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Effects of copper on the acute cortisol response and associated physiology in rainbow trout

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ABSTRACT

The aim of this study was to determine the effects of chronic waterborne copper (Cu) exposure on the acute stress-induced cortisol response and associated physiological consequences in rainbow trout (*Oncorhynchus mykiss*). Trout were exposed to 30 μg Cu/L in moderately hard water (120 mg/L as $CaCO_3$) for 40 days, following which time the acute cortisol response was examined with a series of stressors. At 40 days, a 65% increase in Cu was observed in the gill, but no accumulation was observed in the liver, brain or head kidney. Stressors such as air exposure or confinement did not elicit an increase in circulating cortisol levels for Cu-exposed fish, in contrast to controls. However, this inhibitory effect on the acute cortisol response appeared to have few implications on the ability of Cu-exposed fish to maintain ion and carbohydrate homeostasis. For example, plasma Na^+, Ca^{2+} and glucose levels as well as hepatic glycogen levels were the same post-stress in control and Cu-exposed fish. Trout were also challenged with exposure to 50% seawater for 48 h, where Cu-exposed trout maintained plasma Na^+, glucose and hepatic glycogen levels. However, Cu-exposed fish experienced decreased plasma K^+ levels throughout the Cu exposure and stress tests. In conclusion, chronic Cu exposure resulted in the abolition of an acute cortisol response post-stress. There was no Cu accumulation in the hypothalamus-pituitary-interrenal axis (HPI axis) suggesting this was not a direct toxic effect of Cu on the cortisol regulatory pathway. However, the lack of an acute cortisol response in Cu-exposed fish did not impair the ability of the fish to maintain ion and carbohydrate homeostasis. This effect on cortisol may be a strategy to reduce costs during the chronic stress of Cu exposure, and not endocrine disruption as a result of toxic injury.

1. Introduction

Copper (Cu) is a widely exploited metal in industry, primarily in the production of wire, other electrical products and plumbing pipes (Lander and Reuther, 2004). Although trace amounts of Cu occur naturally in the environment, additional Cu from industrial and domestic wastewater effluents can enter aquatic ecosystems. Global production has increased exponentially over the past 100 years (Lander and Reuther, 2004), and along with increased production comes higher amounts of Cu entering receiving waters, which can impact aquatic biota since Cu is toxic at higher concentrations (Pyle et al., 2005). The levels of Cu that cause toxicity in fish vary between species and life stages, while water chemistry also plays an important role (Taylor et al., 2000). Fish chronically exposed to Cu undergo various physiological and behavioral changes such as a loss of appetite, growth suppression, ionoregulatory disturbance, lower aerobic capacity and higher mortality (Taylor et al., 2000).

Animals possess physiological mechanisms to adapt to changes in their environment. For example, cortisol is a corticosteroid produced by the interrenal cells, which are located in the head kidney of teleosts (the fish homologue of the mammalian adrenal cortex). Circulating cortisol levels are regulated by hypothalamus and pituitary signaling, and rise when fish are exposed to stressors that disrupt homeostasis, such as changes in salinity or toxicant exposure (Hontela, 1997; Richards et al., 2003). This augmented secretion of cortisol in times of stress is generally thought to be an adaptive response, with the neuroendocrine system mediating the link between the environmental challenge and the physiological response (McCormick, 2001). For example, cortisol signaling increases gluconeogenesis in the liver in order to increase the available energy that is required to regain homeostasis (Aluru and Vijayan, 2007). Cortisol signaling is also involved in ionoregulation, with circulating levels increasing when freshwater fish are challenged with either ion-poor water or elevated salinity. Specifically, cortisol appears to cause chloride cell proliferation so as to promote ion uptake in dilute freshwater and ion excretion in seawater (Perry and Wood, 1985; Madsen et al., 1995; Perry, 1997; McCormick, 2001; Richards et al., 2003). Apart from its role in the stress response, cortisol also influences growth and metabolism, immune function and reproduction (Mommsen et al., 1999).

Earlier field studies showed that fish from contaminated environments do not elicit an acute cortisol response when exposed to stressors such as capture from a site (Hontela et al., 1992, 1995).

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Lab studies also showed that impaired acute cortisol secretion with chronic Cu exposure in trout is accompanied by changes in plasma glucose, hepatic glycogen and gill Na^+/K^+-ATPase activity (Levesque et al., 2003; Gagnon et al., 2006). One hypothesis based on *in vitro* evidence is that Cu impairs the secretion of cortisol due to a toxic effect on the interrenal cells (Brodeur et al., 1997; Gagnon et al., 2006).

The main objective of this study was to examine the physiological consequences of the loss of acute cortisol response in the face of a stressor. We hypothesized that chronic Cu exposure would impair acute cortisol secretion as observed in past studies (Laflamme et al., 2000; Gagnon et al., 2006), perhaps due to Cu accumulation in the HPI axis causing toxic injury. This would in turn affect the ability of the fish to mobilize carbohydrate stores and maintain ion homeostasis when faced with stressors such as air exposure, isolation or 50% seawater exposure. However, the results of the present experiments do not support this sequence of events, and have led us to propose an alternative explanation.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*; mean length = 12.7 cm, mean mass = 25 g) were purchased from Humber Springs Hatchery (Orangeville, ON, Canada) and maintained in four 150-L tanks with 50 fish per tank on a feeding ration of 2% body weight per day of dried pellet feed (Martin Mills Inc., ON, Canada). Tanks were aerated, received a water flow of 2 L/min, and were maintained at 12 °C with 12 h light/12 h dark photoperiod. Water was Hamilton dechlorinated tap water originating from Lake Ontario (pH 8.0, Na^+ 13.8, Cl^- 24.8, Ca^{2+} 40.0, all in mg/L; dissolved organic carbon 1.3 mg C/L; hardness 120 mg $CaCO_3$/L; Cu < 2 μg/L).

2.2. Cu exposure

After an acclimation period of 4 weeks, a concentrated Cu solution ($CuSO_4.5H_2O$ in 0.1% trace metal grade nitric acid, Sigma-Aldrich, Oakville, Canada) dripped at 0.5 mL/min into a head tank to achieve a nominal initial waterborne concentration of 20 μg Cu/L in two of the four tanks. The drip rate was then progressively increased, with the goal of reaching 40 μg Cu/L by day 30 (see Fig. 1A); the rationale here was to avoid initial mortality and allow the fish to gradually acclimate to the toxicant. Water samples for dissolved Cu measurements (0.45 μm filtration) were taken every few days. Based on our previous experience with trout in the same water quality, the Cu exposure range was chosen to invoke a physiological response in the fish, but to be below the level that would cause mortality (McGeer et al., 2000a). Daily feeding (2% ration) was continued throughout the 40-day exposure. Each set of fish was non-terminally sampled for weight measurements on days 0, 20, 30, and 40 by bulk weighing the fish and dividing the weight by the number of fish in the tank.

Control and Cu-exposed fish were terminally sampled on days 0, 20 and 40 of the experiment, using rapid anesthesia with an overdose of MS-222. On these days, the blood (plasma), brain, liver, gills, kidney and head kidney (identified as the anterior-most portion of the kidney, where the tissue bifurcates) were sampled from 4 fish in each of the four experimental tanks. To minimize the potential for netting-induced changes in circulating cortisol when sampling a tank, 4 fish were netted all at once and immediately transferred to freshwater containing 0.5 g MS-222 (neutralized). Within 1 min blood was collected from all fish by caudal severance, after which time tissues were sampled. With 2 tanks per treatment, $N = 8$ samples for control and Cu-exposed treatments. Blood samples were collected in vials containing 10 μL heparinized Cortland's saline and centrifuged for 5 min at 5000*g* to separate the plasma which was removed and stored at −70 °C. Tissues were stored at −70 °C until analysis.

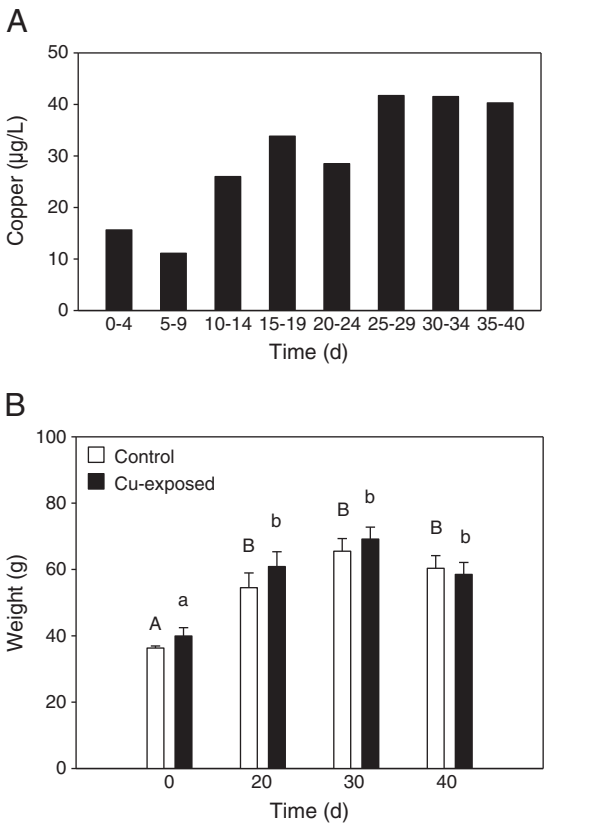

Fig. 1. A) Dissolved water Cu concentrations in the exposure treatment (control values were <2 μg/L). B) Fish weights over the 40 days. Open bars represent control fish and filled bars represent Cu-exposed fish. Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc test ($P < 0.05$). Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between Cu-exposed treatments. Data are means ± SEM. $N = 50$.

2.3. Stress tests

After 40 days, control and Cu-exposed trout were subjected to three stress tests (while the Cu exposure and control exposure conditions were maintained). The first stressor was an acute air exposure: 7 control and 7 Cu-exposed fish were individually netted and held out of the water for 1 min, then returned to aerated recovery tanks (separate from the exposure tanks) for 1 h and sampled as above [1 h post-stress is the time when cortisol levels are reported to reach their peak in rainbow trout (Gravel et al., 2008)].

The second stress test was isolation and confinement, in which 7 control and 7 Cu-exposed fish were placed in separate 150-mL darkened plastic boxes that received fresh water (50 mL/min) and aeration. After 24 h, fish were sampled as above.

The third stress test was a salinity challenge designed to evaluate the effects of Cu exposure on the ability of these fish to acclimate to 50% seawater (Instant Ocean), by analyzing plasma cortisol and cation (Na^+, K^+ and Ca^{2+}) concentrations. Control and Cu-exposed trout (32 fish each) were split into four treatment groups (8 fish each). To test whether physiological impacts associated with Cu exposure were specifically due to an impairment of cortisol secretion, fish in each of the control and seawater transfer groups were injected with either sham implants or cortisol implants (containing 250 mg/kg hydrocortisone 21-hemisuccinate; Sigma Diagnostics, St. Louis, MO, USA). The implants

were injected 24 h prior to the start of the salinity challenge test, using the methodology of De Boeck et al. (2001a). The implantation procedure entailed lightly anesthetizing the fish with neutralized MS-222 (100 mg/L), which was followed by an intraperitoneal injection with either coconut oil or a mixture of coconut oil and cortisol. Coconut oil (~27 °C) with cortisol (250 μg/g fish) or without cortisol was injected in a volume of 10 μL/g fish (De Boeck et al., 2001a). The fish were then placed on ice for 10–15 s to allow the coconut oil to solidify. Fish were then transferred to 55-L static tanks with aeration for 24-h recovery.

After the 24-h recovery period, the tanks were drained to a very low level without air-exposing the fish and replenished with freshwater or 50% seawater, to start the salinity challenge. After the 48-h test, trout were sampled as described above.

2.4. Analysis

The blood plasma was collected through centrifugation of heparinized blood samples and was analyzed for cortisol, glucose, Na^+, K^+ and Ca^{2+}. Cortisol levels were measured using a validated ELISA kit (Caymann Chemicals, Ann Arbor, MI, USA).

Cu, Na^+, K^+ and Ca^{2+} analyses were performed on tissues after they were digested by adding five times their volume of 1 N trace metal grade nitric acid and incubating at 60 °C for 48 h. Cu concentrations in liver, gill, brain, plasma, kidney, head kidney and water samples were analyzed using a Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS; Varian SpectrAA-220 with graphite tube atomizer [GTA-110], Mulgrave, Australia), with the reference standard TM15 (Environment Canada certified reference material, recovery was >90%). The levels of Na^+, K^+ and Ca^{2+} were analyzed in all tissues using flame atomic absorption spectrophotometry (SpectraAA 220FS) and certified commercial standards (Fisher Scientific). Glucose and glycogen levels were determined in the liver and plasma by an enzymatic process which uses hexokinase and amyloglucosidase (Infinity Reagents), as described by Hontela et al. (1995).

Na^+/K^+ ATPase activity was determined in triplicate samples using homogenates of gill that had been dissected and stored at −70 °C following the methods of McCormick (1993). Activity was calculated as the activity without an inhibitor (oubain; Sigma-Aldrich, Oakville, ON, Canada) minus the activity with the inhibitor. The assay measured NADH depletion in 96-well plates at 340 nm (SpectraMAXPlus: Molecular Devices, Menlo Park, CA, USA). Measurements were taken over 30 min at 15 s intervals. Activities were normalized to the protein content of the homogenate, as determined using BSA standards (Sigma-Aldrich) and Bradford's reagent (Sigma-Aldrich; Bradford, 1976).

2.5. Statistics

Statistical analysis was performed with SigmaPlot 10.1. Student's *t* tests (two-tailed) were used to determine differences between control and Cu-exposed fish. One-Way ANOVA was routinely used to detect variation among multiple treatment groups and where the F value indicated significance, Fishers LSD post hoc test was used to identify specific significant differences. All data were checked for homogeneity of variances and normality of distribution, and where necessary were transformed using natural logarithm or square root functions. All data are presented as means ± SEM (*N*, number of fish) on non-transformed data. Changes were considered significant at $P<0.05$.

3. Results

3.1. Water and fish parameters

A nominal Cu concentration of 30 μg/L was used. The initial waterborne dissolved Cu concentration measured in the exposure tanks was approximately 15 μg Cu/L, and this was raised progressively to 40 μg Cu/L by 25 days (Fig. 1A). The mean exposure concentration over the 40 days was 30.1 ± 3.9 μg Cu/L. In the control tanks, Cu levels remained below 2 μg Cu/L. There were no mortalities in the control treatment and two mortalities in the Cu treatment (or 2%) over the first 20 days of the exposure. However, there was an additional instance of mortality, at rates of 9% and 6% in the control and Cu treatments at 25 days, due to an unexpected elevation in water chlorine.

Over the 40-day exposure there was no difference in growth rates between control and Cu exposed fish; both groups increased their mean mass by about 60% from about 38 g to 60 g (Fig. 1B).

3.2. Tissue Cu accumulation

Levels of Cu in the liver, even prior to Cu exposure, were 25–100 times greater than in any of the other tissues sampled, including the gills, and did not significantly accumulate Cu over the course of the exposure (Fig. 2A). The gills accumulated Cu, exhibiting 65% increases above controls at 40 days (Fig. 2B). Neither the kidney (Fig. 2C), the brain (Fig. 2D), nor the head kidney (Fig. 2E) accumulated Cu over the course of the exposure. The brain exhibited a biphasic pattern, falling at 20 days, then rising significantly above pre-exposure concentrations at 40 days (Fig. 2D). Note however, that exactly the same fluctuations occurred in the controls, so there was no differential accumulation of Cu in the brain as a result of chronic waterborne Cu exposure.

3.3. Plasma cortisol levels

At 40 days of the exposure, basal plasma cortisol levels were not significantly different, but were 43% higher in Cu-exposed fish compared to controls ($P=0.13$, Fig. 3A). After acute air exposure, cortisol levels in control fish increased almost 3-fold from basal levels (Fig. 3B). However, cortisol levels in Cu-exposed fish did not increase above unstressed levels after air exposure (Fig. 3B). In the second stress test, cortisol levels in control fish increased from basal cortisol levels after 24 h isolation (Fig. 3C). Again, in comparison to unstressed levels of cortisol, Cu exposed fish did not mount a significant cortisol response following the stress of isolation (Fig. 3C).

At the conclusion of the 48 h salinity challenge, plasma cortisol levels in Cu-exposed fish were not different than control fish in either 50% sea water or fresh water, nor were there cortisol differences between fish in fresh water or 50% sea water (Fig. 3D). Fish that received cortisol implants had approximately 7 to 22-fold increases in the levels of plasma cortisol compared to the sham-implanted fish (Fig. 3D). Cu-exposed and control fish which received cortisol implants had similar cortisol levels (Fig. 3D).

3.4. Plasma ions

Plasma Na^+ levels remained constant over the 40-day exposure and did not differ between control fish (167.7 ± 1.7 mM) and Cu-exposed fish (167.1 ± 6.4 mM) (data not shown). In addition, plasma Na^+ was not different between control and Cu-exposed fish after acute air exposure. Exposure to 50% seawater for 48 h did not change plasma Na^+ levels in any treatment, regardless of whether the fish had received cortisol implants (data not shown). Plasma ion levels were not measured after the isolation stress test, due to insufficient sample volumes.

Similar to Na^+, plasma Ca^{2+} levels (approximately 2.0 mM) also remained constant over the 40-day exposure period, with no significant difference between levels in control and Cu- exposed fish. In addition, acute air exposure did not have an effect on plasma Ca^{2+} levels, nor were there any significant differences in any treatment after the 50% seawater experiment (data not shown).

In control fish, plasma K^+ concentration declined by about 50% on day 20 relative to the pre-exposure value, and then increased to

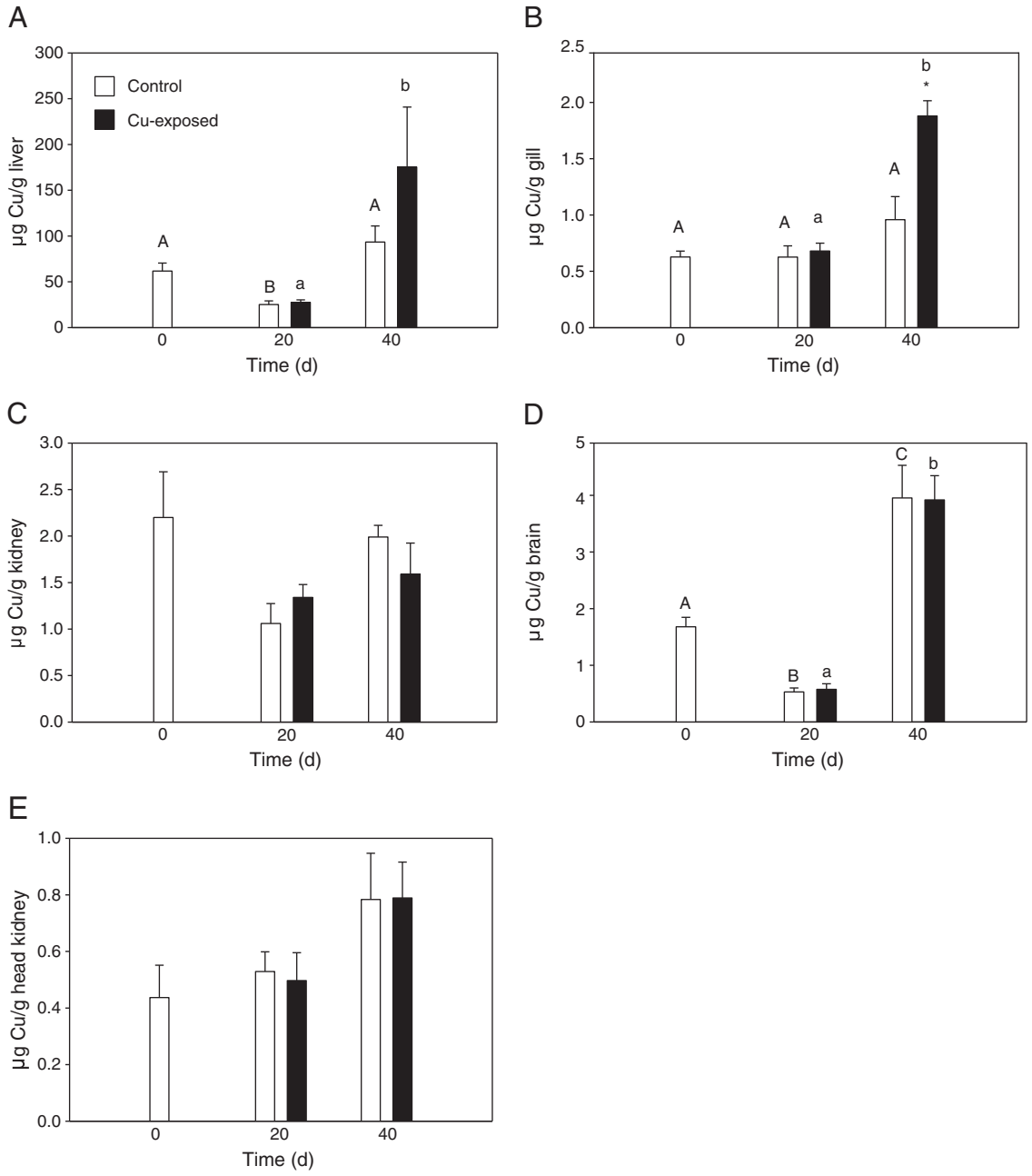

Fig. 2. Cu accumulation in rainbow trout A) liver B) gill C) kidney D) brain and E) head kidney over the 40 day experiment. Open bars represent control fish and filled bars represent Cu-exposed fish. An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between Cu-exposed treatments. Data are means ± SEM. A–C) $N=7$, and D) $N=3$–7.

intermediate levels on day 40 (Fig. 4A). In addition, chronic Cu exposure alone affected plasma K^+ concentrations, which were lower than in control fish by 26% on day 20 and by 21% on day 40 of the exposure (Fig. 4A). Plasma K^+ concentrations in the sham implanted, Cu-exposed fish in both freshwater and 50% seawater were not significantly different by themselves, but when the two treatments were combined, the difference from controls was significant ($P=0.002$) (Fig. 4C).

K^+ levels in the liver, brain and gill (Table 1) were many-fold higher than in plasma (Fig. 4). In control fish, the same relative pattern of variation in plasma K^+ that was observed over the 40-day exposure (Fig. 4A) was also observed in the liver (Table 1). In contrast, brain and gill K^+ remained constant over the 40 days in the control treatment (Table 1). Although plasma K^+ decreased with chronic Cu-exposure (Fig. 4A), liver K^+ increased by 41% at day 40 of the exposure. K^+ levels in the brain and gill were not affected by Cu exposure over the 40-day period (Table 1).

3.5. Plasma glucose levels

There were no significant differences in plasma glucose between control and Cu-exposed fish at 40 days (Fig. 5A), and after the 1 min air exposure (Fig. 5B) or 24 h isolation stress tests (Fig. 5C). However, both treatments experienced an increased in glucose levels following the stressors. After the 50% seawater experiment, plasma glucose levels generally remained constant regardless of salinity or Cu exposure (Fig. 5D).

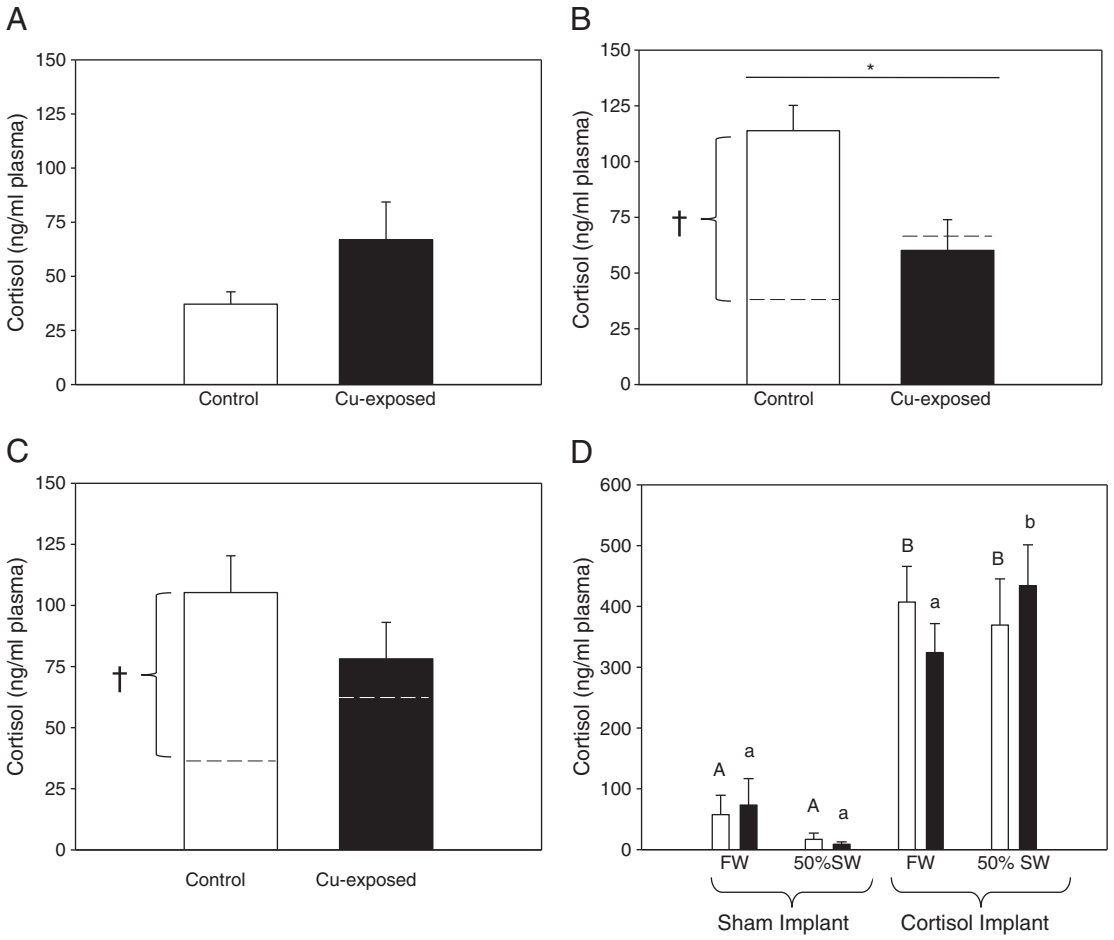

Fig. 3. Cortisol levels in rainbow trout, A) at rest on day 40 of the 30 μg/L Cu exposure B) after the stress of air exposure, following the 40 day experiment, C) after the stress of 24 h confinement, following the 40 day experiment and D) after a 48 h salinity challenge following the 40 day experiment; in freshwater (FW) or 50% seawater (SW) and with sham or cortisol implants. An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P<0.05$). A dagger represents a significant difference from unstressed (basal) cortisol levels as determined with a Student's *t*-test ($P<0.05$). The dashed lines in panels B) and C) represent the unstressed (basal) cortisol levels in panel A). Values with different letters indicate a significantly higher circulating cortisol levels in the fish that received a cortisol implant compared to sham implanted fish as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between Cu-exposed treatments. Data are means ± SEM. A–C) $N=7$ and D) $N=3$–7.

3.6. Hepatic glucose and glycogen levels

Hepatic glucose and glycogen levels of Cu exposed fish did not differ from control fish over the 40-day chronic Cu exposure (Table 2). Hepatic glycogen levels did not change after air exposure or 24 h isolation stress tests (data not shown).

3.7. Na^+/K^+ ATPase activity

Na^+/K^+ ATPase activity was analyzed only in gill samples from the 48 h sea water exposure. In fresh water, Cu-exposed fish had higher Na^+/K^+ ATPase activities than controls, in both the sham (13.5 fold increase) and cortisol implant (3-fold increase) treatments (Fig. 6). Activity in control fish gills was also increased approximately 7-fold with 50% seawater exposure (Fig. 6). However, exposure to 50% seawater actually decreased Na^+/K^+ ATPase activity in Cu-exposed fish in both the sham and cortisol implant treatments (Fig. 6).

4. Discussion

Chronic Cu exposure abolished the stress-induced acute cortisol response, as hypothesized and previously shown in a number of studies with fish exposed to various toxicants in the laboratory and in the field (e.g. Hontela et al., 1992; Gagnon et al., 2006). However, contrary to our prediction, Cu-exposed trout were still able to maintain plasma ions and carbohydrate homeostasis in the face of multiple stressors. We suggest that the loss of an acute cortisol response may be an advantageous physiological adaptation to the chronic stress of Cu exposure, rather than an involuntary toxic effect of the metal.

4.1. Cu accumulation

The mean waterborne Cu exposure concentration of 30 μg/L chosen for this study is environmentally realistic, and is similar to the levels observed in waters contaminated by mining activity (Taylor et al., 2003; Pyle et al., 2005). After 40 days of Cu exposure, rainbow trout experienced moderate increases in gill Cu burden, which was the only tissue that accumulated the metal, similar to previous studies with chronic Cu exposure in trout (McGeer et al., 2000b; Taylor et al., 2000). The liver did not accumulate significant amounts of Cu, similar to rainbow trout exposed to 20 μg/L (Taylor et al., 2000). The brain and head kidney (tissues of the HPI axis) did not accumulate Cu over the 40 day exposure. Another study examining chronic waterborne Cu exposure in rainbow trout also found no significant Cu accumulation in the head kidney (Gagnon et al., 2006), however, they did find modest Cu accumulation in the kidney, contrary to the present study. These

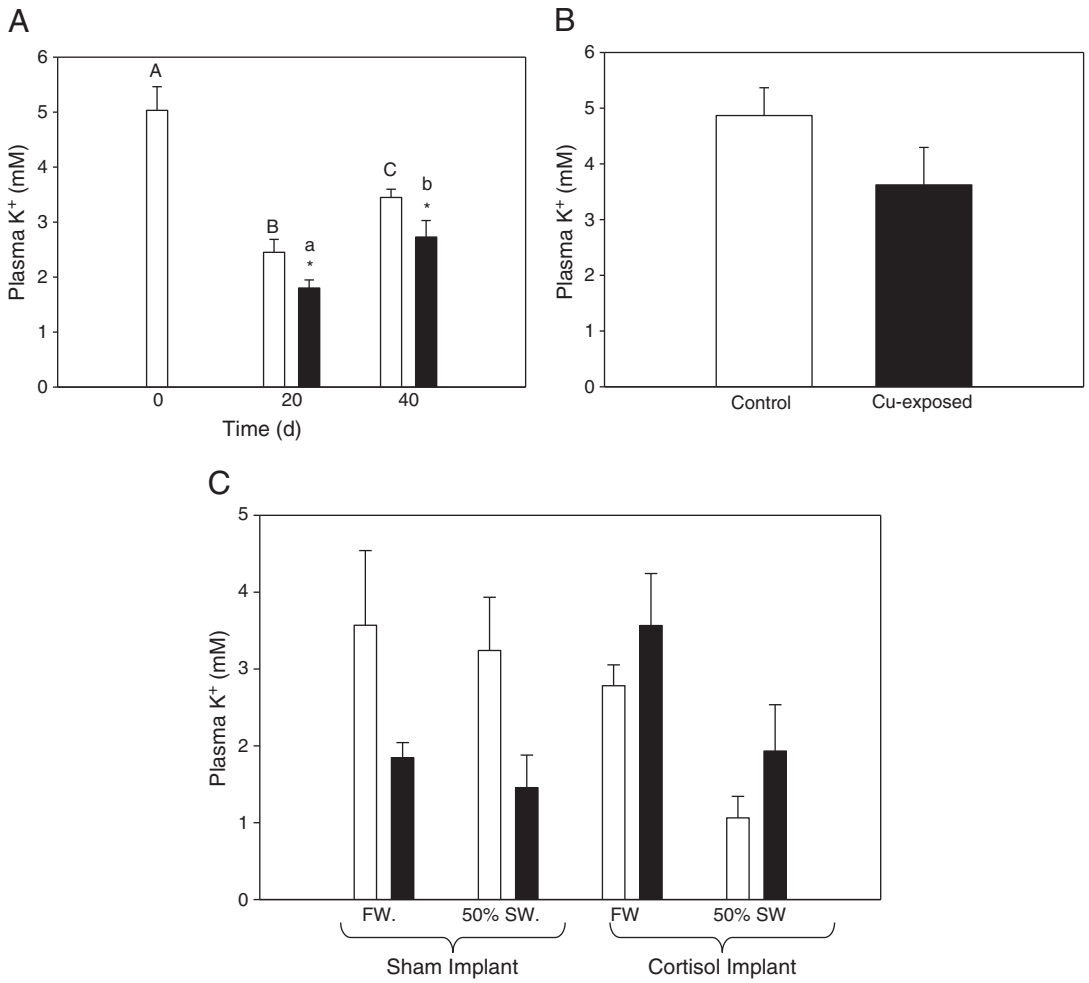

Fig. 4. Plasma K^+ levels in rainbow trout A) over the 40 day experiment. B) after the stress of air exposure, following the 40 day experiment and C) after a 48 h salinity challenge following the 40 d experiment; in freshwater (FW) or 50% seawater (SW) and with sham or cortisol implants. Open bars represent control fish and filled bars represent Cu-exposed fish. An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's *t*-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc test. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between Cu-exposed treatments. Data are means ± SEM. A, B) $N=7$ and C) $N=3$–7.

data suggest that the effect of chronic Cu exposure on acute cortisol secretion is not a direct toxic effect of Cu accumulation in the HPI axis.

4.2. Cortisol

In previous studies examining the effects of waterborne Cu on unstressed or basal cortisol, results have been variable and include: 1) no effect of Cu on circulating cortisol in rainbow trout (Gagnon et al., 2006), 2) increased basal cortisol with Cu exposure in zebrafish (Craig et al., 2009) and tilapia (Wu et al., 2008) and 3) decreased basal cortisol levels with Cu exposure in eel (Oliveira et al., 2008). In the present study, the 43% rise in mean cortisol was not statistically significant ($P=0.13$). A moderate increase in cortisol levels with chronic Cu-exposure, may serve to up regulate the copper transporter ctr1 in the gill to promote Cu elimination (Craig et al., 2007). In fact, many of the changes in gene expression that occur with chronic Cu exposure may be specifically due to cortisol signaling (Craig et al., 2009).

Control trout experienced an elevation in circulating cortisol concentration after an acute air exposure, similar to previous studies (e.g. Norris et al., 1998; Gravel et al., 2005; Gagnon et al., 2006; Aluru and Vijayan, 2007). However, fish that were chronically exposed to Cu did not increase cortisol levels above their basal concentrations. A lack or diminished cortisol response with contaminant exposure has been previously observed (Hontela et al., 1992; Gravel et al., 2005; Gagnon et al., 2006) and theorized to be due to direct Cu toxicity on the HPI axis. *In vitro* evidence showed that cortisol secretion by the interrenal cells in trout is decreased with the addition of high levels of Cu to the medium ($EC_{50}=11{,}438$ μg Cu/L; Gagnon et al., 2006). In the present study, Cu levels were not elevated in the head kidney, remaining at about 800 μg Cu/kg in both control and Cu-exposed trout (Fig. 2E), suggesting direct toxicity is not the mechanism of cortisol impairment. Additionally, basal cortisol levels were not lower in Cu-exposed fish compared to controls, further suggesting there was not a direct toxic effect of Cu on cortisol.

Table 1
Tissue K^+ levels in rainbow trout in liver, brain and gill (μmol/g tissue) during the 40 days, 30 μg/L Cu exposure.

	Time	Liver	Brain	Gill
Control	0 day	150.1 ± 7.5^{a}	106.8 ± 4.5^{a}	49.2 ± 6.7^{a}
	20 days	62.1 ± 2.3^{b}	93.8 ± 8.1^{a}	62.6 ± 5.3^{a}
	40 days	94.5 ± 10.2^{c}	90.1 ± 2.8^{a}	63.4 ± 2.5^{a}
Cu-exposed	20 days	62.6 ± 10.0^{a}	85.4 ± 2.6^{a}	63.8 ± 6.5^{a}
	40 days	$133.4 \pm 6.1^{b*}$	95.1 ± 9.8^{a}	62.3 ± 6.2^{a}

Values with different letters are significantly different within a treatment, as determined by an ANOVA followed by Fisher LSD post hoc test ($P<0.05$). An asterisk (*) indicates a significant difference between control and Cu-exposed fish tissue at the same time point, as determined by a Student's *t*-test ($P<0.05$). Data are means ± SEM. $N=7$.

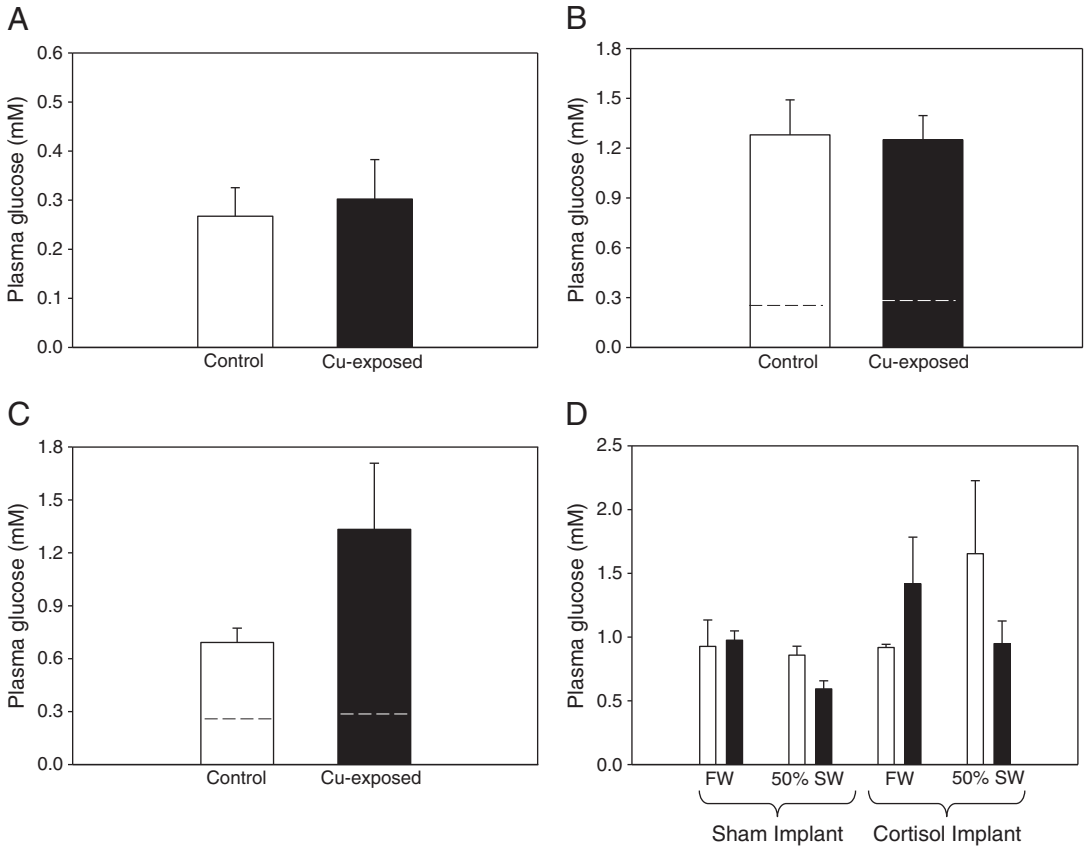

Fig. 5. Plasma glucose levels in rainbow trout A) on day 40 of the 30 μg/L Cu exposure (basal) B) after the stress of air exposure, following the 40 day experiment C) after the stress of 24 h confinement, following the 40 day experiment and D) after a 48 h salinity challenge; in freshwater (FW) or 50% seawater (SW) and with sham or cortisol implants. An asterisk (*) indicates a significant difference from control levels in the same treatment as determined by a Student's *t*-test ($P<0.05$). Data are means ± SEM. A–C) $N=7$ and D) $N=3$–7. Dashed lines in panels B) and C) represent the unstressed (basal) plasma glucose levels in panel A).

Alternatively, a decreased acute cortisol response may be of adaptive value that occurs during the metal-acclimation process. Acclimation to waterborne Cu by both toxicological (Dixon and Sprague, 1980) and physiological criteria (Laurén and McDonald, 1987a, 1987b) is a process that has been well documented (McDonald and Wood, 1993). A reprogramming of the HPI axis stemming from the stress of chronic Cu exposure may be part of this process. Previous studies with trout have shown that acute stressor exposure or acute artificial elevation of cortisol levels can reduce the cortisol response when fish are faced with stressors at a later date (Auperin and Geslin, 2008). In addition, rainbow trout fed cortisol-supplemented pellets for 10 weeks did not increase plasma cortisol after exposure to a handling stressor (Barton et al., 1987). However, these fish were still able to increase plasma glucose levels post-stress, to the same extent as control fish (Barton et al., 1987). The impacts of cortisol generation are multifaceted and very expensive, manifested as a "higher cost of living" (Gregory and Wood, 1999; De Boeck et al., 2001b). It may be an adaptive cost-saving measure not to raise plasma cortisol any higher in response to an acute stressor when an individual is already suffering from a chronic stressor. Notably, the Cu-exposed trout of the present study exhibited no decrement of growth relative to the controls despite the fixed ration, suggesting that the cost of living in these fish was not chronically elevated. But how are cortisol response-inhibited fish able to maintain cortisol related functions?

Table 2
Hepatic glucose and glycogen (mmol/kg) on day 40 of the 30 μg/L Cu exposure.

	Control	Cu-exposed
Hepatic glucose	4.3 ± 1.0	5.1 ± 0.9
Hepatic glycogen	104.0 ± 19.7	125.0 ± 19.6

There are no significant differences (Student's *t*-test, $P<0.05$). Data are means ± SEM. $N=7$.

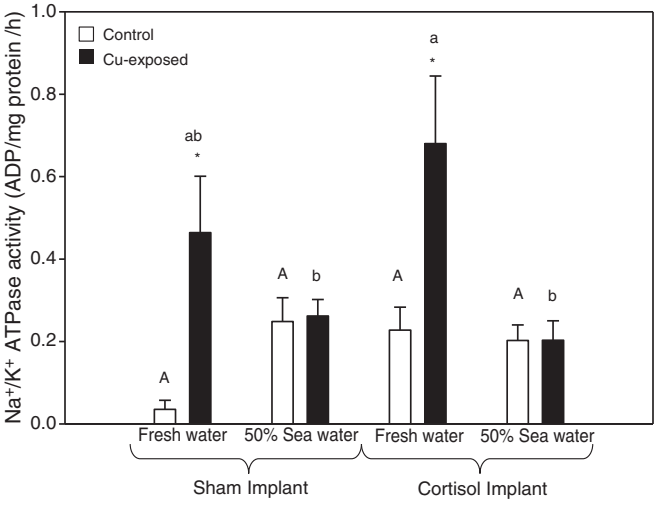

Fig. 6. Gill Na^+/K^+ATPase levels in control and Cu exposed rainbow trout after exposure to 50% seawater with and without cortisol implants for 48 h. Open bars represent control fish and filled bars represent Cu-exposed fish. An asterisk (*) indicates a significant difference from control levels in the same treatment as determined with a Student's *t*-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between Cu-exposed treatments. Data are means ± SEM. $N=3$–5.

The ability to maintain the hyperglycemic response and carbohydrate homeostasis may be due to the activation of other signaling pathways (Handy, 2003) involving catecholamines (Weber and Shanghavi, 2000; Van Heeswijk et al., 2006) or serotonin (Tubío et al., 2010). Much more work is required to examine the mechanisms of stress adaptation in fish, a topic that receives a great deal of attention in mammalian (human) studies (e.g. Matthews and Phillips, 2010).

Cortisol signaling is mediated primarily by the glucocorticoid receptor (GR) and also the mineralocorticoid receptor (Bury and Sturm, 2007). Cortisol regulates the expression of GR (Aluru and Vijayan, 2007), and therefore changes in GR abundance or sensitivity may be one mechanism of cortisol-adaptation. This was previously observed in rainbow trout where waterborne Cu exposure decreased the abundance of gill GR-immunoreactive cells by 73% (Dang et al., 2000). These studies along with the present work indicate that chronic stressors such as Cu exposure can alter the signaling of the stress axis, and the loss of a cortisol response may be an adaptive measure as opposed to endocrine disruption stemming from toxic injury.

Transfer to 50% seawater was expected to increase cortisol levels (in control fish at least), due to the roles of this hormone in the physiological acclimation to seawater (Richards et al., 2003; Yada et al., 2008; Ojima et al., 2009). However, a previous study with rainbow trout (Richards et al., 2003) reported that the response was highest at 24 h whereas sampling in the present study took place at 48 h. In addition even though cortisol levels were highest at 24 h in the Richards et al. study, it was not a significant increase. Similarly, the cortisol results of the present study were variable. Besides cortisol, other hormones such as prolactin, natriuretic peptides and many more are involved in seawater acclimation, which could explain the present lack of a rise in cortisol upon seawater transfer (Sakamoto and McCormick, 2006; Takei, 2008).

4.3. Ionoregulation

Plasma Na^+ and Ca^{2+} in Cu-exposed fish were unaffected on days 20 and 40 of the exposure. However, decreases in these ions have been observed in rainbow trout during the early stages of Cu exposure (~4 days), after which time levels return to control concentrations (Laurén and McDonald, 1987a; McGeer et al., 2000a). In contrast, plasma K^+ was lower in Cu-exposed trout at days 20 and 40 (Fig. 4). To our knowledge, this is the first time that a decrease in plasma K^+ has been observed in fish exposed to waterborne Cu. The mechanism underlying the perturbation in plasma K^+ is unknown, however, gill Na^+/K^+ATPase activity was higher in Cu-exposed fish. This enzyme may be up-regulated to maintain Na^+ levels, but could also theoretically drive K^+ out of the plasma into the gill cells. Alternatively, liver K^+ levels were elevated at 40 days, in contrast to the decrease in plasma K^+ concentration. The liver is the main site of Cu elimination via the bile (Grosell et al., 1998) and perhaps the increase in liver K^+ is due to a link with biliary Cu excretion. In humans, the copper transporter (CTR1; encoded by the SLC31A1 gene) in the plasma membrane of cells is stimulated by higher K^+ concentrations (Lee et al., 2002), although the exact relationship between Cu and K^+ transport is not clear. It is also interesting to note that the pattern of variations in plasma K^+ levels over the 40-day exposure was nearly identical to that in liver K^+ levels, while brain and gill K^+ remain relatively constant (Fig. 4 and Table 1).

Cu-exposed fish were able to maintain plasma Na^+ levels after exposure to 50% seawater. In addition, although plasma K^+ levels were lower in Cu-exposed fish compared to controls prior to the seawater transfer, K^+ remained lower after 48 h exposure to increased salinity. This suggests that if there was a lack of cortisol response in Cu-exposed fish, similar to the other stress tests, it did not have an effect on plasma cation homeostasis.

In control fish, the temporal changes in Cu (liver; Fig. 2A) and brain (Fig. 2D), as well as in plasma K^+ (Fig. 4A) were unexpected and limited to certain tissues and these two ions. Changes in the Cu-exposed fish were similar. The underlying reasons for these changes are not known; presumably, these are normal developmental effects. Nevertheless, it is interesting that the changes occurred in a similar pattern for both Cu and K^+.

Cu exposure stimulated Na^+/K^+ ATPase activity by 7-fold, to a much greater extent than did 50% seawater (Fig. 6). Na^+/K^+ ATPase activity has often been upregulated with seawater transfer in previous studies (eg. Yada et al., 2008). This effect of Cu may be due, at least in part, to the modest stimulatory effect on basal cortisol concentration, which in turn can stimulate Na^+/K^+ ATPase activity (McCormick, 2001; Ojima et al., 2009). Alternately or additionally, it may reflect an over-compensation elicited as part of the damage-repair response, as discussed subsequently.

There are a number of studies that have found different effects of Cu on the activity of Na^+/K^+ ATPase in the fish gill. For example, enzyme activity in rainbow trout and common carp decreased with Cu exposure (Laurén and McDonald, 1987b; De Boeck et al., 2001b; Gagnon et al., 2006), while other studies have observed an increase in activity with Cu exposure (Pelgrom et al., 1995; McGeer et al., 2000a; present study). This has often been interpreted as a compensatory mechanism, as changes in Na^+/K^+ ATPase activity are thought to be a key mechanism in the recovery from metal-induced ionic losses (McDonald and Wood, 1993; Hogstrand et al., 1995).

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