	56.0 ± 4.4		24.2 ± 5.3	a	39.0 ± 5.0
	4 µg Cu L^{-1}	63.0 ± 4.0		12.1 ± 1.0	b	36.5 ± 1.9
	5 µg Cu L^{-1}	71.4 ± 3.1	b	8.7 ± 1.8	a,b,c	33.3 ± 6.4
	11 µg Cu L^{-1}	63.4 ± 4.9	a	16.1 ± 2.2	a,d	43.4 ± 4.6
Day 96	Control	66.2 ± 5.2		13.7 ± 1.7		36.8 ± 9.5
	4 µg Cu L^{-1}	58.8 ± 5.0		9.9 ± 1.4	b	33.2 ± 4.2
	5 µg Cu L^{-1}	60.1 ± 3.1		12.1 ± 1.1		41.1 ± 2.9
	11 µg Cu L^{-1}	75.8 ± 3.1	c,d	8.4 ± 0.7	b,d	45.1 ± 1.4

Table 4.5 Physical and chemical characteristics of gut fluid collected from killifish 8 hours after feeding in both FW and SW (mean ± SEM). "*" indicates a significant difference between the two salinities. (n = 5)

	FW (0.16 ppt)	SW (35 ppt)	
pH	7.37 ± 0.14	7.69 ± 0.15	
$[Na^+]$ (mM)	115.3 ± 5.5	62.6 ± 5.4	*
$[K^+]$ (mM)	7.5 ± 0.9	5.7 ± 0.6	
$[Mg^{2+}]$ (mMl)	3.4 ± 0.3	106.6 ± 11.5	*
$[Ca^{2+}]$ (mM)	2.3 ± 0.3	10.9 ± 0.9	*
HCO_3^- (mM)	29.0 ± 4.9	43.8 ± 4.5	*
$[Cl^-]$ (mM)	32.5 ± 6	30.7 ± 3.1	
$[SO_4^{2-}]$ (mM)	3.3 ± 0.9	40.3 ± 4.9	*

Table 4.6 Calculated Cu speciation in the gut fluid of killifish in FW and SW using the values presented in table 4.4 (Millero and Pierrot, 1998).

	FW gut fluid	SW gut fluid	FW	SW
Cu^{2+}	1.59%	0.14%	1.34%	4.93%
$CuOH^{+}$	0.19%	0.04%	2.17%	4.10%
$Cu(OH)_2$	0.02%	0.02%	2.28%	2.50%
$CuHCO_3^{+}$	0.84%	0.08%	0.11%	0.09%
$CuCO_3$	66.83%	22.16%	84.92%	68.35%
$Cu(CO_3)_2^{2-}$	30.42%	77.50%	9.12%	19.23%
$CuHS^{-}$	0.00%	0.00%	0.00%	0.00%
$Cu(HS)_2$	0.00%	0.00%	0.00%	0.00%
$CuSO_4$	0.13%	0.07%	0.05%	0.79%

Figure 4.1 [Cu] in oyster tissue after 0, 63, and 96 days of exposure to control (clear bar), 4 μg Cu L^{-1} (cross hatched bar), 5 μg Cu L^{-1} (gray bar), 11 μg Cu L^{-1} (gray cross hatched bar). Note the break in the y-axis. See table 4.1 for physical chemical characteristics of the water and table 4.2.a for the measured [Cu]. "a" indicates a significant difference from day 0, “b” indicates a significant difference from day 0 and the simultaneous control, and "c" indicates a significant difference from day 0, day 63, and the simutaneous control. (*n* = 5)

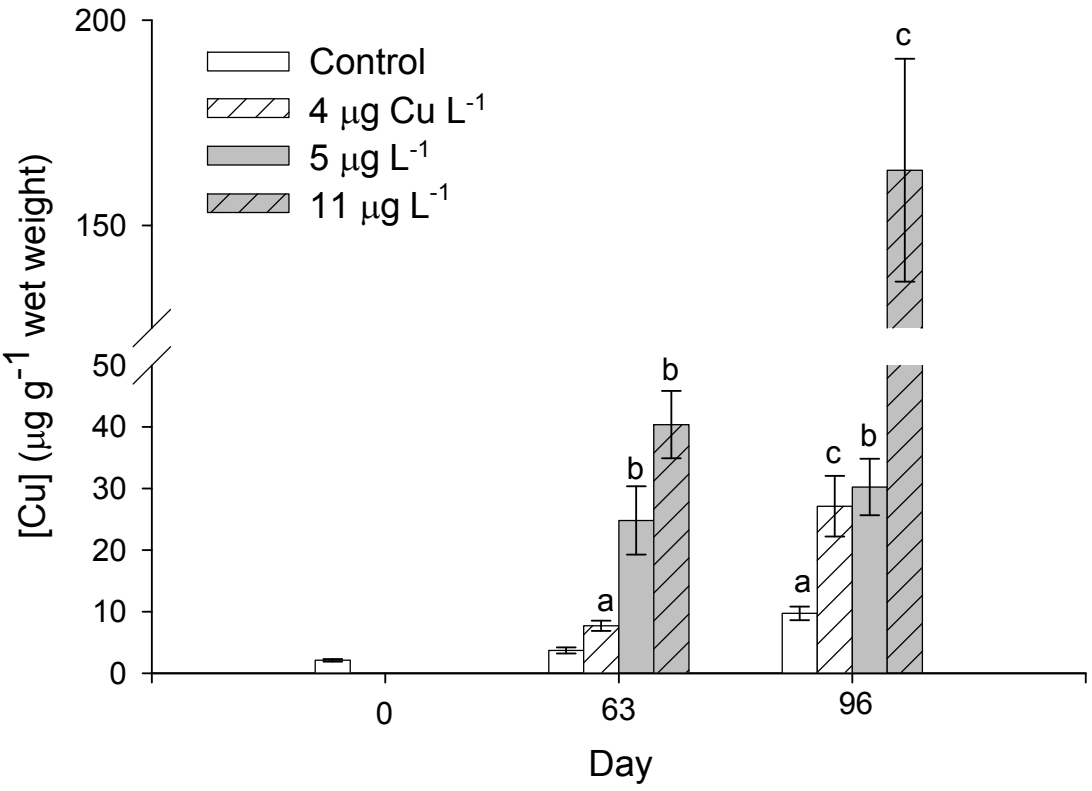


Figure 4.2.a Gill [Cu] in female killifish exposed to a control oyster diet and a high Cu diet (hatched bars, see table 4.3 for [Cu]) for 40 days in FW (clear bars) and SW (grey bars). "*" indicates significant differences from the simultaneous control. (*n* = 5)

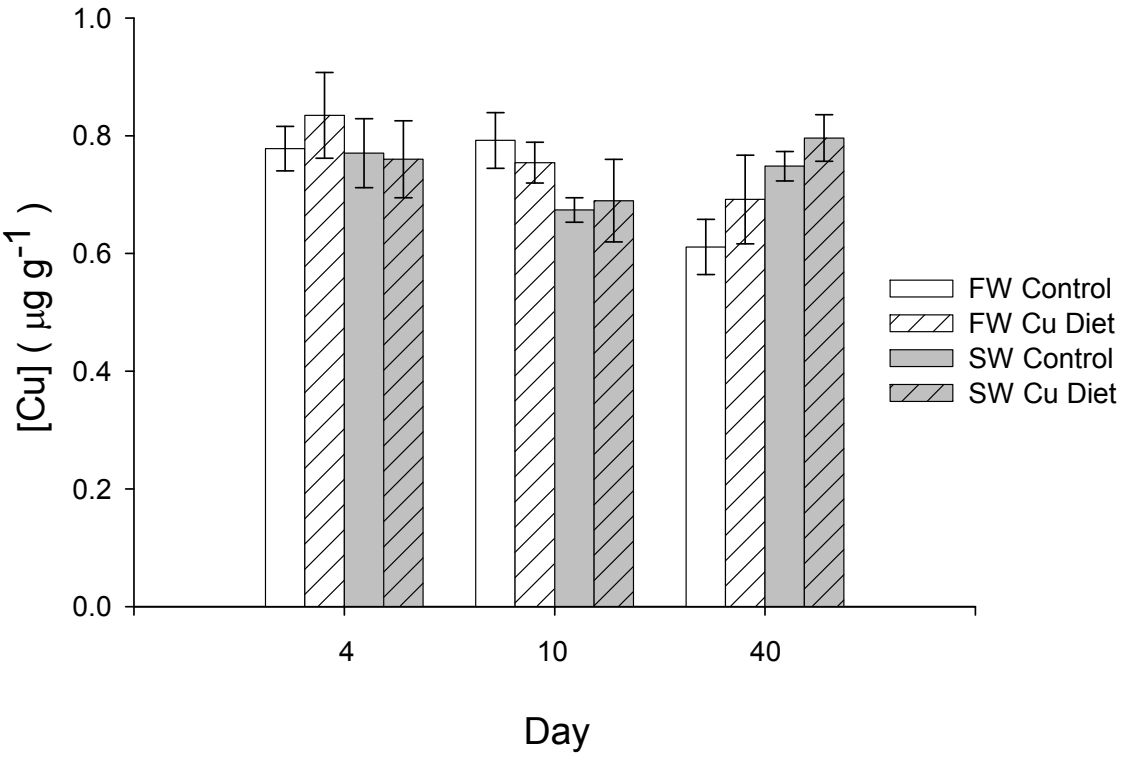


Figure 4.2.b Gill [Cu] in male killifish exposed to a control oyster diet and a high Cu diet (hatched bars, see table 4.3 for [Cu]) for 40 days in FW (clear bars) and SW (grey bars). "*" indicates significant differences from the simultaneous control. (*n* = 5)

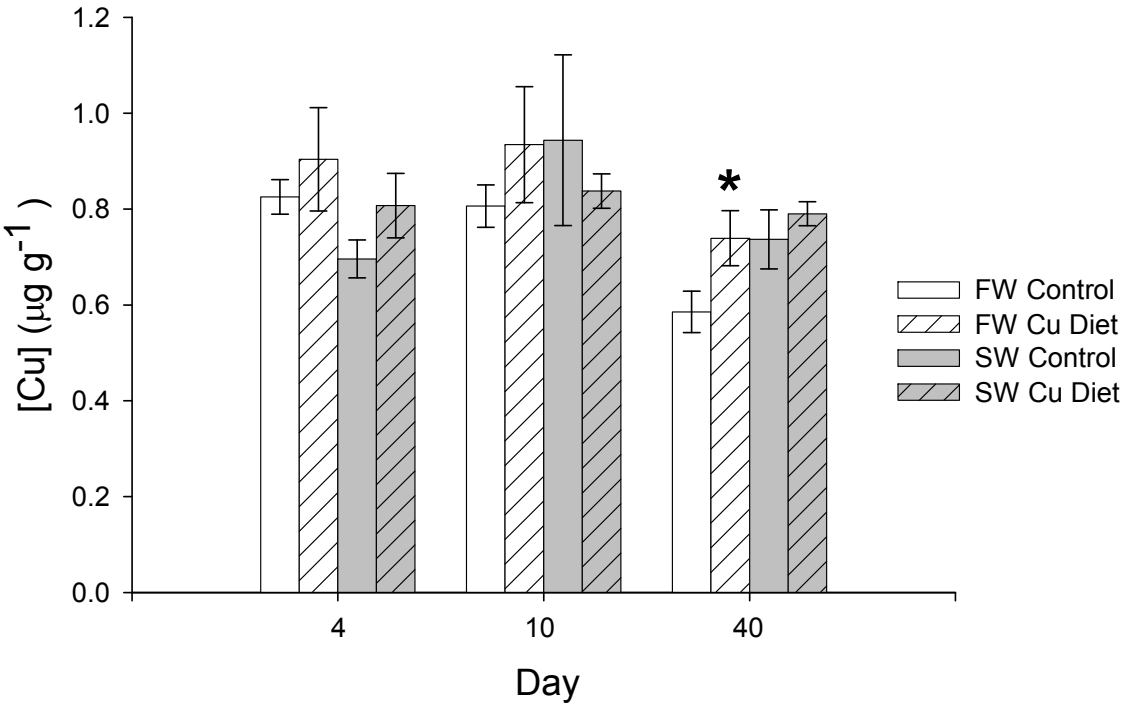


Figure 4.3.a Intestinal [Cu] in female killifish exposed to a control oyster diet and a high Cu diet (hatched bars, see table 4.3 for [Cu]) for 40 days in FW (clear bars) and SW (grey bars). "*" indicates significant differences from the simultaneous control. (n = 5)

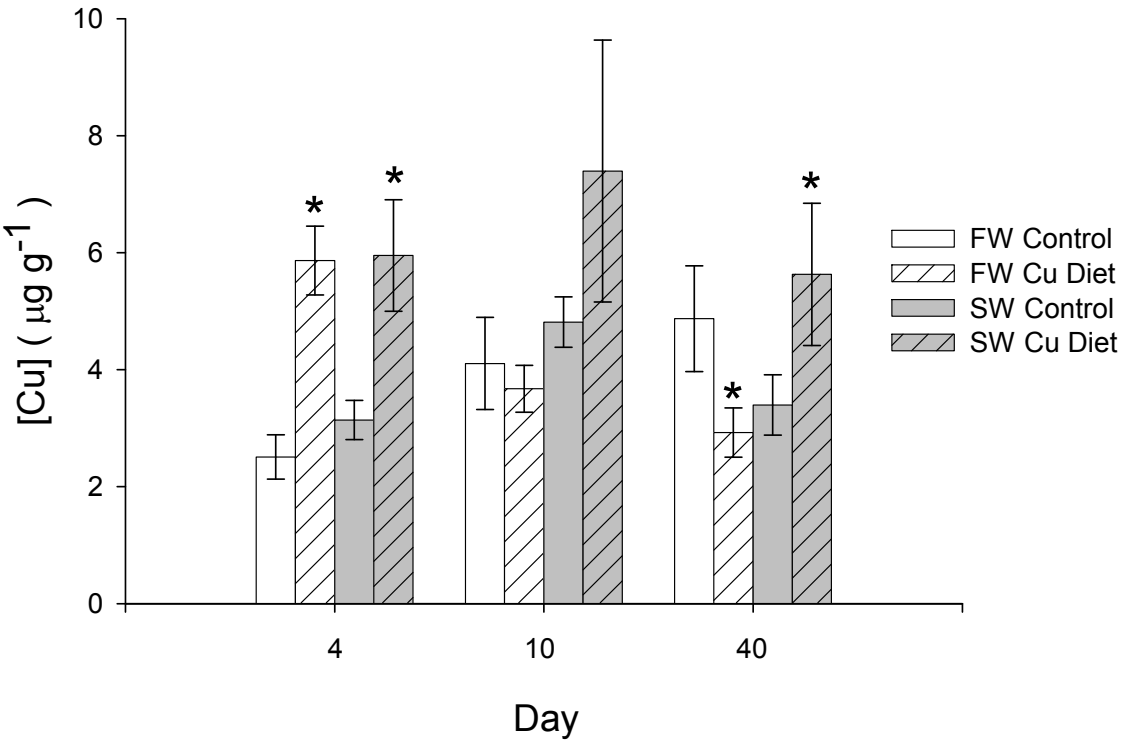


Figure 4.3.b Intestinal [Cu] in male killifish exposed to a control oyster diet and a high Cu diet (hatched bars, see table 4.3 for [Cu]) for 40 days in FW (clear bars) and SW (grey bars). "*" indicates significant differences from the simultaneous control. (n = 5)

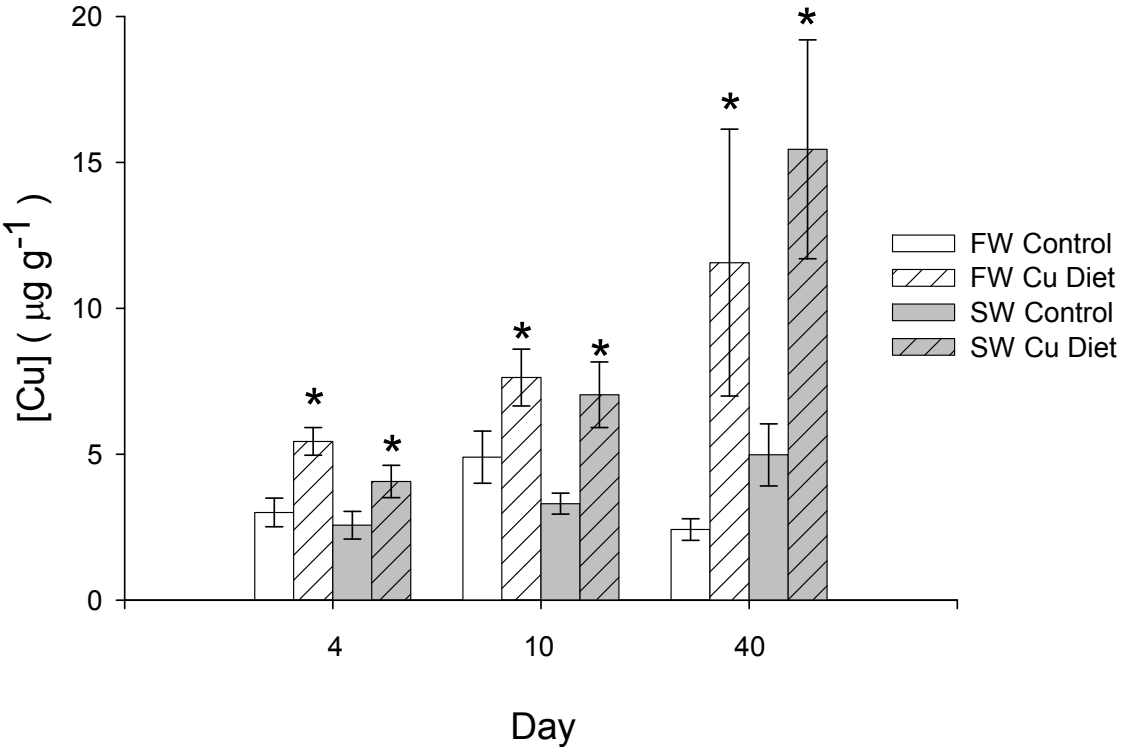


Figure 4.4 [Cu] (μg g^{-1} wet weight) in the gill (**a**), intestine (**b**), and liver (**c**) of fish as a function of total dietary Cu ration (mg kg^{-1}) which is the product of the daily dietary dose and the days of exposure. Data were compiled from the following sources: black circle = Kamunde and Wood (2003), clear circle = Kamunde et al. (2001), black inverted triangle = Kamunde et al. (2002b), clear inverted triangle = Miller et al. (1993), black square = current study, clear square = Handy (1992), black diamond = Berntssen et al. (1999a), clear diamond = Berntssen et al. (1999b), black trangle = Murai et al. (1981), clear triangle = Overnell and McIntosh (1988), black hexagon = Julshamn et al. (1988),clear hexagon = Knox et al. (1984), grey circle = Knox et al. (1982). Dry weight values were corrected to wet weight assuming 80% water in the tissue for Berntssen et al. (1999a and b). Values from Kamunde and Wood (2003) for the intestine are the average of the values given for the four sections of the gut.

Figure 4.4.a

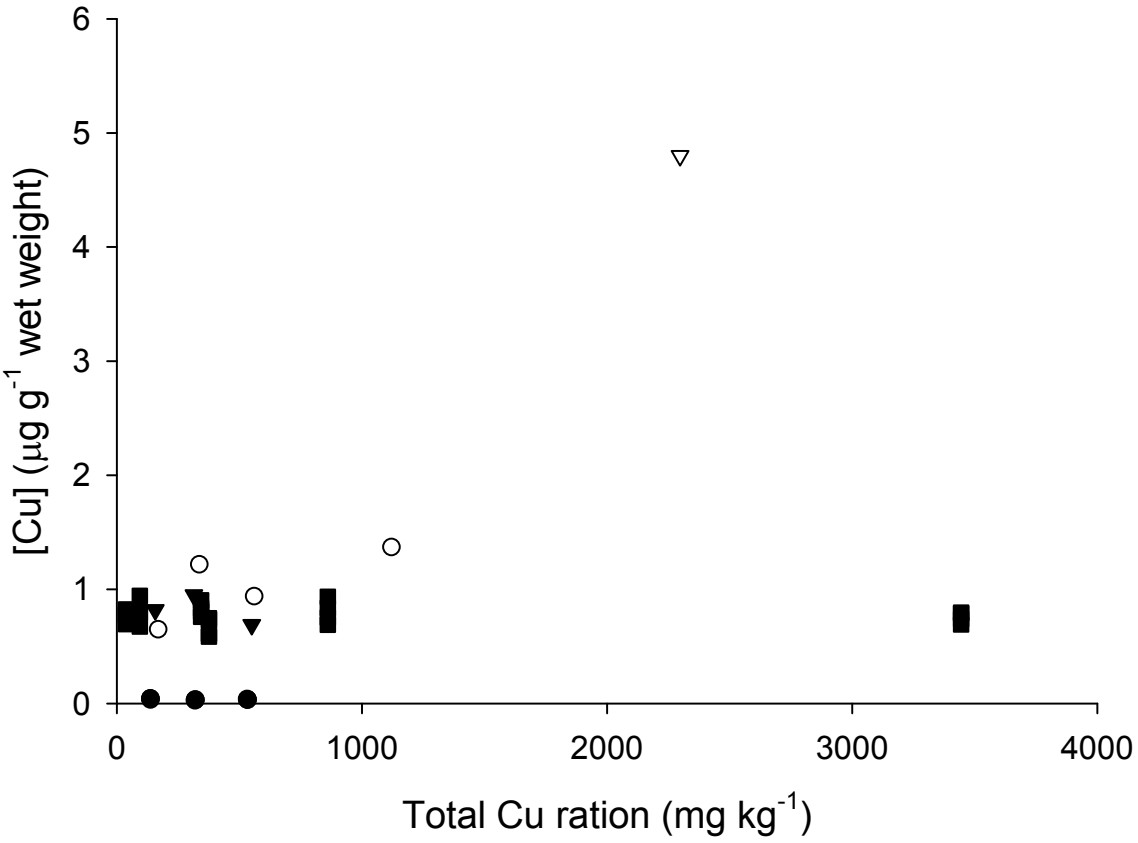

6
5
4
3
2
1
0
[Cu] (μg g-1 wet weight)
0
1000
2000
3000
4000
Total Cu ration (mg kg-1)

Figure 4.4.b Intestine

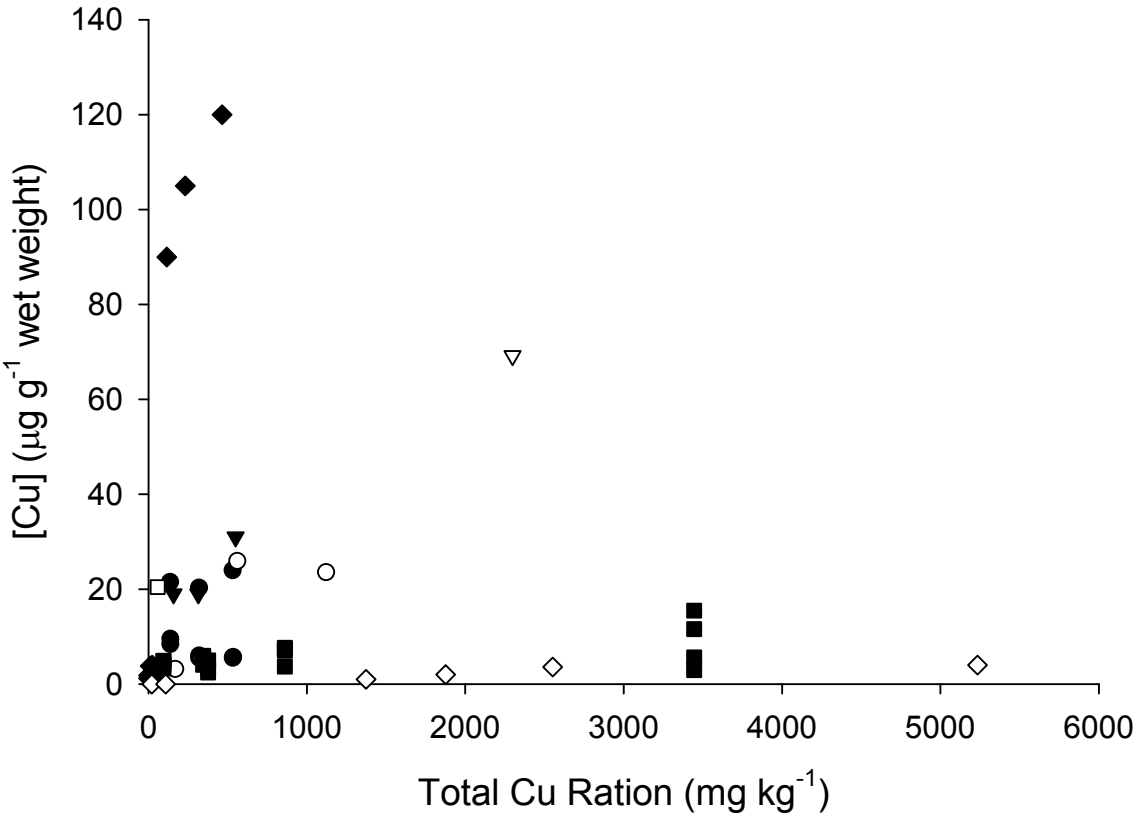
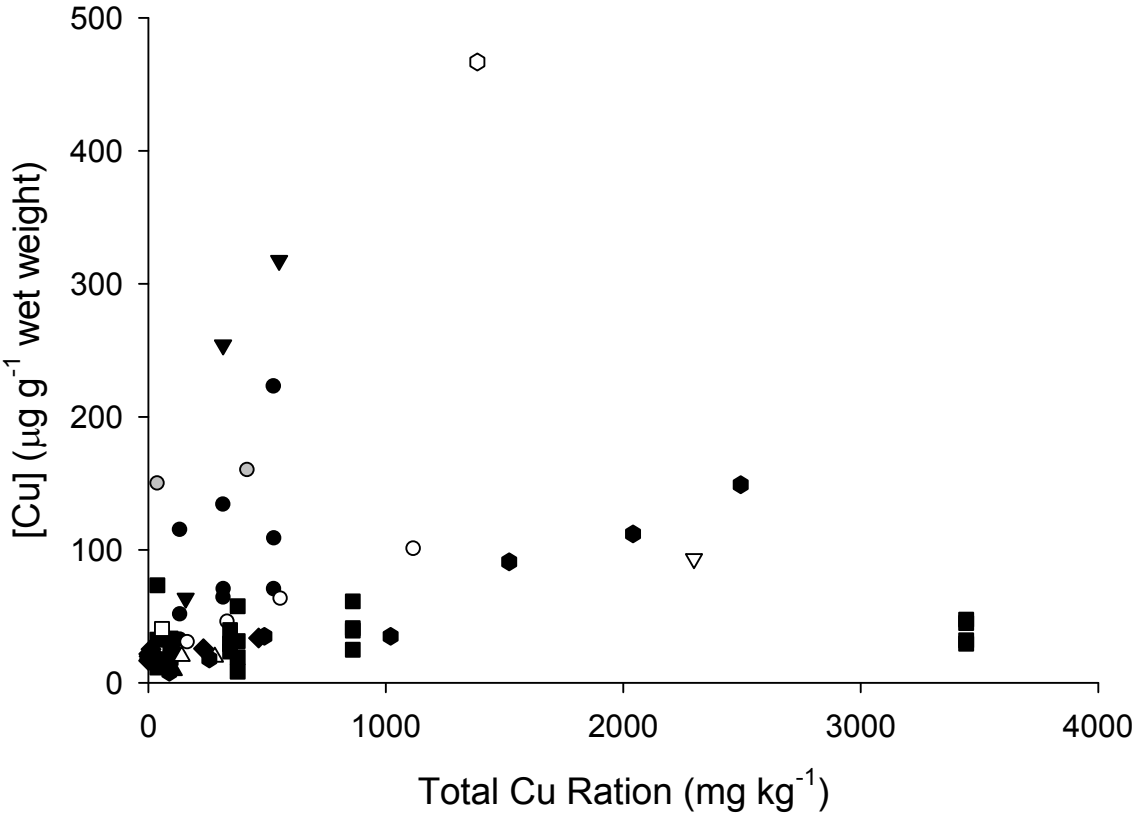


Figure 4.4.c Liver



CHAPTER 5

MOLECULAR CHARACTERIZATION OF WATERBORNE AND DIETARY COPPER EXPOSURE IN FRESHWATER AND SEAWATER IN THE COMMON KILLIFISH, *FUNDULUS HETEROCLITUS*, USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION

5.1 Background

Copper in both water and diet has been demonstrated to be toxic to fish and other aquatic organisms (USEPA, 2003; Bielmeyer et al., 2006). However, the effects of copper differ depending upon the route of entry and also the salinity at which the organism is exposed (chapter 3; Bielmeyer et al., 2006). During exposure Cu often remains elevated in the organisms even though there are naturally evolved mechanisms, conserved from yeast to humans, to control the uptake and distribution of copper (Bertinato and Abbé, 2004; Harris, 2000, Peña et al., 1999). Although the physiological responses differ based on both the route of uptake and salinity, the molecular responses may be similar because of the ubiquity of copper homeostatic mechanisms. The present experiments were focused on the molecular response of the killifish (*Fundulus heteroclitus*) intestine's response to copper exposure from both dietary and waterborne exposures in fresh water (FW) and sea water (SW).

Cellular Cu uptake is mediated by copper transporter 1 (CTR1) and in the intestine the divalent metal transporter (DMT1) is also thought to be involved (Bertinato and Abbé, 2004; Harris, 2000, Peña et al., 1999; Clearwater et al., 2002). An alternate route of uptake for waterborne Cu to enter the fish in FW is through Na^+ uptake pathways where Cu^{2+} has been observed to substitute for Na^+ in the gill which may also be true for the esophagus of fish (Grosell and Wood, 2002). In marine fish, the Na content of SW

ingested for osmoregulation is reduced by ~66% as fluid is moving along the esophagus, stomach and intestine (Marshall and Grosell, 2005) and it is possible that free Cu^{2+} could substitute for Na^{+} in these uptake pathways. However, recent evidence demonstrates that in FW in the mid and posterior intestine Na^{+} stimulates Cu uptake perhaps by stimulating acidification of the boundary layer aiding Cu uptake through CTR1 and DMT1 (Nadella et al., 2007). Once copper enters the cell, usually it is bound to various proteins. Among these proteins, metallothionein (MT) and glutathione (GSH) are both thought to keep the copper available for use by the organism while rendering it non-toxic (Bertinato and Abbé, 2004; Harris, 2000, Peña et al., 1999).

Both the newly accumulated copper and the GSH/MT bound copper can be utilized by copper chaperones (Bertinato and Abbé, 2004; Harris, 2000, Peña et al., 1999). Copper chaperones are necessary to incorporate copper into the copper containing enzymes. Of the three know chaperones, CCS is responsible for transporting copper to Cu/Zn super oxide dismutase, COX17 transports copper to cytochrome c oxidase through the intermediates SCO1 and SCO2, while antioxidant protein 1 (ATX1) interacts with copper transporting ATPases (ATP7A and ATP7B). Copper is excreted from the cell by the copper ATPase, ATP7A, in the intestine and most extrahepatic tissues while ATP7B dominates in hepatic tissue.

Cu has been observed to accumulate during waterborne exposures in the intestine at higher salinities (Chapter 2) due to the need of fish to drink as salinity increases above the isoosmotic point of the fish's plasma (for detailed reviews see Marshall and Grosell, 2005). This accumulation shows a trend to increase with increasing inwardly directed ionic gradients from the intestinal fluid to the plasma (Figure 2.2). It has also been noted

to accumulate at salinities below the isoosmotic point but not in FW (Figure 2.2). During dietary exposures Cu accumulates equally well in the intestine in both FW and SW and is a major site of accumulation during dietary exposures (Chapter3).

Copper exposure also causes changes at the cellular level. Dietary Cu exposure has been noted to cause increased apoptosis and stimulate cell proliferation in the intestine (Berntssen et al., 1999; Kamunde et al., 2001). Dietary metal has also been noted to cause intestinal impaction suggesting that the muscle cells surrounding the intestine are experiencing toxic effects from metal exposure (Woodward et al., 1995).

Because of the diversity of physiological responses to Cu exposure and the ubiquity of Cu metoblolic genes, we hope to identify molecular markers that would be indicative of Cu exposure. These biomarkers would potentially not only indicate that an organism was exposed to Cu (biomarker of exposure) but also would indicate the downstream physiological consequence of this exposure (biomarkers of effect) (Fowle and Sexton, 1992; van der Oost et al. 2003). The advantages to this approach are that molecular markers are thought to be more sensitive indicators of a potential problem than their corresponding physiological responses (van der Oost et al. 2003).

Suppression subtractive hybridization (SSH) is a technique that was developed in order to identify differentially expressed genes from two pools of RNA (Diatchenko, 1996). The technique is versatile in that no foreknowledge of the genes of interest is necessary for the identification of differentially expressed genes. Through repeated subtractions one can directly compare the experimental conditions of interest to identify subsets of genes that are responding specifically to the variables being tested. By using

this technique it is possible that genes related to the physiological effects and cellular effect of Cu exposure may be identified for potential use as biomarkers.

The present study utilized SSH to identify genes related to Cu exposure in the intestine of the *Fundulus heteroclitus,* killifish. Specifically, killifish were exposed to normal Cu in the diet (Control), waterborne Cu, or elevated dietary Cu in both FW and SW. Killifish were used because they are euryhaline (Wood and Marshall, 1994) and both dietary and waterborne Cu exposures have been characterized in FW and SW (Chapter 2, 3, and 4). In addition, there is a considerable amount of gene sequence data available for this organism since it has been utilized in microarray studies (Oleksiak et al., 2002; Oleksiak et al., 2005).

5.2 Materials and methods

5.2.1 Fish and exposures

Fundulus heteroclitus (1.2-12.8 g) were obtained from the Whitney Lab and acclimated to laboratory conditions for at least one month prior to exposure and to the appropriate salinity for two weeks. Fish were kept in 20 gallon aquaria under a 16:8 light :dark photoperiod. Three exposures were performed in both FW and SW (28ppt) (for detailed water chemistry see Table 2.1) under static conditions for ten days and twelve fish were included in each exposure (6 male and 6 female).

The three exposure conditions in both FW and SW were 1) controls that received a commercial pellet diet (8.8 ± 2.8 μg Cu g^{-1}), 2) a dietary Cu exposure that were fed a diet of copper rich oysters homogenized in agar (58.2 ± 0.4 μg Cu g^{-1}) a waterborne copper exposure at 60 μg Cu L^{-1} (nominal) that was also fed the commercial control

pellet diet. Dissolved waterborne copper concentrations for all treatment and salinities are listed in table 5.1 and presented as initial and final values. Tanks were fed on a total biomass basis at 5% of the total fish mass per day in all treatments and all food was generally consumed within 1 hour.

All fish were starved for 24 hours prior to sampling at 2 and 10 days post exposure. Prior to sampling, fish were killed with an overdose of MS-222 and three male and three female fish from each treatment were sampled. Intestinal tissue was excised and flash frozen in liquid nitrogen. The tissue samples were stored at -80º C prior to RNA extraction.

5.2.2 Cu analyses

Cu measurements were made using a Varian Model 220 Z graphite furnace atomic absorption spectrometer (Mulgrave, Australia). Dietary [Cu] were determined after digesting the oyster or pellets in 1 N trace metal grade nitric acid (Fisher Scientific, Pittsburg, PA, USA) overnight at 80º C and all samples were appropriately diluted prior to measurement. All water samples were acidified upon collection using trace metal grade nitric acid (1% acid). Cu in SW samples was extracted to remove matrix effects using a solvent extraction and back extraction into 1% trace metal grade nitric acid prior to dilution and measurement as outlined previously (Chapter 2).

5.2.3 Subtraction, cloning, and sequencing

RNA was extracted as described below. In total, 9 libraries were generated and the subtractions were performed to obtain differentially expressed genes at times and under appropriate conditions for future experiments. Subtractions were performed using Clontech PCR-Select cDNA subtraction kit (Mountain View, CA) and products from the subtracted library were cloned using an Invitrogen TOPO TA cloning kit (Carlsbad, CA). In all cases, the subtractions produced genes from the driver RNA that were more highly expressed than in the tester RNA. Detailed descriptions of individual subtractions are presented in table 5.2.

Clones from the subtracted libraries were grown up overnight in LB broth at 37º C and 2 uL of the culture was amplified by PCR using M13 forward and reverse primers that were obtained from Eurofins MWG Operon (Huntsville, AL) and Integrated DNA Technologies, Inc.(Coralville, IA) at a concentration of 20 µM per reaction. Each reaction (25µL) also contained 1 x PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 units of Taq polymerase. The reaction conditions were 50 cycles of 94º C for 15 seconds, 56º C for 30 seconds, and 72º C for 1 minute. The resulting PCR products were purified using AMPure (Agencourt Biosciences, Beverly, MA).

The purified PCR products were then sequenced using Applied Biosystems BigDye Terminator v. 3.1 Cycle Sequencing Kit (Carlsbad, CA). Each 10 µL reaction was composed of 0.5 µL BigDye® v3.1, 2.15 µL BigDye® v3.1 sequencing buffer (5X), 1.0 µL primer (3.2 µM), 1.0 µL purified PCR product, and 5.35 µL sterile water. The

reaction conditions were 36 cycles of 94º C for 15 seconds, 50º C for 20 seconds, and 60º C for 4 minutes. The reactions were cleaned using CleanSEQ (Agencourt Biosciences) and sequenced on an Applied Biosystems 3730 DNA Analyzer.

5.2.4 RNA extraction

RNA extraction was performed by homogenizing tissue samples (Ultra-Turrax 8.01 homogenizer, IKA Werke GmbH and Co., Staufen, Germany) in a guanadinium isothiocyanate buffer followed by extraction using acidic phenol and chloroform isoamyl alcohol. RNA samples were purified using the Qiagen RNAeasy Mini kit (Valencia, CA, USA) in accordance with the manufacturer's protocols. RNA quantity was assessed spectrophotometrically using a Nanodrop ND-1000 V. 3.2.1(Wilmington, DE, USA) and quality was assessed using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). All RNA was of good quality as indicated by 260/280 ratios of ~2 and 18S/28S ribosomal RNA ratios of >1.

5.3 Results

5.3.1 Cu Concentrations

The [Cu] in FW and SW were very similar (Table 5.1). At both time points, the [Cu] in the SW elevated dietary Cu treatment and the control were slightly higher than in the corresponding FW treatments due to the higher background concentrations of Cu in SW. The [Cu] also increased over time in the control and elevated dietary Cu treatment which was expected in the static exposure. The increases in concentration were similar in

magnitude between salinities and the increase was greater in the elevated Cu diet treatment. This could be because of greater Cu excretion by the fish receiving the high Cu diet and/or leaching of Cu from the food.

The waterborne [Cu] were near the nominal concentration of 60 µg Cu L^{-1} and at the end of the ten day exposure had decreased (table 5.1). The magnitude of the decrease was similar between the two salinities and the final [Cu] were the same.

5.3.2 Libraries and sequencing

Eight libraries were generated from the intestinal RNA of the exposed fish (Table 5.2). Eight libraries were generated because a rapid reduction in novel sequences obtained for each plate of 48 clones was observed after sequencing ~192 clones (Figure 5.1). The first 48 clones sequenced from a new library generated 4-20 novel sequences while additional sequencing of a library beyond 192 clones provided only 1-4 new sequences per 48 clones. Overall, 768 clones were successfully sequenced and 329 (42.8%) of these sequences were able to be annotated using BLAST searches.

The libraries generated in these experiments contained 109 unique genes (table 5.3). A vast majority of these genes came from libraries enriched in sequences that were upregulated due to Cu exposure. Tables 5.4-5.9 list genes that were found in the individual libraries where a specific time and type of Cu exposure was driving the changes in expression and contain genes that may be shared between more than one library. Table 5.10 lists seven genes that were found to differ in expression between two individual control fish.

5.4 Discussion

5.4.1 Type of Cu exposure, Time, Salinity, and upregulated genes

The data presented in tables 5.4-5.10 suggest that there are a wide variety of genes that are affected by Cu exposure and that these genes are unique to the type of exposure (waterborne or dietary), the length of the exposure, the sex of the fish, and the salinity in which they were exposed. In fact, there are no genes that are upregulated at both 2 and 10 days post exposure in the libraries generated from the dietary Cu exposure in SW, the waterborne Cu exposure in SW, and the dietary Cu exposure in FW. Within the fish exposed to elevated dietary Cu, only 4 genes are shared between the two salinities which correspond to 29% of the genes found to be upregulated in SW and 17% of those found in the FW libraries (Figure 5.2). A similar situation is observed when comparing the different types of exposure, where again only 4 genes are shared between the waterborne (15%) and the dietary (12.5%) exposures (Figure 5.3). Thus, either the variables being compared are truly unique or there is a technical issue that is skewing the data toward novelty ("false positives").

In this case, the latter seems true. These experiments were designed to obtain a large amount of genes that respond to Cu exposure. When it was observed that genes could be identified more efficiently by performing more subtractions to generate additional libraries rather than exhaustively sequencing a single pooled library (figure 5.1), we introduced a bias that creates the impression that differing conditions generate unique subsets of genes. It is much more likely that, with additional sequencing, the amount of overlap between the sets of genes would increase and that genes which are contained in only one library might even become rare.

In addition, it is impossible to conclude that the 102 genes identified in these experiments respond to Cu exposure even though the subtractions were designed to enrich the pools of RNA for genes that responded to Cu exposure. In fact, one of the subtractions performed generated a library in which genes that differed in expression between two individuals were revealed. Other researchers have noted that there is a large amount of variation in gene expression between individuals (for a review of this see Whitehead and Crawford, 2006). Studies have reported in *Fundulus heteroclitus* that anywhere from 18% (Oleksiak et al., 2002) to 94% (Oleksiak et al., 2005) of genes examined by microarray analysis were statistically different between two individuals.

In this experiment, we found that 7 genes (6.4%) were differentially expressed between two individuals. This number seems small, but is similar to the proportion of clones that were sequenced from the library formed by subtracting the one individual from a second relative to the total number of clones sequenced (7.1%). In addition to this, 57% of the genes identified as differing between two individuals were also identified in other libraries (Figure 5.2 and 5.3). This suggests that some of the genes identified in he libraries apparently enriched for genes that respond to Cu exposure may actually only differ between individuals and are perhaps not affected by Cu exposure.

5.4.2 Cu related gene expression and mechanistic support

In spite of the concerns presented in the previous section, we can safely conclude that many of our genes are upregulated in response to Cu exposure. More than 39% of the genes were identified from library 1 in table 5.2. This library was more extensively sequenced than the other libraries. Because of this, it contains a large proportion of the

genes. It is also the library that is least subject to influences of individual variation because the tester and driver pools of RNA contain a large number (18) of individuals. The pooling of samples will therefore average out the genes with small (~2 fold) among individual variable expression causing the pool to be enriched for sequences that are consistently upregulated across individuals due to Cu exposure. It will still contain genes that differ greatly among individuals.

The genes idenitified from all of the libraries are related to a wide variety of functions within the organism and some of these functions are summarized in table 5.11. Several of the genes have more than one function and are therefore counted several times for the purpose of this table. These functions were selected because there is additional evidence which suggests that they may be responding to Cu exposure.

One of the major ways in which metals cause cellular injury is through the generation of free radicals by free metal ions (Peña et al., 1999). In order to prevent this, metal binding proteins chelate metals preventing the generation of free radicals (Bertinato and Abbé, 2004; Harris, 2000; Peña et al. 1999). The subtractions identified 15 genes (Table 5.3 and 5.11) which have metal binding properties among these is metallothionein A which is known to bind Cu to prevent the formation of free radicals (Bertinato and Abbé, 2004; Harris, 2000; Peña et al. 1999). In addition to this, two genes, glutathione S transferase (Hayes et al., 2004) and ferritin (Balla et al., 1992), that respond to oxidative stress were also upregulated which could also be the result of Cu exposure.

Dietary Cu exposure has been observed to cause an increase in apoptosis within the intestine and an increase in cell proliferation (Berntssen et al., 1999; Kamunde et al., 2001).We do see an upregulation of four genes involved in apoptosis. However, even

though we found 11 genes that are involved in cell differentiation and growth, four of these are negative regulators of cell proliferation. Therefore, it is unclear as to whether or not there is a strong signal for cell proliferation in our data set.

Dietary metal exposure has also been observed to cause intestinal impaction suggesting decreased motility in the gut (Woodward et al., 1995). We found 8 genes that are necessary for muscular function to be upregulated and another 12 cytoskeletal genes in our libraries. These genes could be upregulated in an attempt to restore gut motility.

Cu is also known to cause increases in plasma cortisol levels which lead to several downstream changes in fish (Monteiro et al., 2005; Aluru and Vijayan, 2009). Cortisol can increase the level of transcription of genes involved in protein synthesis (Wenedelaar, 1997; Mommsen et al., 1999) which may explain the upregulation of the 22 genes observed in this study. Protein synthesis also requires energy and we observed the upregulation of 13 genes involved in ATP synthesis. Cortisol also increases the concentration of glucose in the plasma (Mommsen et al., 1999) and we found 4 genes involved in carbohydrate metabolism to be upregulated . Cortisol has also been established in mammals to increase muscle proteolysis and cause lysosomal activation (Aluru and Vijayan, 2009). We observed the upregulation of 6 proteolytic enzymes which could be a result of cortisol signaling.

It is also interesting to note that there were two genes involved in reproduction that were upregulated in this study: vitellogenin (Matsubara et al., 2003) and gonadotropin releasing prehormone (Swapna and Senthilkumaran, 2007). It is interesting because naturally incorporated Cu diets have been shown in invertebrates to affect reproduction at very low Cu concentrations (Bielmeyer et al., 2006).

Finally, these subtractions indicate that many of the genes encoded by the mitochondrion are upregulated by Cu exposure. In fact, 8 of the 13 protein coding genes from the mirochondrion were identified as well as both of the rRNA genes (Zardoya et al., 1995). This is remarkable considering the small size of the mitochondrial genome relative to the entire genome.

5.4.3 Gene Frequency

For the most part, we see no trends in the frequency at which a gene was found. Many of the genes identified were unique sequences within a given library and the vast majority of genes appeared 5 or less times. This is likely due to the low sequencing effort on most of the libraries (Table 5.2) because of the goal to find as many novel sequences as possible. Trends in gene frequency may have been noticeable had the sequencing effort on each individual library been higher. The only gene that it is interesting to note is the trypsinogen-like precursor which was found at a high frequency (Tables 5.5, 5.6, and 5.10) in several libraries.

5.4.4 Conclusions

SSH is a technique that can relatively rapidly generate a list of genes that respond to stressors (Diatchenko, 1996). However, it is important to be careful with the interpretation of the results because of inter individual genetic variation and incomplete sequencing of subtracted libraries. The list of genes generated by this study seems to include a gene set representing a coordinated response to Cu toxicity. At the cellular level, we observed upregulation of metal chelators and free radical defense. While at the

tissue level, apoptotic genes are upregulated along with genes that may be involved in tissue repair. At the more systemic level, there are a many genes from our libraries that respond to cortisol and thus may represent a general stress response caused by elevated cortisol.

Table 5.1 Measured concentrations of [Cu]. Initial samples were taken on day 0 and the final on day 10. (n=4)

Treatment	Initial [Cu]	Final [Cu]
FW Control	1.80 ± 0.19	4.82 ± 0.18
FW Dietary Cu	3.36 ± 0.34	10.98 ± 0.17
FW Waterborne Cu	59.14 ± 0.88	37.75 ± 0.73
SW Control	4.99 ± 1.16	7.67 ± 0.79
SW Dietary Cu	4.30 ± 0.38	13.40 ± 1.05
SW Waterborne Cu	63.97 ± 1.83	37.77 ± 5.29

Table 5.2 Subtractions performed on pools of RNA from the exposures. Each tester and driver pair provides genes upregulated in the tester pool of RNA.

	Tester				Driver					
Library	Salinity	Sex	Day	Copper	Salinity	Sex	Day	Copper	*n*	No. of clones sequenced
1	FW, SW	Male, Female	2, 10	Diet, Water	FW, SW	Male, Female	2, 10	Control	36	432
2	SW	Female	2	Water	SW	Female	2	Control	6	48
3	SW	Male	10	Water	SW	Male	10	Control	6	44
4	SW	Female	10	Diet	SW	Female	10	Control	6	110
5	SW	Male	2	Diet	SW	Male	2	Control	6	48
6	FW	Female	10	Diet	FW	Female	10	Control	6	48
7	FW	Male	2	Diet	FW	Male	2	Control	6	48
8	SW	Male (1)	2	Control	SW	Male (2)	2	Control	2	52

Table 5.3 List of unique genes identified from the libraries in table 5.2. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Function
beta-succinyl CoA synthetase	0	gb|AF087890.1|	ATP production
cardiomyopathy associated 3	0	gi|61696133|ref|NM_152381.3|	Cytoskeleton
cornichon homolog (Drosophila) (CNIH)	0	gi|57165418|ref|NM_001009551.1|	Vescicle transport, Immune response
cytidine and dCMP deaminase domain containing 1 (CDADC1)	0	ref|NM_030911.1|	Zinc binding
endonuclease domain containing 1, transcript variant 3 (ENDOD1)	0	gi|113423076|ref|XM_937098.2|	Apoptosis, DNA damage, Metal binding
eukaryotic translation termination factor 1	0	BC088358.1	Translation
golgi autoantigen, golgin subfamily a, 5	0	gi|18606387|gb|BC023021.1|	Golgi vescicle trafficking
lysosomal associated protein transmembrane 4 beta	0	gb|BC031021.1|	Lysosome, Transport
microtubule-associated protein 4 (MAP4)	0	gi|47519638|ref|NM_002375.3|	Microtubule assembly, Cell cycle progression
myosin regulatory light chain MRCL3	0	gi|16741042|gb|BC016372.1|	Muscle, Ca binding
myosin, heavy chain 1, skeletal muscle, adult (MYH1)	0	gi|115527081|ref|NM_005963.3|	Muscle
N-acetyltransferase 5 (NAT5)	0	NM_016100.3	
protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta) (PPP3CB)	0	ref|NM_021132.1|	Fe binding, Zn binding, Signal transduction
protein phosphatase 6 regulatory subunit 3 (PP6R3)	0	gi|74100788|gb|DQ111954.1|	
putative aminopeptidase Fxna (Fxna)	0	gi|42491371|ref|NM_184050.2|	Proteolysis, Zn binding

Tale 5.3 (continued)

Name	E-value	GenBank ref ID	Function
pyruvate dehydrogenase kinase, isozyme 4 (PDK4)	0	ref|NM_002612.3|	Carbohydrate metabolism, Signal transduction
SERPINE1 mRNA binding protein 1 (SERBP1)	0	gi|66346684|ref|NM_015640.3|	mRNA stability
sestrin 1	0	gi|109731078|gb|BC113569.1|	DNA damage, negative regulator of cell proliferation
titin	0	gi|110349718|ref|NM_133378.3|	Muscle
WD repeat domain 77 (WDR77)	0	gi|20127622|ref|NM_024102.2|	Signaling, mRNA processing
DEAH (Asp-Glu-Ala-His) box polypeptide 9 (DHX9)	5.00E-180	ref|NM_001357.3|	Translation initiation, Cell growth
ribosomal protein L5 (RPL5)	1.00E-178	ref|NM_000969.3|	Translation
DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 (DDX1)	8.00E-167	gi|4826685|ref|NM_004939.1|	Translation initiation, Cell growth
NDRG family member 2 (NDRG2), transcript variant 3	4.00E-158	NM_201536.1	Cell differentiation
high density lipoprotein binding protein (vigilin)	3.00E-157	NM_203346.1	Lipid Binding
cytochrome c oxidase subunit I	2.00E-151	gi|52430369|gb|AY735174.1|	ATP production
eukaryotic translation initiation factor 4 gamma, 3 (EIF4G3)	2.00E-148	gi|115529454|ref|NM_003760.3|	Translation
ATP-dependent metalloprotease YME1L	2.00E-143	emb|AJ132637.1|HSA132637	Zn binding, proteolysis
cytochrome b	3.00E-142	gi|52001216|gb|AY725226.1|	ATP production
nebulin-related anchoring protein (NRAP)	2.00E-138	NM_006175.3	Cytoskeleton, Muscle
ferritin, light polypeptide (FTL)	3.00E-133	gb|BC021670.1|	Fe binding

Tale 5.3 (continued)

Name	E-value	GenBank ref ID	Function
guanine nucleotide exchange factor p532	2.00E-130	AK226162.1	Protein transport located in the golgi
nebulin	2.00E-122	gi|1470067|gb|U58109.1|MMU58109	Cytoskeleton, Muscle
inositol monophosphatase domain containing 1	4.00E-116	ref|NM_017813.2|	Mg binding
eukaryotic translation initiation factor 5 (EIF5)	1.00E-108	NM_183004.3	Translation
ubiquitin-binding protein homolog (UBPH)	5.00E-102	NM_019116.2	Protein degradation
ribosomal protein L27	4.00E-93	gi|52430375|gb|AY735177.1|	Translation
desmuslin (DMN)	6.00E-93	gi|112382238|ref|NM_015286.5|	Cytoskeleton, Muscle
WNK lysine deficient protein kinase 1	3.00E-91	gi|12711659|ref|NM_018979.1|	Ion transport, Cell signaling
vitellogenin 1 (Vtg I)	3.00E-87	gb|U07055.2|FHVTGI	Reproduction
beta-actin	3.00E-84	gi|16798645|gb|AF435092.1|AF435092	Cytoskeleton
cytochrome c oxidase subunit II	1.00E-81	gi|52430385|gb|AY735182.1|	ATP production, Cu binding
ribosomal protein S13	6.00E-79	gi|67764863|gb|AY909421.1|	Translation
protein phosphatase 2, regulatory subunit B	4.00E-66	gi|41282171|ref|NM_194412.2|	Negative regulator of cell growth and division
gonadotropin releasing preprohormone 1 (GnRH1)	1.00E-61	gi|22037881|gb|AF467291.1|	Reproduction, Cell signaling, Negative regulation of cell proliferation
cytochrome c oxidase subunit III	1.00E-59	gi|52430361|gb|AY735170.1|	ATP production
beta-2 microglobulin type 2	2.00E-57	gi|37785818|gb|AY217454.1|	Immune response
gelsolin (amyloidosis, Finnish type) GSN gene	4.00E-52	gb|DQ892187.2|	Actin binding, Ca binding
ubiquitin	7.00E-52	gi|37779121|gb|AY190746.1|	Protein degradation
selenoprotein T, 1a (selt1a)	1.00E-50	gi|40254665|ref|NM_178290.3|	Se binding, Redox homeostasis

Tale 5.3 (continued)

Name	E-value	GenBank ref ID	Function
metallothionein A	1.00E-49	gi|82697987|gb|AY375239.2|	Metal binding
transducer of ERBB-2	9.00E-46	gi|10121755|gb|AF266238.1|AF266238	Cell signaling, Negative regulator of transcription
chitinase	2.00E-45	gi|56713231|emb|CAD59687.1|	Digestion, Carbohydrate metabolism, Immune response
NADH dehydrogenase subunit 5 gene	5.00E-45	gi|55669308|gb|AY655543.1|	ATP production
ribosomal protein L4	1.00E-44	gi|37779079|gb|AY190725.1|	Translation
non-LTR retrotransposon reverse transcriptase	4.00E-44	gi|28894315|gb|AY212388.1|	
annexin A2	5.00E-43	gi|54288764|gb|AY639382.1|	Cell growth, Ca Binding
eukaryotic translation elongation factor-1 gamma	8.00E-43	gi|67772017|gb|AY909455.1|	Translation
Rex3 retrotransposon, Rex2 retrotransposon, and Rex1 retrotransposon	2.00E-42	gi|34762039|gb|AY298859.1|	
CArG box-binding factor	4.00E-42	gb|L36663.1|MUSCARGBF	Transcription
12S small subunit ribosomal RNA gene	1.00E-40	gi|55507101|gb|AY655515.1|	Translation
retinol binding protein 2a	4.00E-37	gi|23308502|ref|NM_153004.1|	Lipid Binding, Transport
alpha tubulin	3.00E-36	gi|10242283|gb|AF263276.1|AF263276	Cytoskeleton
Ubiquinol-cytochrome c reductase complex 14 kDa protein (Complex III subunit VI)	4.00E-34	gi|61217194|sp|Q5RC24|UCR6_PONPY	ATP production
ubiquinol-cytochrome c reductase binding protein	9.00E-34	gi|5454152|ref|NP_006285.1|	ATP production

Tale 5.3 (continued)

Name	E-value	GenBank ref ID	Function
fructose-1,6-bisphosphatase	2.00E-33	gi|11932951|emb|AJ300657.1|OCU300657	Carbohydrate metabolism
40S ribosomal protein S24	3.00E-33	gi|37779110|gb|AAP20215.1|	Translation
28S ribosomal RNA	4.00E-33	ref|NR_003287.1|	Translation
glutamate dehydrogenase	7.00E-29	emb|AJ556995.1|SSA556995	Nitrogen metabolism
adenine nucleotide translocator	1.00E-28	gi|71725703|gb|AAZ38997.1|	ATP transport
fructose-1,6-bisphosphate aldolase	2.00E-28	gi|587523|emb|X82278.1|SAFBPAL	Carbohydrate metabolism, Muscle maintainence
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	2.00E-24	gi|49904605|gb|AAH76339.1|	ATP production
apolipoprotein A-IV1 (apoa-iv1)	7.00E-24	ref|NM_001078591.1|	Lipid metabolism
heat shock 70kDa protein 8 (HSPA8)	2.00E-23	gi|37682086|gb|AY422994.1|	Protein folding
tRNA-Arg and tRNA val	8.00E-23	gi|28845|emb|X64282.1|HSARGICG	
reverse transcriptase-like protein	6.00E-22	gi|32394793|gb|AAN15747.1|	
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1) (GALNT1)	9.00E-22	gi|13124890|ref|NM_020474.2|	Protein trafficking
protein p61 (Xyes)	2.00E-21	gi|64483|emb|X54970.1|XHCYES	
60S ribosomal protein L35	4.00E-21	gi|132917|sp|P17078|RL35_RAT	Translation
asparaginyl endopeptidase (AEP), legumain	1.00E-20	gb|EF198070.1|	Proteolysis
glutathione-S-transferase	1.00E-20	gi|10121683|gb|AAG13321.1|	Reactive oxygen defense

Tale 5.3 (continued)

Name	E-value	GenBank ref ID	Function
C-type lectin 2	1.00E-19	gi|27530674|dbj|AB060538.1|	Immune response
NADH dehydrogenase subunit 2	2.00E-19	gi|62721939|gb|AY902131.1|	ATP production
ATP synthase 8	7.00E-19	gi|37779900|gb|AY191552.1|	ATP production
Nod 3	4.00E-18	gi|68438101|ref|XP_688444.1|	Negative regulation of transcription, Cell death
capping protein (actin filament) muscle Z-line, alpha 1	8.00E-18	gi|47086649|ref|NP_997862.1|	Cytoskeleton, Muscle
metallopanstimulin	1.00E-17	emb|AJ001134.1|SRMETPANS	Translation
N-myc-like protein	3.00E-17	gi|50952870|gb|AY583215.1|	Transcription
fatty acid translocase/CD36	5.00E-17	gi|3273897|gb|AAC24876.1|	Apoptosis, Lipid metabolism
ATP synthase F0 subunit 6	1.00E-15	gi|119380377|gb|ABL73405.1|	ATP production
nucleotide-binding oligomerization domain protein	1.00E-14	gi|68361190|ref|XP_688350.1|	Inflammatory response, Apoptosis
Fatty acid binding protein 6 (bile acid-binding protein)	3.00E-14	gi|50754747|ref|XM_414486.1|	Lipid metabolism
Trypsinogen-like protein 3 precursor	3.00E-14	gi|20140449|sp|O93267|TRP3_PSEAM	Digestion, Proteolysis
type II keratin	2.00E-12	gi|45239060|gb|AY547504.1|	Cytoskeleton, Positive regulator of cell proliferation, Cell differentiation
ribosomal protein L7	7.00E-11	gi|95020349|gb|AAZ23151.2|	Translation
Keratin, type I cytoskeletal 18	8.00E-11	gi|109086237|ref|XR_011513.1|	Cytoskeleton, Muscle
villin 1	1.00E-10	gi|54020823|ref|NP_001005657.1|	Actin binding, Ca binding, Brush border membrane

Tale 5.3 (continued)

Name	E-value	GenBank ref ID	Function
plastin 1 (I isoform)	2.00E-09	gi|39645699|gb|BC063742.1|	Actin binding, Ca binding
UDP-glucuronosyltransferase (UDPGT)	3.00E-09	gi|2501483|sp|Q91280|UGT3_PLEPL	Digestion, Estrogen metabolism
apolipoprotein A-IV4	5.00E-09	gi|74096418|ref|NM_001032721.1|	Lipid metabolism
5S rRNA	7.00E-09	gi|64492|emb|X05089.1|XL5SRNA	Translation
apolipoprotein A-IV3	2.00E-07	gi|74096415|ref|NP_001027892.1|	Lipid metabolism
ribosomal protein S21	2.00E-07	gi|50418439|gb|BC077662.1|	Translation
transmembrane 4 superfamily member 4	6.00E-07	gi|14789880|gb|BC010814.1|	Negative regulator of cell proliferatrion
choriogenin L [Oryzias melastigma]	7.00E-06	gi|124494532|gb|ABN13414.1|	
40S ribosomal protein S30	2.00E-05	gi|15294077|gb|AAK95215.1|	Translation
fatty acid binding protein 6	4.00E-05	gi|8393345|ref|NM_017098.1|	Lipid metabolism, Bile metabolism
40S ribosomal protein S25	2.00E-04	gi|51316652|sp|Q6PBI5|RS25_BRARE	Translation
techylectin	8.00E-04	gi|67772055|gb|AY909474.1|	

Table 5.4 Genes upregulated in female killifish exposed to 60 µg Cu L^{-1} in SW for 2 days. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
sestrin 1	0	gi|109731078|gb|BC113569.1|	1	DNA damage, Negative regulator of cell proliferation
titin	0	gi|110349718|ref|NM_133378.3|	5	Muscle
endonuclease domain containing 1, transcript variant 3 (ENDOD1)	0	gi|113423076|ref|XM_937098.2|	1	Apoptosis, DNA damage
myosin, heavy chain 1, skeletal muscle, adult (MYH1)	0	gi|115527081|ref|NM_005963.3|	1	Muscle
myosin regulatory light chain MRCL3	0	gi|16741042|gb|BC016372.1|	1	Muscle, Ca binding
golgi autoantigen, golgin subfamily a, 5	0	gi|18606387|gb|BC023021.1|	1	Golgi vescicle trafficking
WD repeat domain 77 (WDR77)	0	gi|20127622|ref|NM_024102.2|	1	Signaling, mRNA processing
putative aminopeptidase Fxna (Fxna)	0	gi|42491371|ref|NM_184050.2|	1	Proteolysis, Zn binding
microtubule-associated protein 4 (MAP4)	0	gi|47519638|ref|NM_002375.3|	2	Microtubule assembly, Cell cycle progression
cornichon homolog (Drosophila) (CNIH)	0	gi|57165418|ref|NM_001009551.1|	1	Vescicle transport, Immune response
Cardiomyopathy associated 3 (CMYA3)	0	gi|61696133|ref|NM_152381.3|	2	Cytoskeleton
SERPINE1 mRNA binding protein 1 (SERBP1)	0	gi|66346684|ref|NM_015640.3|	1	mRNA stability
protein phosphatase 6 regulatory subunit 3 (PP6R3)	0	gi|74100788|gb|DQ111954.1|	1	
DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 (DDX1)	8.00E-167	gi|4826685|ref|NM_004939.1|	1	Translation initiation, Cell growth

Table 5.4 (continued)

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
eukaryotic translation initiation factor 4 gamma, 3 (EIF4G3)	2.00E-148	gi|115529454|ref|NM_003760.3|	1	Translation
nebulin	2.00E-122	gi|1470067|gb|U58109.1|MMU58109	2	Cytoskeleton, Muscle
desmuslin (DMN), transcript variant B	6.00E-93	gi|112382238|ref|NM_015286.5|	2	Cytoskeleton, Muscle
WNK lysine deficient protein kinase 1	3.00E-91	gi|12711659|ref|NM_018979.1|	1	Ion transport, Cell signaling
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1) (GALNT1)	9.00E-22	gi|13124890|ref|NM_020474.2|	1	Protein trafficking

Table 5.5 Genes upregulated in male killifish exposed to 60 µg Cu L^{-1} in SW for 10 days. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
ribosomal protein L5 (RPL5)	1.00E-178	ref|NM_000969.3|	1.09	Translation
DEAH (Asp-Glu-Ala-His) box polypeptide 9 (DHX9)	2.00E-171	NM_001357.2	1.09	Translation initiation, Cell growth
ferritin, light polypeptide (FTL)	3.00E-133	gb|BC021670.1|	1.09	Fe binding
guanine nucleotide exchange factor p532	2.00E-130	AK226162.1	1.09	Protein transport located in the golgi
ubiquitin-binding protein homolog (UBPH)	5.00E-102	NM_019116.2	2.18	Protein degradation
CArG box-binding factor	4.00E-42	gb|L36663.1|MUSCARGBF	1.09	Transcription
Trypsinogen-like protein 3 precursor	3.00E-14	gi|20140449|sp|O93267|TRP3_PSEAM	15.27	Digestion, Proteolysis
choriogenin L [Oryzias melastigma]	8.00E-06	gi|124494532|gb|ABN13414.1|	9.82	

Table 5.6 Genes upregulated in female killifish exposed to elevated dietary Cu in SW for 10 days. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
eukaryotic translation termination factor 1 (ETF1)	1.00E-180	ref|NM_004730.1|	0.6	Translation
DEAH (Asp-Glu-Ala-His) box polypeptide 9 (DHX9)	5.00E-180	ref|NM_001357.3|	0.6	Translation initiation, Cell growth
inositol monophosphatase domain containing 1	4.00E-116	ref|NM_017813.2|	0.6	Mg binding
vitellogenin 1 (Vtg I)	3.00E-87	gb|U07055.2|FHVTGI	1.1	Reproduction
apolipoprotein A-IV1 (apoa-iv1)	7.00E-24	ref|NM_001078591.1|	1.1	Lipid metabolism
chitinase [Oncorhynchus mykiss]	1.00E-23	gi|56713231|emb|CAD59687.1|	3.3	Digestion, Carbohydrate metabolism, Immune response
metallopanstimulin	1.00E-17	emb|AJ001134.1|SRMETPANS	0.6	Translation
type II keratin	9.00E-13	AY547504.1	1.7	Cytoskeleton, Positive regulator of cell proliferation, Cell differentiation
Trypsinogen-like protein 3 precursor	3.00E-06	gi|20140449|sp|O93267|TRP3_PSEAM	11.2	Digestion, Proteolysis
apolipoprotein A-IV3 [Takifugu rubripes]	2.00E-05	gi|74096415|ref|NP_001027892.1|	0.6	Lipid metabolism

Table 5.7 Genes upregulated in male killifish exposed to elevated dietary Cu in SW for 2 days. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
beta-succinyl CoA synthetase	0	gb|AF087890.1|	2	ATP production
pyruvate dehydrogenase kinase, isozyme 4 (PDK4)	0	ref|NM_002612.3|	4	Carbohydrate metabolism, Signal transduction
eukaryotic translation initiation factor 5	3.00E-108	gb|BC007728.2|	4	Translation
gelsolin (amyloidosis, Finnish type) GSN gene	4.00E-52	gb|DQ892187.2|	2	Actin binding, Ca binding

Table 5.8 Genes upregulated in female killifish exposed to elevated dietary Cu in FW for 10 days. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
N-acetyltransferase 5 (NAT5)	0	NM_016100.3	1	
eukaryotic translation termination factor 1	2.00E-148	BC088358.1	5	Translation
ATP-dependent metalloprotease YME1L	2.00E-143	emb|AJ132637.1|HSA132637	2	Zn binding, Proteolysis
titin (TTN), transcript variant N2-A	6.00E-74	NM_133378.3	4	Muscle
cytochrome c oxidase subunit II	6.00E-54	AY735182.1	1	ATP production, Cu binding
partial cytb gene for cytochrome b	1.00E-36	AM269883.2	1	ATP production
Chitinase	6.00E-35	gi|56713231|emb|CAD59687.1|	3	Digestion, Carbohydrate metabolism
glutamate dehydrogenase	7.00E-29	emb|AJ556995.1|SSA556995	4	Nitrogen metabolism
asparaginyl endopeptidase (AEP), legumain	1.00E-20	gb|EF198070.1|	1	Proteolysis
Nod 3	4.00E-18	gi|68438101|ref|XP_688444.1|	2	Negative regulation of transcription, Cell death
Trypsinogen-like protein 3 precursor	3.00E-14	gi|20140449|sp|O93267|TRP3_PSEAM	3	Digestion, Proteolysis

Table 5.9 Genes upregulated in male killifish exposed to elevated dietary Cu in FW for 2 days. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
lysosomal associated protein transmembrane 4 beta	0	gb|BC031021.1|	1	Lysosome, Transport
cytidine and dCMP deaminase domain containing 1 (CDADC1)	0	ref|NM_030911.1|	1	Zn binding
high density lipoprotein binding protein (vigilin)	3.00E-157	NM_203346.1	2	Lipid binding
nebulin-related anchoring protein (NRAP)	2.00E-138	NM_006175.3	1	Cytoskeleton, Muscle
eukaryotic translation initiation factor 5	1.00E-106	gb|BC007728.2|	2	Translation
Ubiquinol-cytochrome c reductase complex 14 kDa protein (Complex III subunit VI)	4.00E-34	BC086921.1	2	ATP production
ubiquinol-cytochrome c reductase binding protein	9.00E-34	gi|5454152|ref|NP_006285.1|	2	ATP production
60S ribosomal protein L35	5.00E-21	gi|119608008|gb|EAW87602.1|	2	Translation
capping protein (actin filament) muscle Z-line, alpha 1	8.00E-18	gi|47086649|ref|NP_997862.1|	4	Cytoskeleton, Muscle
ATP synthase F0 subunit 6	1.00E-15	gi|119380377|gb|ABL73405.1|	1	ATP production
Apolipoprotein A-IV1	6.00E-11	gi|118344584|ref|NP_001072059.1|	2	Lipid metabolism
Choriogenin L	0.002	gi|59859131|gb|AAX09343.1|	3	

Table 5.10 Genes whose expression differs between two male control fish in SW. E-values were obtained from BLAST and the gene functions from the GENE database.

Name	E-value	GenBank ref ID	Frequency per 48 sequences	Function
protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta) (PPP3CB)	0	ref|NM_021132.1|	0.9	Fe binding, Zn binding, Signal transduction
NDRG family member 2 (NDRG2), transcript variant 3	4.00E-158	NM_201536.1	0.9	Cell differentiation
eukaryotic translation initiation factor 5 (EIF5)	1.00E-108	NM_183004.3	0.9	Translation
NOD3	1.00E-17	gi|68438101|ref|XP_688444.1|	1.8	Negative regulator of transcription, Cell death
Trypsinogen-like protein 3 precursor	4.00E-13	gi|20140449|sp|O93267|TRP3_PSEAM	15.7	Digestion, Proteolysis
UDP-glucuronosyltransferase (UDPGT)	3.00E-09	gi|2501483|sp|Q91280|UGT3_PLEPL	0.9	Digestion, Estrogen metabolism
choriogenin L	0.006	gi|124494532|gb|ABN13414.1|	2.8	

Table 5.11 Summary of selected functions / locations of the genes identified from the libraries described by table 5.2.

Function / Location	Number of genes identified
Protein synthesis	22
Metal binding	15
ATP production	13
Cytoskeleton	12
Cell growth / differentiation	11
Muscle	8
Lipid metabolisn	8
Signaling	6
Proteolysis	6
Immune response	5
Carbohydrate metabolism	4
Apoptosis	4
Reproduction	3
Digestion	3
DNA damage	2
Free radical defense	2
Nitrogen metabolism	1

Figure 5.1 Number of unique genes discovered as a function of the number of clones sequenced. Each bar represents 48 sequences. The clear bars are from library 1 in table 5.2 and the solid bar is from library 2 in table 5.2.

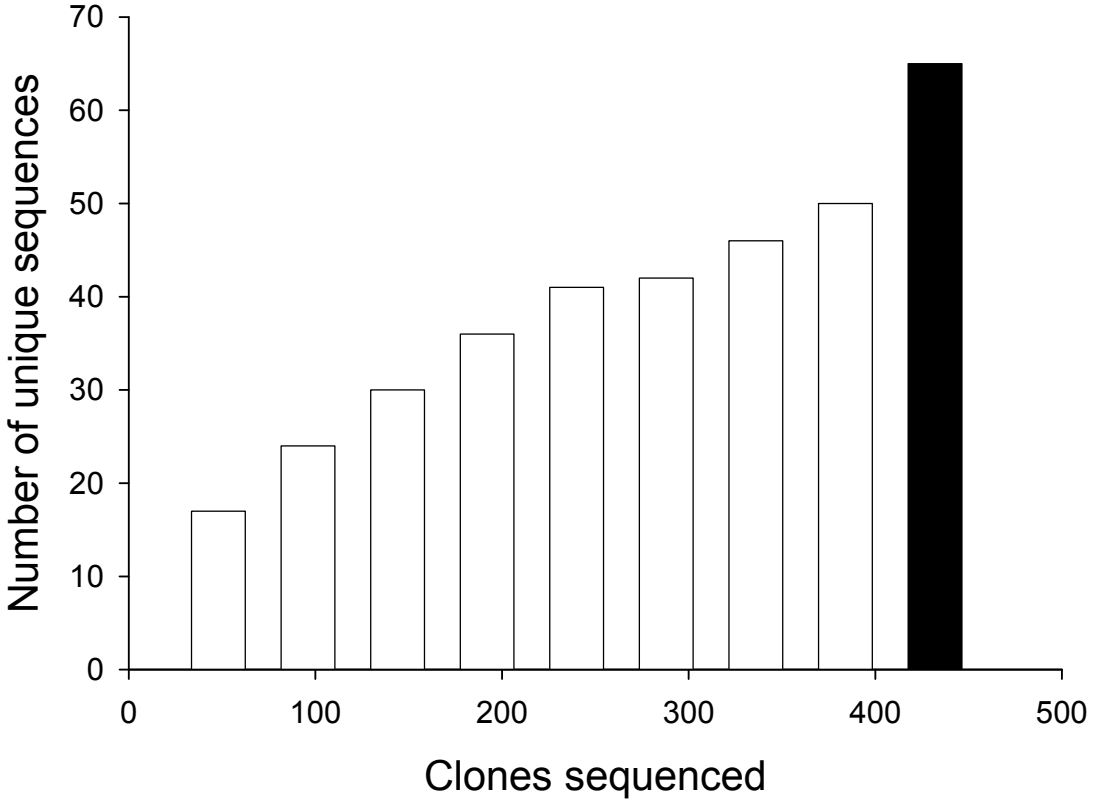


Figure 5.2 Venn diagram of differentially expressed genes shared between fish exposed to dietary Cu in FW, dietary Cu in SW, and those differing between two control individuals.

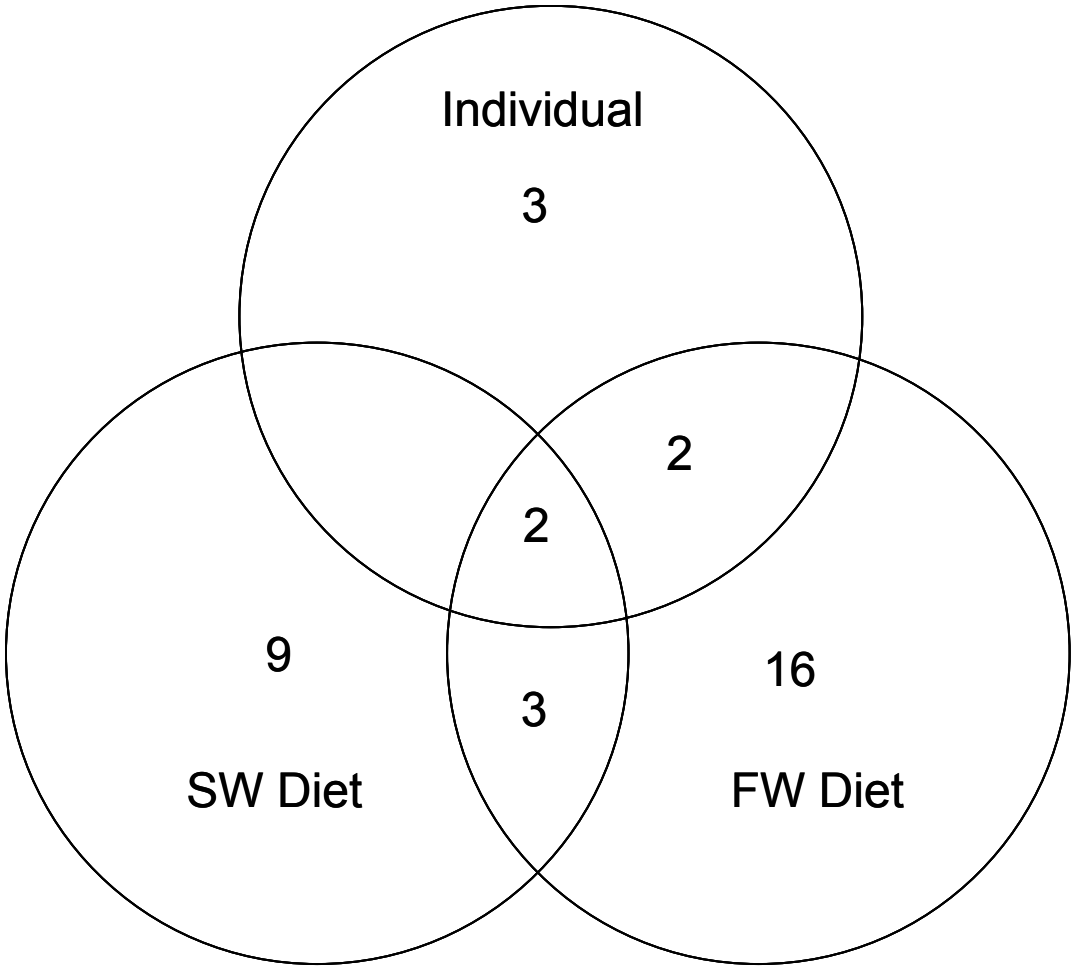
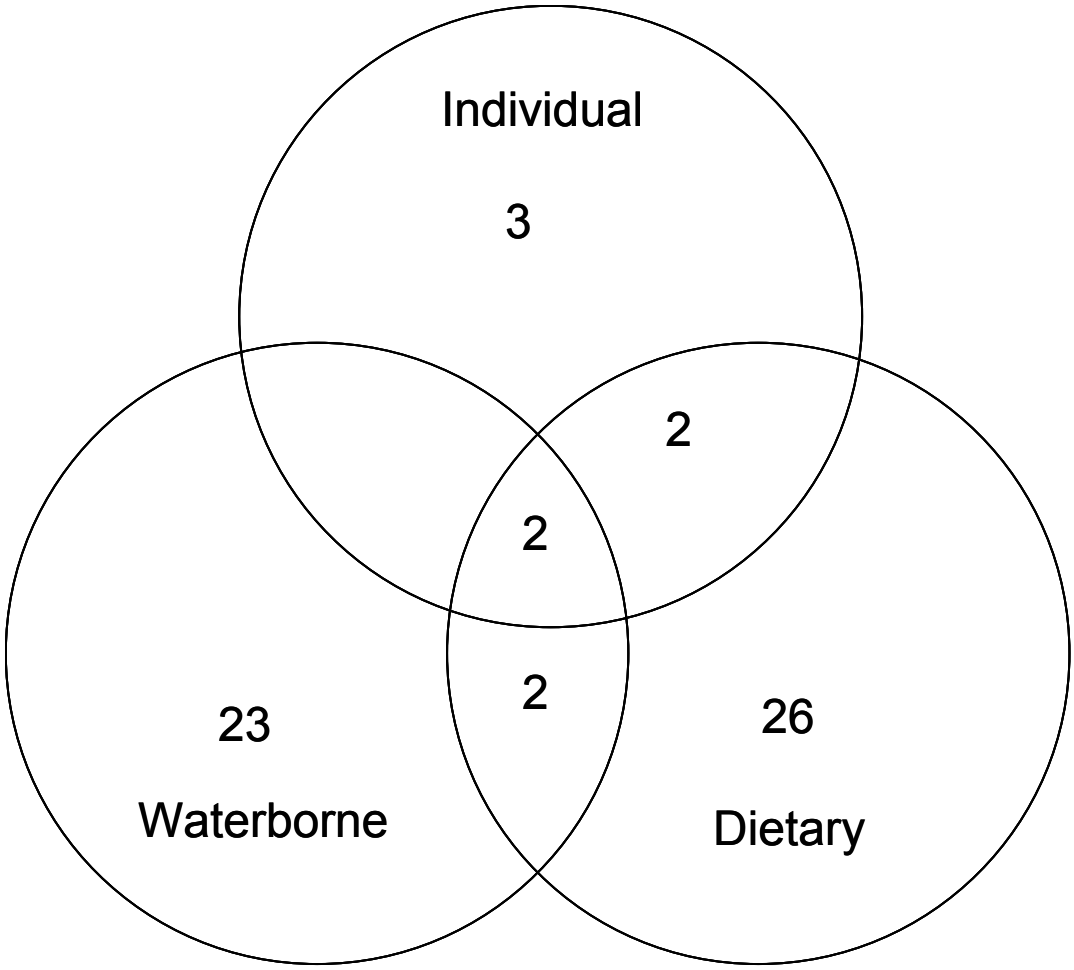


Figure 5.3 Venn diagram of differentially expressed genes shared between fish exposed to dietary Cu in FW or SW, waterborne Cu in SW, and those differing between two control individuals.



CHAPTER 6

CONCLUSIONS

6.1 Physiology is important

Current aquatic water criteria in FW for Cu are established using the BLM (USEPA, 2003). The BLM has no biological input parameters except the gill biotic ligand which is allowed to vary based on calibration tests from four organisms (fathead minnows (*Pimephales promelas*), *Ceriodaphnia dubia, Daphnia pulex,* or *Daphnia magna*) (Paquin et al., 2002; Hydroqual, 2005). This is a very limited approach because it allows for prediction of toxicity only in organisms for which there are toxicity tests which measure the appropriate parameters. This approach does not account for the >3 orders of magnitude variation that is seen in FW toxicity data (Grosell et al., 2002)

It has been noted that size and thereby Na^+ turnover rate in the organisms explains a large amount (54%) of this variation (Grosell et al., 2002). Na^+ turnover rates are correlated with size ($R^2 = 0.65$) (Grosell et al., 2002) and thus an easy adjustment would be to add a factor allowing for size to be incorporated into the BLM. This concept seems to apply to SW as well where size explains 48% of the four order of magnitude variation within SW toxicity (Grosell et al., 2007).

The BLM also suggests that as the free Cu ion concentration decreases Cu toxicity should decrease (Paquin et al., 2002). In chapter 2, we demonstrate that this may not be true because we observe the highest toxicity in FW and SW even though speciation calculations predict that the lowest concentrations of free Cu ion would be found in FW and SW (Table 2.6). A more recent study using *Fundulus heteroclitus*

demonstrates this as well. In addition to the lack of correlation between free Cu ion concentration and toxicity, it also demonstrates that as the Na^+ gradient from the inside of the fish to the outside decreases the Cu tolerance increases (Grosell et al. 2007). Currently the BLM can only predict Cu toxicity in FW, but adding a parameter to account for the difference in Na^+ gradient could allow the salinity range over which the BLM is valid to be expanded.

Finally, in chapter 3, we observed that the Cu induced mortality in FW was likely to be a result of an ionoregulatory disturbance while in SW mortality could perhaps have been caused by the inhibition of ammonia excretion. Ionoregulatory disturbances and increases in plasma ammonia have often been reported for waterborne Cu exposures in both FW and SW (Grosell et al., 2002; Grosell et al., 2003; Grosell et al., 2004a; Wilson and Taylor, 1993; Larsen et al., 1997; Stagg and Shuttleworth, 1982a). Generally, both ammonia excretion inhibition and osmoregulatory disturbance was observed when measured, but at times a lack of ionoregulatory disturbance was observed (Grosell et al., 2003). This could indicate that Cu toxicity may also be influenced by an organism's relative ability to respond to ionoregulatory disturbance and to tolerate elevated ammonia.

6.2 Environmental regulation and intermediate salinities

As stated above, Cu tolerance increases in an ionorelator, *Fundulus heteroclitus*, as the Na^+ gradient increases (Grosell et al., 2007). Therefore, current environmental regulation overprotects ionoregulators at intermediate salinities because the criteria values for intermediate salinities are currently the same as SW criteria values (USEPA,

2002; USEPA, 2003). Even if the FW value were selected, there still would be an even greater overprotection of ionoregulators because ionoregulators are least sensitive to Cu at intermediate salinities (Chapter 3; Grosell et al., 2007).

In addition to this, it will be important to evaluate these intermediate salinities for organisms with other ionoregulatory strategies. This is especially true because of the observations of Bielmeyer et al. (2005) in sea urchins. In their study, they found that not only were adult sea urchins sensitive Cu but also that the mechanism of toxicity was most likely acid-base balance disturbance. Sea urchins are osmoconformers, therefore the composition of their coelomic fluids are similar to their surrounding environment. As salinity decreases from SW (35 ppt), the buffering capacity also decreases. This would potentially act to exacerbate a Cu induced acidosis due to the lack of buffering making osmoconformers more sensitive at intermediate salinities than at high salinities.

6.3 Environmental regulation and diet

Recently the importance of diet has been demonstrated in several different manners. First it has been observed that naturally incorporated Cu diets are extremely toxic at concentrations below the water quality criteria (Bielmeyer et al. 2006). Secondly, the composition of the diet has been shown to ameliorate Cu toxicity. A diet containing elevated Na^+ was shown to be protective of waterborne Cu accumulation (Kamunde et al., 2003). Finally, it has been observed that Na enhances Cu uptake from the diet (Kjoss et al., 2005a; Kjoss et al., 2005b; Nadella et al., 2007). Therefore, it is necessary to improve regulation to adapt new toxicity testing that includes both dietary and waterborne Cu which would be more environmentally realistic.

6.4 Chemistry, chemistry, chemistry

The EPA has adopted the use of the BLM for establishing FW Cu water quality criteria (USEPA, 2003) and would like to have a similar model available for SW and intermediate salinities. It has as its inputs a set number of parameters which can be measured in exposure waters (Hydroqual, 2005). It is necessary that reviewers and editors insist that these parameters be reported in new publications relating to metal exposures. This is important because without this information the BLM can not be improved to better protect aquatic organism. In addition to this, it is also important, with the new insight into the effects of diet, to require the reporting of the composition of the diet in all metal publications.

6.5 Overall conclusions

Cu accumulation and toxicity are processes that are influenced not only by water chemistry but also by physiology. In order to develop better water quality criteria, both of these factors should be considered in any model designed to predict Cu toxicity. The work presented here demonstrated that the principles used to develop the BLM may not necessarily hold true at higher salinities because of the profound changes in fish physiology that occur as salinity increases (Chapter 2,3, and 4), dietary Cu does not appear to be accumulated as the free ion (Chapter 4), and there is a coordinated response to Cu exposure at the molecular level that involves cellular, tissue, and systemic responses (Chapter 5). Hopefully, my work has illustrated the need for additional study at intermediate and high salinities preferably using a comparative approach that examines

organisms with differing ionoregulatory strategies (Chapter 2 and 3). It is also hoped that the necessity to further examine trophic transfer of naturally incorporated dietary Cu along a food chain at different salinities is evident (Chapter 4) and that the data obtained will aid in the establishment of more versatile aquatic water quality criteria for Cu.

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