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Influence of 96h sub-lethal copper exposure on aerobic scope and recovery from exhaustive exercise in killifish (*Fundulus heteroclitus*)

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ABSTRACT

Production of industrial effluents have led to increased copper (Cu) pollution of aquatic ecosystems, impacting the physiology of aquatic vertebrates. Past work has shown that Cu exerts its toxicity by disruption ion regulation and/ or increasing oxidative stress. However, it remains unclear how Cu may influence aerobic metabolism and hypoxia tolerance, two possible targets of its toxicity. To address this issue, we exposed freshwater acclimated killifish (*F. heteroclitus*) to a 96 h Cu exposure at a target concentration of 100 μg L^{-1}. We determined resting oxygen consumption ($\dot{M}O_2$), $\dot{M}O_{2max}$ after exhaustive exercise, and followed $\dot{M}O_2$ for 3 h in post-exercise recovery in water with either no Cu or 100 μg L^{-1} Cu. We assessed hypoxia tolerance by determining the critical oxygen tension (P_{crit}). It was found that killifish exposed to combined 96 h Cu exposure and Cu present during metabolic measurements, showed a significant decrease in $\dot{M}O_{2max}$ and in aerobic scope ($\dot{M}O_{2max}$ - $\dot{M}O_{2rest}$), compared to control fish. However, changes in blood and muscle lactate and muscle glycogen were not consistent with an upregulation of anaerobic metabolism as compensation for reduced aerobic performance in Cu exposed fish. Hypoxia tolerance was not influenced by the 96 h Cu exposure or by presence or absence of Cu during the P_{crit} test. This study suggests that Cu differentially influences responses to changes in oxygen demand and oxygen availability.

1. Introduction

Coastal waters may experience increased levels of metals such as copper (Cu) due to natural and anthropogenic influences. Although Cu is an essential micronutrient, in high concentrations it can have toxic effects on aquatic organisms (Harris and Gitlin, 1996). Past studies on fishes have shown that Cu can exert its toxic effects by disrupting ion homeostasis and growth (reviewed in Grosell, 2012). Moreover, due to its reactive nature, excess Cu increases reactive oxygen species (ROS) leading to cellular oxidative stress (Craig et al., 2010; Harris and Gitlin, 1996; Ransberry et al., 2015). Environmentally relevant levels of Cu in freshwater (15-20 μg L^{-1}) have also been shown to disrupt mitochondrial function and capacity for aerobic ATP production in both zebrafish (Craig et al., 2007, 2010) and trout (Sappal et al., 2016) following acute (48 h and 24 h, respectively) and chronic (21 days – zebrafish only) Cu exposure. To add to these tissue-level toxic effects, fishes also demonstrated hypoxic-like effects on ventilation from exposure to Cu but under normoxic conditions (De Boeck et al., 2007; Martin et al., 2005). Thus, excess water-borne metals may impact tolerance to hypoxia and the ability of fish to regulate ventilation in response to declining O_2 availability or increase in O_2 demand. For example, when common carp (*Cyprinus carpio*) were exposed to sublethal levels of Cu (0.08-0.81 mg L^{-1} Cu) for 24 h they showed a decreased tolerance to hypoxia (Malekpouri et al., 2016), and a higher critical PO_2 (P_{crit}), than seen without Cu (Fangue et al., 2008; McBryan et al., 2013; Steffensen, 1985).

How Cu influences metabolism is still unclear, but metals can affect metabolic rate (Grosell, 2012) and Cu can decrease routine oxygen consumption ($\dot{M}O_2$) (brown trout, *Salmo trutta,* and common carp, *C. carpio*; Beaumont, 2003; De Boeck et al., 1995). In contrast, previous research by our group found resting $\dot{M}O_2$ to be unaffected by a 96 h sub-lethal Cu exposure (50 − 200 μg L^{-1} Cu) in fresh-water acclimated killifish (*F. heteroclitus*; Ransberry et al., 2015).

Given that aerobic metabolism is a potential target of metal toxicity (Grosell, 2012), acute and longer-term exposures to Cu likely influence aerobic activities where O_2 demand increases, such as with locomotion.

Abbreviations: Cu, copper; CrP, creatine phosphate; Cr, creatine; P_{crit}, critical oxygen tension; G-6-P, glucose-6-phosphate; $\dot{M}O_{2max}$, maximal oxygen consumption; $\dot{M}O_2$, oxygen consumption; ROS, reactive oxygen species; RI, regulation index

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The influence of Cu on resting or routine $\dot{M}O_2$ is a good measure of effects on basal metabolic costs in non-active fish. However, $\dot{M}O_{2max}$, which can be defined as the maximum oxygen consumption during the initial recovery phase following an exhaustive bout of exercise (Healy and Schulte, 2012), has been shown to also be influenced by excess Cu (*C. carpio*; Malekpouri et al., 2016). The range of aerobic metabolism from rest to $\dot{M}O_{2max}$, termed the aerobic scope ($\dot{M}O_{2max}$ – resting $\dot{M}O_2$), represents the availability of energy above the minimal requirements for survival (Clark et al., 2013; McBryan et al., 2013) and affects organismal performance and fitness (Clark et al., 2013). Currently, it is unclear how Cu may exert its toxic effects to influence resting $\dot{M}O_2$, $\dot{M}O_{2max}$ and aerobic scope.

The purpose of this study was to determine how 96 h Cu exposure affects resting $\dot{M}O_2$ and $\dot{M}O_{2max}$ in fresh water-acclimated killifish. We also examined how Cu affects metabolic recovery from exhaustive exercise. We tested the hypothesis that 96 h Cu expopsure will decrease $\dot{M}O_{2max}$ and aerobic scope, and that there will be an increased contribution of anaerobic metabolism for exercise in the presence of Cu. We predicted that exposure to Cu would also increase the time required for killifish to fully recover from exhaustive exercise.

To test this hypothesis, we exposed freshwater-acclimated adult killifish to a target concentration of 100 μg Cu L^{-1} for 96 h and determined resting $\dot{M}O_2$ and $\dot{M}O_{2max}$ after exhaustive exercise in water containing either no added Cu or 100 μg L^{-1} Cu. $\dot{M}O_2$ was monitored for 3 h post exercise to determine metabolic recovery. Changes in blood lactate and white muscle metabolites were also determined at rest, immediately post exercise (0 h recovery) and 3 h of recovery from exhaustive exercise. To assess the potential influence of Cu on overall hypoxia tolerance we also determined P_{crit} on a separate group of fish.

2. Materials and methods

2.1. Experimental animals

All procedures were approved by the McMaster University Animal Research Ethics Board (AREB) in accordance with the Guidelines of the Canadian Council on Animal Care (CCAC). Wild-caught killifish (*F. heteroclitus*) were acquired from Aquatic Research Organisms (ARO, Hampton, New Hampshire, USA) and acclimated to freshwater (FW) for two weeks before experimentation as previously described (Ransberry et al., 2015). Briefly, water salinity was reduced from 100 % SW to FW gradually over the two weeks through the addition of dechlorinated Hamilton tap water (water quality has been reported previously - Craig et al., 2007, 2010; Ransberry et al., 2015, 2016). Fish were fed daily with commercial fish food (Big Al's Aquarium Superstore, Woodbridge, ON), and maintained under a constant photoperiod (12 h:12h light:-dark) and at approximately 18 °C. Fish were fasted for 24 h prior to the start of the experiments and throughout the 96 h exposure period.

2.2. Chronic copper exposure

A total of 160 killifish (4.22 ± 0.09 g, range 1.60–7.14 g) were randomly divided into two groups, control and 96 h Cu exposure. Killifish for each group were removed from FW acclimated tanks and quickly transferred into individual 8 L tanks, at a density of 5 fish per tank, in either a control exposure (no added Cu), or a 96 h exposure to a nominal concentration of 100 μg L^{-1} Cu (actual concentration 96 ± 10 μg L^{-1} total Cu, 88 ± 6 μg L^{-1} dissolved Cu). Stock Cu solutions were made from $CuSO_4$ dissolved in 1 % HNO_3. The level of Cu was chosen to be within a range known to have toxicity effects on freshwater acclimated killifish but also to not cause mortality (Ransberry et al., 2016). Control fish were held in clean Hamilton tap water, which contains low levels (0.98 ± 0.11 μg L^{-1}) of total Cu. The tanks were continuously aerated and underwent 100 % water renewal daily, and Cu in exposure water was allowed to equilibrate for 24 h prior to fish exposure as described previously (Ransberry et al., 2015). To determine Cu levels, water samples were taken before water changes and total and dissolved Cu were analyzed by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS, Spectra AA 220Z, Varian Palo Alto, CA) as described previously (Craig et al., 2007; Ransberry et al., 2015).

2.3. Resting and post-exercise metabolic rate

After 96 h of control or Cu exposure, fish were weighed and placed in glass metabolic chambers filled with water containing either no added Cu or 100 μg L^{-1} for physiological measurements. The glass metabolic chambers (5 cm × 15 cm) were submerged in a 32 L tank filled to 26 L with dechlorinated tap water containing either no added Cu or 100 μg L^{-1} to match the respirometry conditions. The metabolic chamber was connected to a circuit pump and fish were allowed to adjust to the system overnight with continuous flushing of the chambers. The following morning resting oxygen consumption ($\dot{M}O_2$) was measured by intermittent respirometry with water recirculating through the metabolic chamber to allow O_2 concentration to decline with consumption by the fish, followed by flushing of the chamber to restore O_2 levels. Water O_2 was determined by a recirculating circuit flowing past a fibre-optic sensor (PreSens, Regensburg, Germany), and resting $\dot{M}O_2$ was calculated as the average $\dot{M}O_2$ determined over 6 flush cycles (Borowiec et al., 2015) using pumps regulated by AutoResp software (Loligo Systems, Denmark). The killifish were then removed from the respirometry chamber and placed in a bucket filled with 2 L of water containing either no added Cu or 100 μg L^{-1} in aerated dechlorinated Hamilton tap water, to match the same conditions used for resting $\dot{M}O_2$ measurements for each fish. After a 15-min adjustment period, the killifish were chased by hand until they were unresponsive to touching of their caudal fin, indicating they had reached exhaustion (Clark et al., 2013; Reidy et al., 1995). Once exhausted, the killifish were immediately placed back into the metabolic chambers, and $\dot{M}O_2$ was measured. This was assumed to be maximum $\dot{M}O_2$ (Clark et al., 2013; Reidy et al., 1995). Post-exercise $\dot{M}O_2$ was then recorded in recovery at 4-min intervals for a total of 3 h.

2.4. Tissue sampling

Skeletal muscle and blood were sampled from different killifish at rest, immediately after exhaustive swimming (0 h), and at 3 h in recovery from exercise in the conditions described above. To obtain accurate resting samples, fish that had not been exercised were anesthetized by adding neutralized tricaine methanesulfonate (MS222, 100 ppm) to the water, and then quickly euthanizing by cephalic concussion. The caudal fin was quickly removed using a razor blade, and a blood sample was obtained using a capillary tube. Whole blood lactate was determined using a Lactate Pro LT Portable Blood Lactate Analyzer (Arkray, Kyoto, Japan). The white epaxial muscle below the dorsal fin was excised and quickly crushed between liquid N_2-cooled aluminum blocks. All samples were kept at −80 °C prior to analysis.

2.5. Muscle metabolites

Skeletal muscle concentrations of adenosine triphosphate (ATP), creatine phosphate (CrP), creatine (Cr), pyruvate, glucose-6-phosphate (G-6-P), lactate, and glycogen were determined at rest and at 0 h and 3 h post-exercise. Metabolites were extracted and measured following standard methods (Bergmeyer et al., 1974) adapted to a 96-well format for the Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA) as described previously (Borowiec et al., 2018; Lau et al., 2017; Le Moine et al., 2011). Muscle samples (~25 − 60 mg) were powdered using liquid N_2-cooled mortar and pestle, weighed and extracted through homogenization in 6 % perchloric acid. Aliquots of the homogenate were removed and stored at −80 °C to determine lactate, G-6-P, and glycogen. The remaining homogenate was centrifuged at 10,000 x g for 10 min at 4 °C and the supernatant was

removed and neutralized using 3 M K_2CO_3. The neutralized supernatant was then centrifuged at 10,000 x g for 10 min at 4 °C. This supernatant was removed and used to determine ATP, CrP, Cr, and pyruvate.

Assay were carried out using the following conditions for ATP and CrP (in mM): 20 Tris (pH 8.0), 5 $MgCl_2$, 5 glucose, 2 NAD^+, 1U ml^{-1} glucose-6-phosphate dehydrogenase (G6PDH from *L. mesenteriodes*, Roche Diagnostics), and for CrP only: 2 ADP and 1.5U ml^{-1} hexokinase (HK from *S. cerevisiae*, Roche Diagnostics). The Reaction for ATP was started with the addition of 1U of HK, and for CrP with creatine phosphokinase (CPK from rabbit muscle, Sigma, Oakville, ON). For Cr determinations (in mM): 500 triethanolamine (TEA, pH 7.5), 5 ATP, 5 PEP, 0.15 NADH, 5 $MgCl_2$, 1U ml^{-1} lactate dehydrogenase (LDH from rabbit muscle, Roche Diagnostics), 1U ml^{-1} pyruvate kinase (PK from rabbit muscle, Sigma), and the reaction was started with 2.5U CPK. For pyruvate (in mM): 500 TEA, 5 EDTA (pH 7.6), 0.15 NADH, and started with 5U LDH. For lactate, G-6-P, and glycogen content, samples were thawed and mixed with 50 µl of 1 M K_2CO_3 and 100 µl 400 mM acetate buffer (pH 4.8). Half the mixture was set aside to measure lactate, G-6-P and free glucose content. These samples were neutralized with 1 M K_2CO_3 and centrifuged at 10,000 x g for 10 min at 4 °C. Assay conditions (in mM) for lactate were 2 NAD^+ in glycine/hydrazine buffer (pH 9.2, Sigma) using 8U LDH to start the reaction. For G-6-P the conditions were (in mM): 20 imidazole (pH 7.4), 1 ATP, 0.5 NADP, and 5 $MgCl_2$ with the reaction initiated with 1 U G6PDH. For free glucose: 1U HK suspended in TRA buffer (Roche Diagnostics) was added to each G-6-P assay.

The remaining 125 µl of homogenate was mixed with 7 µl of amyloglucosidase (4 U μl^{-1}; Roche Diagnostics) and incubated at 40 °C for 2 h. After incubation, samples were neutralized with 1 M K_2CO_3 and assayed for glucose content. Assay conditions for glucose were (in mM): 20 imidazole (pH 7.4), 1 ATP, 0.5 NADP, 5 $MgCl_2$, 1U G6PDH suspended in TRA buffer (300 mM TEA – HCl, 4.05 $MgSO_4$, pH 7.5; Roche Diagnostics), and the reaction was initiated with 1U HK suspended in TRA buffer.

2.6. Critical oxygen tension (P_{crit}) and regulation index (RI)

In a different set of individuals and after 96 h exposure, killifish were placed in metabolic chambers (see Section 2.3) with either no added Cu or 100 µg L^{-1} Cu and allowed to adjust overnight. The following morning, $\dot{M}O_2$ was measured for 2 min at 100 % air saturation. Water PO_2 was then progressively lowered using an oxygen regulating system (Qubit, Kingston, ON) at increments of 10 % O_2 and two $\dot{M}O_2$ measurements were taken at each PO_2. This process was repeated until 10 % O_2 saturation was reached. Critical oxygen tension (P_{crit}) was calculated using the REGRESS software and based on the intersect between two lines of best fit calculated by the program (Yeager and Ultsch, 1989). Regulation index value (RI) was determined and assigned to an animal a relative measure of regulatory ability by finding the area under the $\dot{M}O_2$ versus PO_2 curve that is greater than a linear trend (Mueller et al., 2011). To determine RI a $\dot{M}O_2$ versus PO_2 graph was fit with a curve with the highest r^2 using Prism 6 (GraphPad Software, San Diego, CA). The curve that best fit the data was one of three models: a straight line, quadratic, or a one-phase association. The curve was then compared with a linear regression that represented how $\dot{M}O_2$ would decline if the fish showed complete conformity. The area between these two curves was calculated and expressed as a proportion of the area bound by the straight line and horizontal line at maximum $\dot{M}O_2$.

2.7. Statistical analysis

All statistical analysis was carried out using Sigma Stat 3.5 (Systat Software Inc., San Jose, CA), GraphPad Prism or SPSS (IBM). Differences in $\dot{M}O_2$ over time in recovery from exhaustive exercise were analyzed for each group separately using a Repeated Measures Analysis of Variance (ANOVA) and Bonferroni *post hoc* multiple comparisons. Resting $\dot{M}O_2$, maximum $\dot{M}O_2$, and aerobic scope (as maximum – resting) were corrected for body mass by fitting an allometric regression (Borowiec et al., 2015). Residuals from these regressions were analyzed using a 2-way ANOVA with 96 h Cu exposure and test condition for physiological measurements (no added Cu versus with 100 µg L^{-1} Cu) as main factors. These data are presented as values independent of the predicted variation due to body mass (Klingenberg, 2016) by adding the residuals of the allometric regression to $\dot{M}O_2$ for the average sized fish. A one-way ANOVA followed by a *post hoc* test (Holm-Sidak) was used to test the significance of differences observed in critical oxygen tension and regulation index. A 2-way ANOVA was used to test the significance of differences observed in blood lactate and muscle metabolite levels in response to exercise. For all tests, a p-value ≤ 0.05 was considered statistically significant. Results are presented as means ± SEM.

3. Results

3.1. Post exercise recovery and aerobic scope

To assess the influence of 96 h Cu exposure and test condition for physiological measurements on whole organism metabolism, we measured $\dot{M}O_2$ at rest, immediately after exhaustive exercise (0 h), and over a 3 h period in recovery (Fig. 1). Mass-specific resting metabolic rate varied between the experimental groups from 5.4 ± 0.5 µmoles $g^{-1} h^{-1}$ in controls, 5.1 ± 0.4 µmoles $g^{-1} h^{-1}$ for controls measured in water with 100 µg L^{-1} Cu, 5.6 ± 0.4 µmoles $g^{-1} h^{-1}$ for 96 h Cu exposed fish, and 4.2 ± 0.4 µmoles $g^{-1} h^{-1}$ after 96 h Cu exposure and measured in water with 100 µg L^{-1} Cu. However, after accounting for differences in body mass (see Section 2.7), resting $\dot{M}O_2$ showed no significant main effect of 96 h Cu treatment ($F_{1,46} = 0.28$, $P = 0.60$), Cu in the test condition for physiological measurements ($F_{1,46} = 1.59$,

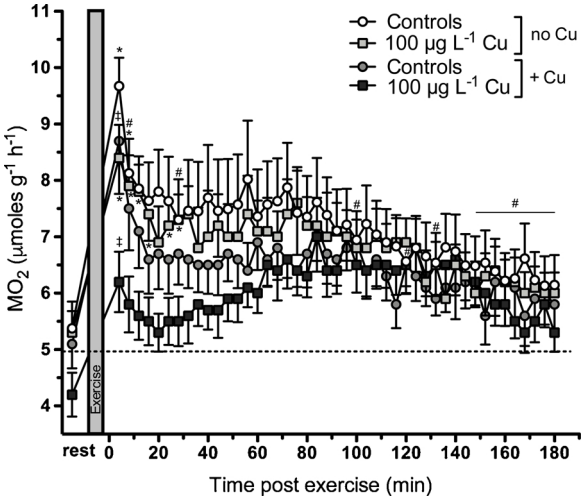

Fig. 1. Rates of oxygen consumption ($\dot{M}O_2$ in µmoles $g^{-1} h^{-1}$) at rest and during 3 h recovery from exhaustive exercise (exercise period denoted by the grey rectangle) in freshwater acclimated adult killifish (*Fundulus heteroclitus*) exposed to control conditions or to a target of 100 µg L^{-1} Cu for 96 h, and $\dot{M}O_2$ in both groups measured either in water with no added Cu or 100 µg L^{-1} Cu. Data are presented as means ± SEM of n = 15 for controls, n = 11 for controls tested with Cu, n = 13 for 96 h Cu exposed fish, and n = 11 for 96 h Cu exposed fish tested with Cu. (*) denotes a significant difference from resting $\dot{M}O_2$ within an experimental group. (#) denotes a significant difference from $\dot{M}O_2$ immediately post exercise (Repeated Measures Analysis of Variance and Bonferroni *post hoc* multiple comparisons). (‡) denotes a significant difference of $\dot{M}O_2$ immediately post exercise relative to at rest (pairwise t-test). The dashed line is drawn at the combined average resting $\dot{M}O_2$ for all groups. Body mass for controls, 4.24 ± 0.24 g, controls + Cu, 4.85 ± 0.30 g, 100 µg L^{-1}Cu, 4.88 ± 0.20 g, and 100 µg L^{-1} Cu + Cu, 4.74 ± 0.13 g.

P = 0.21), or an interaction between treatment Cu x Cu in test condition for physiological measurements ($F_{1,46}$ = 2.74, P = 0.11). There was a statistically significant increase in $\dot{M}O_2$ immediately after exercise in control fish ($F_{45,585}$ = 6.14, P < 0.001) and 96 h Cu exposed fish ($F_{45,495}$ = 5.55, P < 0.05) measured in water with no added Cu. In control fish, $\dot{M}O_2$ was significantly greater then resting $\dot{M}O_2$ at 4 min (+80 %) and 8 min post exercise (P < 0.05) and was significantly higher at 4 min than various time points during the 3 h recovery period (P < 0.05). For 96 h Cu exposed fish measured in water with no added Cu, $\dot{M}O_2$ was also significantly elevated at 4 min post exercise (+58 %, P = 0.03), and remained elevated above resting rates at 8, 12, 16, 24 and 28 min in recovery (P < 0.05; Fig. 1). Control fish measured in water with 100 μg L^{-1} Cu showed a 71 % rise in $\dot{M}O_2$ from rest to post exercise, but these values were not significantly different when all post exercise time points were compared (P > 0.05). Similarly, 96 h Cu exposed fish measured in water with 100 μg L^{-1} Cu showed a 48 % rise in $\dot{M}O_2$ that failed to reach statistical significance (P > 0.05, Fig. 1). However, maximum $\dot{M}O_2$ was significantly higher than resting $\dot{M}O_2$ for 96 h Cu exposed fish measured in water with 100 μg L^{-1} Cu and for controls measured in water with 100 μg L^{-1} Cu, when only these 2 time points were compared within a treatment (paired t-test, P < 0.05).

To get a better idea of the capacity for exercise to increase $\dot{M}O_2$ we made the assumption that the $\dot{M}O_2$ immediately after exhaustive exercise was $\dot{M}O_{2max}$ for each individual (Clark et al., 2013; Reidy et al., 1995). We found that for $\dot{M}O_{2max}$ there was a significant main effect of 96 h Cu exposure ($F_{1,46}$ = 5.35, P < 0.05), for Cu in the test condition for physiological measurements ($F_{1,46}$ = 5.31, P < 0.05), and an interaction that approached statistical significance ($F_{1,46}$ = 3.41, P = 0.071). $\dot{M}O_{2max}$ was similar between controls and 96 h Cu exposed fish when measured in water with no added Cu (Fig. 2B). Pairwise comparisons showed a significant reduction (28 %) in $\dot{M}O_{2max}$ in the 96 h Cu exposed fish measured in water with 100 μg L^{-1} Cu from those measured in water with no added Cu (P < 0.05). This $\dot{M}O_{2max}$ was significantly lower than control fish measured in water with 100 μg L^{-1} Cu (p < 0.05).

The difference between resting and maximum $\dot{M}O_2$ (aerobic scope) is an important indicator of performance in fishes (Clark et al., 2013), and we wanted to determine the influence of Cu on this important organismal performance trait. We found that with 96 h Cu exposure, when metabolic rate measurements were made in water with the same Cu level, that aerobic scope was reduced (by 43 %) to a level that approached statistical significance (P = 0.052), compared to control fish measured under the same conditions (Fig. 2D).

3.2. Blood lactate

Resting blood lactate concentrations were unaffected by 96 h Cu exposure ($F_{1,78}$ = 2.95, P = 0.09; Fig. 3). However, immediately post exercise (0 h), blood lactate increased significantly above resting values compared to rest. By 3 h into exercise recovery, lactate concentrations were reduced from 0 h recovery values in all groups ($F_{2,78}$ = 75.33, P < 0.001), but remained above resting levels for control fish (P < 0.05). At 3 h into recovery fish exposed for 96 h Cu had significantly lower blood lactate than controls tested in both water with and without 100 μg L^{-1} Cu (P < 0.05; Fig. 3).

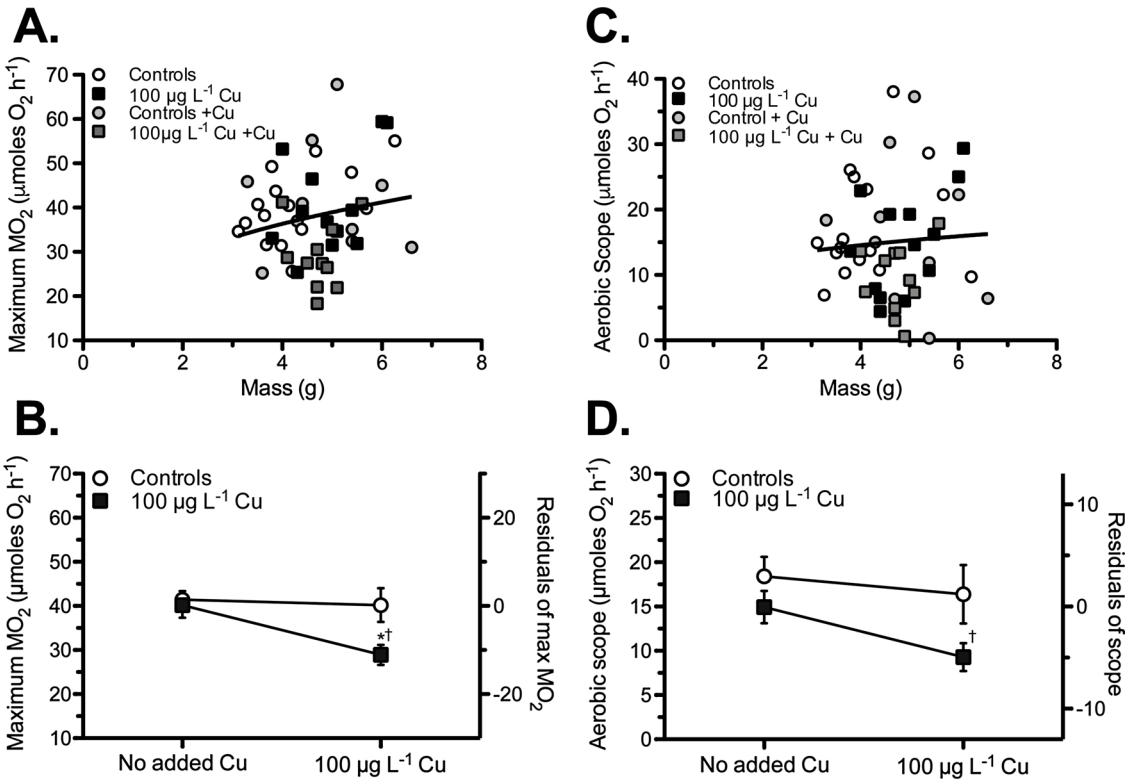

Fig. 2. (A) Maximum rates of oxygen consumption ($\dot{M}O_{2max}$) after exhaustive exercise regressed relative to body mass (allometric relationship, $\dot{M}O_{2max}$ = 23.62 $M^{0.311}$) in freshwater acclimated adult killifish (*Fundulus heteroclitus*) exposed for 96 h to control conditions or to a target of 100 μg L^{-1} Cu, and $\dot{M}O_2$ in both groups measured in water with no added Cu or in water with 100 μg L^{-1} Cu. (B) Residuals for the regression of $\dot{M}O_{2max}$ and body mass, and mass-independent $\dot{M}O_{2max}$ as residual values + predicted $\dot{M}O_{2max}$ for average sized fish, for control and 96 h Cu exposed fish measured in water with no added Cu or 100 μg L^{-1} Cu. (C) Aerobic scope ($\dot{M}O_{2max}$ - $\dot{M}O_{2rest}$) regressed relative to body mass (allometric relationship, Aerobic Scope = 10.71 $M^{0.221}$), (D) Residuals of the regression of aerobic scope and body mass and mass-independent aerobic scope as residual values + aerobic scope predicted for average sized fish. Data are presented at means ± SEM of n = 15 controls, n = 11 for controls tested in Cu, n = 13 for 96 h exposed fish, and n = 11 for 96 h Cu exposed fish tested in Cu. (*) denotes a significant difference from physiological measurements made in water with no added Cu within a treatment group. (†) denotes a significant difference between controls and 96 h Cu exposed fish measured in the same conditions.

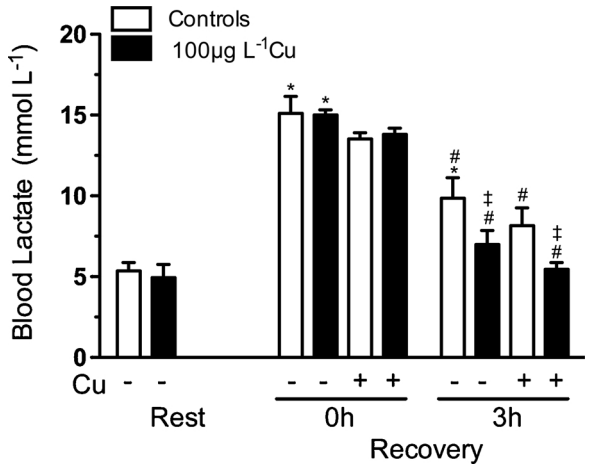

Fig. 3. Blood lactate concentrations at rest, immediately after (0 h), and 3 h of recover from exhaustive exercise in freshwater acclimated adult killifish (*Fundulus heteroclitus*) exposed for 96 h to control conditions (white bars) or to a target of 100 µg L^{-1} Cu (black bars), after metabolic rate measurements in water with no added Cu (-) or in water with 100 µg L^{-1} Cu (+). *represents a significant difference from rest for controls and 96 h exposed fish in each of the measurement conditions (Cu vs. no Cu). #represents a significant difference from 0 h in control and 96 h Cu exposed fish in each of the measurement conditions. ‡represents a significant effect of 96 h Cu exposure within a measurement condition at each time point. Data are presented at means ± SEM, n = 6-11. Body mass at rest: controls, 3.72 ± 0.45 g, 100 µg L^{-1} Cu, 3.86 ± 0.22 g, 0 h recovery: controls, 3.61 ± 0.17 g, controls + Cu, 1.86 ± 0.10 g, 100 µg L^{-1} Cu, 3.23 ± 0.11 g, 100 µg L^{-1} Cu + Cu, 2.45 ± 0.24 g, 3 h recovery: controls, 3.97 ± 0.23 g, controls + Cu, 4.62 ± 0.28 g, 100 µg L^{-1} Cu, 5.15 ± 0.20 g, 100 µg L^{-1} Cu + Cu, 4.67 ± 0.14 g.

3.3. Muscle metabolites

Metabolite levels were measured in white skeletal muscle for each treatment group and were found to be relatively consistent at each time point and across treatments (Figs. 4 and 5). ATP levels in muscle showed a trend to decline immediately after exercise but recovered by 3 h (main effect of time, $F_{2,52} = 10.16$, $P < 0.001$) to levels significantly greater than 0 h in control fish exercised in either water with and without 100 µg L^{-1} Cu ($P < 0.05$; Fig. 4A). Exercise caused a significant decline in CrP levels ($F_{2,51} = 37.86$, $P < 0.001$) (Fig. 4B), which increased by 3 h recovery in all groups except control fish measured in water without added Cu, where levels remained below those at rest. In response to swimming, Cr levels showed a corresponding increase immediately post exercise, which declined by 3 h (time, $F_{2,53} = 29.44$, $P < 0.001$) except in controls measured in water without added Cu (test condition x time, $F_{1,53} = 17.08$, $P < 0.05$). Levels of G-6-P remained relatively stable with exercise except that the presence of Cu during exercise resulted in increased G-6-P in both controls and 96 h Cu exposed fish at 0 h recovery (test condition x time, $F_{1,47} = 5.20$, $P < 0.05$). By 3 h recovery G-6-P was significantly reduced in these 2 groups ($P < 0.05$, Fig. 4D). Similarly, pyruvate levels remained relatively constant immediately after exhaustive swimming, but at 0 h recovery levels were significantly higher in 96 h Cu exposed fish exercised in water with 100 µg L^{-1} Cu compared to controls tested in the same condition and to 96 h Cu exposed fish measured in water with no added Cu (test condition x time, $F_{1,53} = 6.05$, $P < 0.05$). Pyruvate declined by 3 h in all groups except control fish measured in water with no added Cu (Fig. 4E). Muscle lactate showed a significant increase from rest to 0 h post exercise, and then significantly decreased by 3 h to levels similar to those at rest ($F_{2,52} = 129.37$, $P < 0.001$). However, there was no statistically significant difference between experimental groups at any of the time points ($F_{1,52} = 1.48$, $P = 0.23$). There was a significant effect of the presence of Cu ($F_{1,52} = 16.38$, $P < 0.001$), where at 0 h recover, controls measured in water with 100 µg L^{-1} Cu showed a reduction in lactate ($P = 0.001$; Fig. 4F). Glycogen levels showed a significant decline between rest and exercise in 96 h Cu treatment fish measured in water with no added Cu by 3 h recovery (test x time x treatment, $F_{1,48} = 5.53$, $P < 0.05$). Glycogen levels also differed at 0 h recovery due to Cu test condition and Cu treatment group (Fig. 5).

3.4. Critical oxygen tension and regulation index

Critical oxygen tension (P_{crit}) was similar across all experimental groups (range: 30 − 41 Torr; Fig. 6). There was also no significant difference between groups in regulation index (RI) (Fig. 6B).

4. Discussion

4.1. General findings

Our data suggest that excess water-borne Cu affects the ability of killifish to increase $\dot{M}O_2$ to match energy demand with exhaustive exercise. The effect of Cu was not apparent in controls measured in short-term acute Cu present during physiological measurements or in 96 h Cu exposed fish measured in water without added Cu. However, when 96 h Cu exposed fish were measured in water with 100 µg L^{-1} Cu they showed a significant reduction in $\dot{M}O_{2max}$ after exhaustive exercise. Resting $\dot{M}O_2$ was similar in all groups leading to a reduced aerobic scope after 96 h Cu exposure. Surprisingly, the significant reduction in $\dot{M}O_{2max}$ did not result in greater blood or skeletal muscle lactate accumulation post-exercise, suggesting there was no acceleration of glycolysis to support exhaustive exercise. In fact, 96 h Cu exposure fish exercised in acute Cu conditions showed significantly lower blood lactate concentrations after 3 h of recovery compared to those exercised in conditions without added Cu. Although 96 h exposure to Cu and exercise in water with no added Cu did not significantly reduce $\dot{M}O_{2max}$, this exposure did result in $\dot{M}O_2$ remaining above resting levels for longer in the post exercise recovery period. When faced with reduced O_2 availability neither 96 h Cu exposure nor short-term acute Cu exposure during the physiological measurements significantly impacted hypoxia tolerance as indexed with P_{crit}. These data support the hypothesis that 96 h Cu exposure affects aerobic capacity, exercise performance and recovery in killifish, but only when swimming is performed in the presence of Cu.

4.2. MO_{2max}, aerobic scope, and post-exercise recovery

Metal toxicity has been shown to elicit several physiological responses, specifically changes in oxygen consumption rate (Grosell, 2012), which decreases in fish exposed to sub-lethal Cu concentrations (Beaumont, 2003). Our study shows that 96 h Cu exposure decreases $\dot{M}O_{2max}$ while maintaining resting $\dot{M}O_2$, resulting in a decrease in aerobic scope. Previous studies on other fish species have shown that acute Cu exposure impairs maximum sustainable swimming speeds, an indicator of aerobic performance (softwater acclimated trout, Beaumont, 1995; Waiwood and Beamish, 1978). The impairment of swimming performance could also be related to increased metabolic costs of compensating for copper-induced physiological deteriorations, reducing aerobic scope, but we did not detect any changes in resting $\dot{M}O_2$ with Cu. Another possible explanation for this reduced aerobic capacity may be related to hyperammonenia. Ammonia regulates many metabolic pathways and can change membrane potentials by displacing potassium ions in ion-exchange mechanisms, resulting in depolarization of neurons and muscle cells (Beaumont, 2000). Our data show that 96 h Cu exposure did not impact $\dot{M}O_{2max}$ or aerobic scope if physiological measurements are made in water with no added Cu. Perhaps exercise and recovery in 'clean' water may help reverse the impacts of prior metal exposure. This may represent a damage-repair cycle shown in

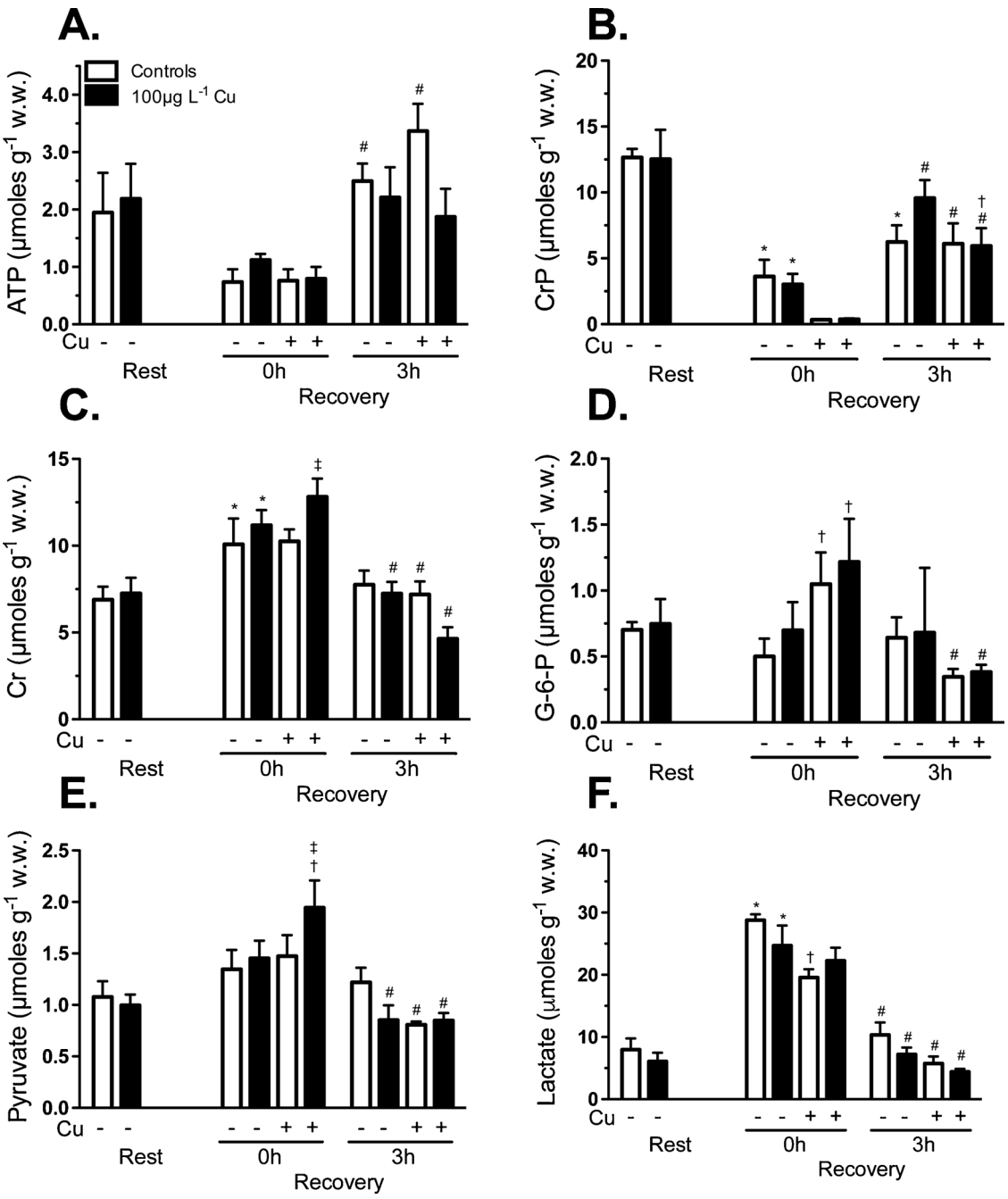

Fig. 4. Metabolite concentrations were determined in white epaxial muscle at rest, immediately after exhaustive exercise (0 h) induced by chasing, and after 3 h of recover from exercise in freshwater acclimated adult killifish (*Fundulus heteroclitus*) exposed for 96 h to control conditions (white bars) or to 100 µg L^{-1} Cu (black bars), and exercised in water with no added Cu (-) or in water with 100 µg L^{-1} Cu (+). (A) ATP, (B) creatine phosphate (CrP), (C) creatine (Cr), (D) glucose-6-phosphate (G-6-P), (E) pyruvate, and (F) lactate. *represents a significant difference from rest within controls or 96 h Cu exposed fish in each exercise condition. #represents a significant difference from 0 h within controls or 96 h Cu exposed fish in each exercise condition. †represents a significant effect of exercise condition within controls or 96 h Cu exposed fish at each time point. ‡ represents a significant effect for controls or 96 h Cu exposed fish within an exercise condition at each time point. (N = 4–5 for all groups except for 3 h in recovery for controls and Cu exposed fish tested in 100 µg L^{-1} Cu, where N = 7).

other species to lead to enhanced Cu tolerance (e.g., Tate-Boldt and Kolok, 2008). However, previous studies used longer exposures (8 days) and to high Cu levels to show increased Cu tolerance in fat head minnow (Tate-Boldt and Kolok, 2008). Moreover, longer residence in uncontaminated water after Cu exposure than used in our study may be necessary for fish to recover from metal exposure. For example, at least 2 days in clean water may be necessary before any improvement in gill tissue lesions occurs, although morphological changes (decrease in filament epithelium height) may begin as early as one day in clean water (Cerqueira and Fernandes, 2002). Our physiological measurements were also unaffected in control fish if exposure to Cu occurred only during the physiological measurement period (Fig. 1 and Fig. 2). This acute Cu exposure used in our physiological test conditions may not have had sufficient length of exposure on their own to impact swimming metabolism, in contrast to studies showing impacts may be evident early during exposure (hours to days) (Beaumont, 2000, 1995).

In recovery from exercise, all groups quickly reduced $\dot{M}O_2$ from $\dot{M}O_{2max}$ and over the 3 h period $\dot{M}O_2$ returned to resting levels (Fig. 1). The exception was the 96 h Cu exposed groups exercised in water without added Cu. This group showed $\dot{M}O_2$ elevated above resting

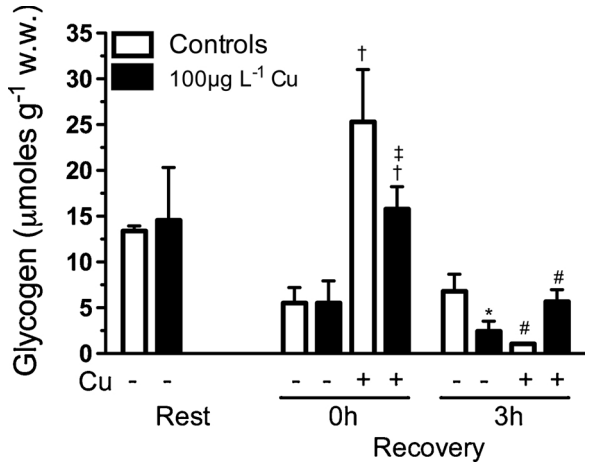

Fig. 5. Glycogen concentrations in white epaxial muscle at rest, immediately after exhaustive exercise (0 h) induced by chasing, and after 3 h of recover from exercise in freshwater acclimated adult killifish (*Fundulus heteroclitus*) exposed for 96 h to control conditions (white bars) or to 100 μg L^{-1} Cu (black bars), and both tested either in water with no added Cu (-) or in water with 100 μg L^{-1} Cu (+). *represents a significant difference from rest within a treatment in each test condition. #represents a significant difference from 0 h within a treatment in each test condition. †represents a significant effect of test condition within a treatment at each time point. ‡ represents a significant effect of treatment within a test condition at each time point. (N = 4–5 for all groups except for 3 h in recovery for controls and Cu exposed fish tested in 100 μg L^{-1} Cu where N = 6–7).

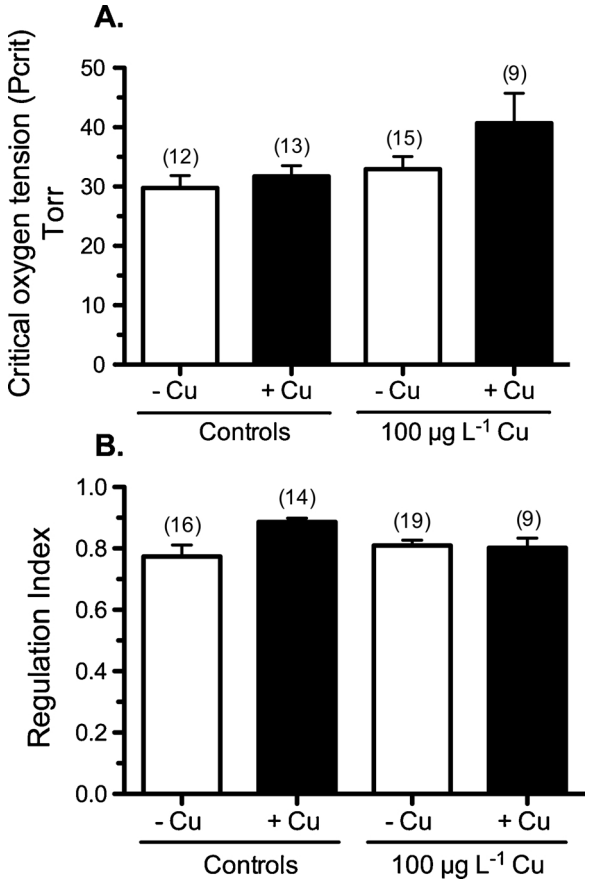

Fig. 6. Critical oxygen tension, P_{crit} (A) and Regulation Index (B) in freshwater acclimated adult killifish (*Fundulus heteroclitus*) exposed for 96 h to control conditions (white bars) or to 100 μg L^{-1} Cu (black bars), and both tested either in water with no added Cu (-Cu) or in water with 100 μg L^{-1} Cu (+Cu). Sample sizes are indicated above each bar. Data are presented at means ± SEM.

levels until 28 min post-exercise. This suggests that 96 h Cu alone is sufficient to increase excess post-exercise oxygen consumption. The influence of body mass on toxicity may also affect these results. However, the underlying mechanisms responsible for these increased demands remain to be fully explored.

4.3. Blood lactate and muscle metabolites

Shortfalls in aerobic ATP production with exercise may be compensated for by an acceleration of glycolysis and an increased reliance on substrate level phosphorylation. Along with potential impacts on O_2 delivery pathways, Cu has been shown to induce changes in the activity of a number of enzymes, impacting metabolic pathways and ATP synthesis rates (Craig et al., 2007; Grosell, 2012). Previous work has shown that chronic Cu exposure in soft water leads to differences in white muscle lactate metabolite levels in exercised fish (Beaumont, 2000). In contrast, we found no difference in post exercise lactate accumulation with 96 h Cu exposure when exercised in water with and without added Cu or in controls exercised in water with added Cu. This suggests that lactate release into the circulation was equivalent between the treatment groups and that lactate was not differentially sequestered in muscle. Consistent with this conclusion is post exercise muscle lactate concentrations that were similar across the 4 groups or even reduced in controls exercised in water with added Cu. Killifish showed a post exercise reduction in CrP with a concurrent increase in Cr, suggesting that 96 h Cu exposed fish did not recruit high-energy phosphates to a greater extent than controls. Cu exposed killifish may accelerate glycolysis during exhaustive exercise but are limited in the ability to oxidize pyruvate or convert it to lactate. This would be consistent with the elevated muscle G-6-P and pyruvate levels immediately after exercise.

After the 3 h of recovery from exercise, combined 96 h Cu exposure and Cu present during physiological measurements showed blood lactate concentrations return to levels lower than 0 h recovery and compared to control fish. This was not due to differences in sequestering of lactate by muscle as lactate levels were reduced in all groups by 3 h of recovery (Fig. 4F). This suggests that 96 h exposure and short-term acute Cu leads to better clearance of lactate from the circulation possibly into the liver as a substrate for gluconeogenesis.

4.4. Hypoxia tolerance

In certain fish species, P_{crit} has been shown to increase in response to chronic Cu exposure, supporting the idea that either oxygen sensing, or oxygen regulation can be affected by Cu (De Boeck et al., 1995). High Cu concentrations have been shown to result in gill histopathologies such as cell swelling and thickening of the lamellae, increasing the blood-water diffusive distance, leading to impaired gas transfer across the gills of rainbow trout (Wilson and Taylor, 1993). This reduced oxygen uptake may result in impaired oxygen delivery during sublethal Cu exposures. The results from this study illustrate that there is no significant difference in hypoxia tolerance between control and 96 h Cu exposed killifish. It was predicted that due to a potentially impaired ability to sense oxygen in the water, Cu exposed killifish would switch from oxy-regulation to an oxy-conforming strategy at a higher P_{crit}. It was also predicted that regulation index (RI) would decrease in copper exposed killifish, suggesting a more oxy-conformation strategy. However, we saw no significant difference in either P_{crit} or RI between treatment groups. The difference in the results from this experiment and those of De Boeck et al. (1995) on common carp may be due to species-specific effects of Cu exposure. Studies have shown that responses to Cu exposure has clear differences among species, with cyprinids evidently responding differently than salmonids (De Boeck et al., 2007). This study shows that killifish are a species where Cu exposure does not influence the ability to sense low water PO_2.

5. Conclusion

In this study, we have shown that a combination of both 96 h Cu exposure and presence of Cu during physiological measurements had a negative impact on metabolic response in killifish, but no observable effect on hypoxia tolerance. It is clear from this study that Cu specifically effects aerobic scope. Our results suggest that even though there is a decrease in aerobic capacity, there is no significant change in anaerobic metabolism to compensate for the decrease in energy supply. This suggests a reduction exercise capacity set by the inability to induce $\dot{M}O_2$ in the presence of Cu. This study also suggests that Cu differentially influences responses to changes in oxygen demand and oxygen availability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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References

Beaumont, M., 2000. Exposure of brown trout, *Salmo trutta*, to a sub-lethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. Aquat. Toxicol. 51, 259–272. https://doi.org/10.1016/S0166-445X(00)00109-0.

Beaumont, M., 1995. Exposure of brown trout, *Salmo trutta*, to sub-lethal copper concentrations in soft acidic water and its effect upon sustained swimming performance. Aquat. Toxicol. 33, 45–63. https://doi.org/10.1016/0166-445X(95)00007-Q.

Beaumont, M.W., 2003. Exposure of brown trout *Salmo trutta* to a sublethal concentration of copper in soft acidic water: effects upon gas exchange and ammonia accumulation. J. Exp. Biol. 206, 153–162. https://doi.org/10.1242/jeb.00060.

Bergmeyer, H.U., Bergmeyer, J., Grassl, M., 1974. Methods of enzymatic analysis. Samples, Reagents, Assessment of Results Vol. 2 Verlag Chemie.

Borowiec, B.G., Darcy, K.L., Gillette, D.M., Scott, G.R., 2015. Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*). J. Exp. Biol. 218, 1198–1211. https://doi.org/10.1242/jeb.114579.

Borowiec, B.G., McClelland, G.B., Rees, B.B., Scott, G.R., 2018. Distinct metabolic adjustments arise from acclimation to constant hypoxia and intermittent hypoxia in estuarine killifish (*Fundulus heteroclitus*). J. Exp. Biol. 221, 1–16. https://doi.org/10.1242/jeb.190900.

Cerqueira, C.C.C., Fernandes, M.N., 2002. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish Prochilodus scrofa. Ecotoxicol. Environ. Saf. 52, 83–91. https://doi.org/10.1006/eesa.2002.2164.

Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J. Exp. Biol. 216, 2771–2782. https://doi.org/10.1242/jeb.084251.

Craig, P.M., Wood, C.M., McClelland, G.B., 2010. Water chemistry alters gene expression and physiological end points of chronic waterborne copper exposure in zebrafish, Danio rerio. Environ. Sci. Technol. 44, 2156–2162. https://doi.org/10.1021/es902995q.

Craig, P.M., Wood, C.M., McClelland, G.B., 2007. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R1882–R1892. https://doi.org/10.1152/ajpregu.00383.2007.

De Boeck, G., De Smet, H., Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, Cyprinus carpio. Aquat. Toxicol. 32, 127–141. https://doi.org/10.1016/0166-445X(94)00086-6.

De Boeck, G., Van der Ven, K., Meeus, W., Blust, R., 2007. Sublethal copper exposure induces respiratory stress in common and gibel carp but not in rainbow trout. Comp. Biochem. Physiol. Part C 144, 380–390. https://doi.org/10.1016/j.cbpc.2006.11.008.

Fangue, N.A., Mandic, M., Richards, J.G., Schulte, P.M., 2008. Swimming performance and energetics as a function of temperature in killifish Fundulus heteroclitus. Physiol. Biochem. Zool. 81, 389–401. https://doi.org/10.1086/589109.

Grosell, M., 2012. Copper. In: In: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology Vol 31. Academic Press, pp. 53–133 Part A.

Harris, Z.L., Gitlin, J.D., 1996. Genetic and molecular basis for copper toxicity. Am. J. Clin. Nutr. 63, 836S–841S. https://doi.org/10.1093/ajcn/63.5.836.

Healy, T.M., Schulte, P.M., 2012. Thermal acclimation is not necessary to maintain a wide thermal breadth of aerobic scope in the common killifish (*Fundulus heteroclitus*). Physiol. Biochem. Zool. 85, 107–119. https://doi.org/10.1086/664584.

Klingenberg, C.P., 2016. Size, shape, and form: concepts of allometry in geometric morphometrics. Dev. Genes Evol. 226, 113–137. https://doi.org/10.1007/s00427-016-0539-2.

Lau, D.S., Connaty, A.D., Mahalingam, S., Wall, N., Cheviron, Z.A., Storz, J.F., Scott, G.R., McClelland, G.B., 2017. Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. 312, R400–R411. https://doi.org/10.1152/ajpregu.00365.2016.

Le Moine, C.M.R., Morash, A.J., McClelland, G.B., 2011. Changes in HIF-1 protein, pyruvate dehydrogenase phosphorylation, and activity with exercise in acute and chronic hypoxia. Am. J. Phyiol. Regul. Integr. Comp. Physiol. 301, R1098–R1104. https://doi.org/10.1152/ajpregu.00070.2011.

Malekpouri, P., Peyghan, R., Mahboobi-Soofiani, N., Mohammadian, B., 2016. Metabolic capacities of common carp (*Cyprinus carpio*) following combined exposures to copper and environmental hypoxia. Ecotoxicol. Environ. Saf. 127, 1–11. https://doi.org/10.1016/j.ecoenv.2016.01.004.

Martin, F., Linden, T., Katschinski, D.M., Oehme, F., Flamme, I., Mukhopadhyay, C.K., Eckhardt, K., Tröger, J., Barth, S., Camenisch, G., Wenger, R.H., 2005. Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. Blood 105, 4613–4619. https://doi.org/10.1182/blood-2004-10-3980.

McBryan, T.L., Anttila, K., Healy, T.M., Schulte, P.M., 2013. Responses to temperature and hypoxia as interacting stressors in fish: implications for adaptation to environmental change. Integr. Comp. Biol. 53, 648–659. https://doi.org/10.1093/icb/ict066.

Mueller, C.A., Joss, J.M.P., Seymour, R.S., 2011. Effects of environmental oxygen on development and respiration of Australian lungfish (*Neoceratodus forsteri*) embryos. J. Comp. Physiol. B. 181, 941–952. https://doi.org/10.1007/s00360-011-0573-3.

Ransberry, V.E., Blewett, T.A., McClelland, G.B., 2016. The oxidative stress response in freshwater-acclimated killifish (*Fundulus heteroclitus*) to acute copper and hypoxia exposure. Comp. Biochem. Physiol. Part C. 179, 11–18. https://doi.org/10.1016/j.cbpc.2015.08.001.

Ransberry, V.E., Morash, A.J., Blewett, T.A., Wood, C.M., McClelland, G.B., 2015. Oxidative stress and metabolic responses to copper in freshwater- and seawater-acclimated killifish, Fundulus heteroclitus. Aquat. Toxicol. 161, 242–252. https://doi.org/10.1016/j.aquatox.2015.02.013.

Reidy, S.P., Nelson, J.A., Tang, Y., Kerr, S.R., 1995. Post-exercise metabolic rate in Atlantic cod and its dependence upon the method of exhaustion. J. Fish Biol. 47, 377–386. https://doi.org/10.1111/j.1095-8649.1995.tb01907.

Sappal, R., Fast, M., Purcell, S., MacDonald, N., Stevens, D., Kibenge, F., Siah, A., Kamunde, C., 2016. Copper and hypoxia modulate transcriptional and mitochondrial functional-biochemical responses in warm acclimated rainbow trout (*Oncorhynchus mykiss*). Environ. Pollut. 211, 291–306. https://doi.org/10.1016/j.envpol.2015.11.050.

Steffensen, J.F., 1985. The transition between branchial pumping and ram ventilation in fishes: energetic consequences and dependence on water oxygen tension. J. Exp. Biol. 114, 141–150.

Tate-Boldt, E.K., Kolok, A.S., 2008. Copper acclimation in juvenile fathead minnows: is a cycle of branchial damage and repair necessary? Aquat. Toxicol. 87, 13–18. https://doi.org/10.1016/j.aquatox.2008.01.001.

Waiwood, K., Beamish, F., 1978. Effects of copper, pH and hardness on the critical swimming performance of rainbow trout (*Salmo gairdneri* Richardson). Water Res. 12, 611–619. https://doi.org/10.1016/0043-1354(78)90141-0.

Wilson, R.W., Taylor, E.W., 1993. The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. J. Comp. Physiol. B. 163, 38–47. https://doi.org/10.1007/BF00309663.

Yeager, D.P., Ultsch, G.R., 1989. Physiological regulation and conformation: a BASIC program for the determination of critical points. Physiol. Zool. 62, 888–907. https://doi.org/10.1086/physzool.62.4.30157935.