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Physiological impacts and bioaccumulation of dietary Cu and Cd in a model teleost: The Amazonian tambaqui (*Colossoma macropomum*)

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ABSTRACT

Increasing anthropogenic activities in the Amazon have led to elevated metals in the aquatic environment. Since fish are the main source of animal protein for the Amazonian population, understanding metal bioaccumulation patterns and physiological impacts is of critical importance. Juvenile tambaqui, a local model species, were exposed to chronic dietary Cu (essential, 500 μg Cu/g food) and Cd (non-essential, 500 μg Cd/g food). Fish were sampled at 10–14, 18–20 and 33–36 days of exposure and the following parameters were analyzed: growth, voluntary food consumption, conversion efficiency, tissue-specific metal bioaccumulation, ammonia and urea-N excretion, O_2 consumption, P_{crit}, hypoxia tolerance, nitrogen quotient, major blood plasma ions and metabolites, gill and gut enzyme activities, and *in vitro* gut fluid transport. The results indicate no ionoregulatory impacts of either of the metal-contaminated diets at gill, gut, or plasma levels, and no differences in plasma cortisol or lactate. The Cd diet appeared to have suppressed feeding, though overall tank growth was not affected. Bioaccumulation of both metals was observed. Distinct tissue-specific and time-specific patterns were seen. Metal burdens in the edible white muscle remained low. Overall, physiological impacts of the Cu diet were minimal. However dietary Cd increased hypoxia tolerance, as evidenced by decreased P_{crit}, increased time to loss of equilibrium, a lack of plasma glucose elevation, decreased plasma ethanol, and decreased NQ during hypoxia. Blood O_2 transport characteristics (P_{50}, Bohr coefficient, hemoglobin, hematocrit) were unaffected, suggesting that tissue level changes in metabolism accounted for the greater hypoxia tolerance in tambaqui fed with a Cd-contaminated diet.

1. Introduction

The Amazon is characterized by its rich and endemic biological diversity which thrives amidst challenging environmental conditions, such as frequent variations in water dissolved oxygen and carbon dioxide concentrations, extremely low pH and ion concentrations in many water bodies, and very high but stable temperatures (Val and Almeida-Val, 1995). The rise in population development in the Amazon has led to an increase in the potential risks for metal contamination in the natural environment. The major sources include urban home and industrial effluents, untreated sewage and the mining of mineral resources. Silva et al. (1999) measured up to 11 mg cadmium/g and 3.8 mg copper/g in the surface sediments nearby the Manaus industrial park, while measured copper (Cu) concentrations in industrial effluents were shown to exceed Brazilian water quality guidelines of < 9 μg/L (CONAMA 357/2005) by about 350-fold (Sampaio, 2000). Pinto et al. (2008) found that the waterborne Cu and Cd concentrations vary seasonally (dry versus wet) in several tributaries of the Rio Negro in the region of Manaus. In "natural" areas, water Cu and Cd concentration were 10–50 and 8–90 μg/L respectively, while in areas under heavy anthropogenic pressures, the measured water Cu was up to 2100 μg/L and Cd was up to 590 μg/L (Geissler, 1999; Pinto et al., 2008; Pinto et al., 2009). Thus, Amazonian fishes are very likely to experience a combination of metal and environmental stressors, such as hypoxia, in their natural environments. To date, the overwhelming majority of available studies on metal toxicity have employed northern temperate

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species such as rainbow trout and fathead minnow as models, and there is the need for a non-temperate, warm water teleost model relevant to tropical ecosystems such as the Amazon.

For the present study, we chose the tambaqui (*Colossoma macropomum*), an omnivore characid from the Serrassalmidae family, as our experimental model. The tambaqui is one of the most consumed local fish species in the Amazonian region (Araujo-Lima and Goulding, 1997) and is economically very important due to its intense artisanal and commercial fisheries, as well as heavy aquacultural exploitation. The tambaqui is native to both the white waters (moderately soft) and black waters (very soft, acidic) of the Amazonian basin and very tolerant of low pH and low ion concentrations (Wood et al., 1998; Wilson et al., 1999; Gonzalez et al., 2006). In the wild, their diet largely consists of fruits and nuts, with high lipid and carbohydrate contents (Araujo-Lima and Goulding, 1997). Additionally, the tambaqui can withstand long periods of hypoxia and hypercarbia (Saint-Paul, 1984; Sundin et al., 2000), and is capable of reducing the passive ion losses commonly experienced by most fish during hypoxia (Robertson et al., 2015). Tambaqui is quickly becoming an important neotropical model species (FAO, 2016; Wood et al., 2017).

The acute toxicities of waterborne Cu and Cd to freshwater fish are now well understood and appear to be associated mainly with the bioaccumulation of these metals in the gills. Although essential, elevated waterborne Cu impairs Na^+ regulation; mortality is due to net Na^+ loss associated with inhibition of branchial Na^+ uptake, elevation of Na^+ efflux, blockade of gill Na^+,K^+-ATPase and carbonic anhydrase activities, and inhibited excretion of ammonia (for a comprehensive review, see Grosell, 2012). Cadmium is not essential, and waterborne Cd toxicity is caused mainly by a disruption of whole body ion homeostasis, especially Ca^{2+} regulation. Cadmium disrupts branchial Ca^{2+} uptake by competition for apical uptake channels and blockade of basolateral Ca^{2+}-ATPase activity in the gills (for a comprehensive review, see McGeer et al., 2012). The tambaqui is unusually resistant to waterborne Cu toxicity, and branchial Na^+ uptake appears to be completely resistant to inhibition by Cu, although Na^+ efflux is elevated at higher concentrations (Matsuo et al., 2005). In contrast, the tambaqui is quite sensitive to waterborne Cd toxicity, exhibiting the classic syndrome of inhibited Ca^{2+} uptake at low concentrations (Matsuo et al., 2005).

Far less is known about the toxicity of foodborne Cu and Cd in fish, despite the fact that dietary exposure is now recognized as a major pathway of metal poisoning, which occurs almost exclusively on a chronic rather than an acute basis (Dallinger et al., 1987; Clearwater et al., 2002; Meyer et al., 2005; DeForest and Meyer, 2015). The uptake of metals via dietary exposure occurs initially with a transfer from the food to the gut tissue, followed by movement through the bloodstream to the liver, and subsequent internal distribution via the circulation, or excretion. Thus the gills are exposed only indirectly, and the gut itself may be a proximate target for toxicity, with similar or different mechanisms than the gills. For example, *in vitro* studies in the rainbow trout found that Cu uptake, in part, is mechanistically linked to Na^+ transport, though there are other pathways involved and considerable regional differences along the gastrointestinal tract (Nadella et al., 2007; Nadella et al., 2011). Similarly, Cd uptake in the gut occurs in part via Ca^{2+} transport pathways, again with considerable regional differences (Ojo and Wood, 2008; Klinck et al., 2009; Klinck and Wood, 2011). Chronic exposure of rainbow trout and other salmonids to Cd- and Cu-enriched diets results in the accumulation of both metals in the gut tissue, potentially impairing nutrient uptake, but in general inhibitory effects on growth appear to be more severe with Cd-contaminated diets (Sorensen, 1991; Handy, 1993; Szebedinszky et al., 2001; Ng and Wood, 2008; Ng et al., 2009) than with Cu-contaminated diets (Lanno et al., 1985; Handy, 1993; Campbell et al., 2002; Miller et al., 1993; Kamunde et al., 2001; Kamunde et al., 2002; Kamunde and Wood, 2003), though some studies showed no effects, or the opposite difference (*e.g.* Berntssen et al., 1999a, 1999b, Lundebye et al., 1999; Berntssen and Lundebye, 2001; Franklin et al., 2005; Kamunde and MacPhail, 2011). Comparison of many of these same studies also suggests differential patterns of tissue-specific bioaccumulation (*e.g.* liver > kidney for Cu, kidney > liver for Cd).

Waterborne Cu can decrease an animals' ability to deal with hypoxia (Bennett et al., 1995); chronic Cu and hypoxia exposures may have either synergistic (Malekpouri et al., 2016) or antagonistic effects (Ransberry et al., 2016). McGeer et al. (2000) concluded that chronic waterborne Cu exposure increased metabolic costs in trout; a decrease in swimming capacity was associated with higher oxygen consumption rates, while similar exposure to waterborne Cd did not appear to exert any metabolic costs. Juvenile yellow perch sampled from historically metal-contaminated lakes (where exposure was likely both waterborne and dietborne) showed reduced scope for aerobic activity and lower resting O_2 consumption, effects that were associated with high levels of liver Cd and Cu accumulation respectively (Couture and Kumar, 2003). Additionally, trout fed a Cu-contaminated diet had higher metabolic rates at all swimming speeds, and this was compensated with a behavioural decrease in spontaneous activity (Handy et al., 1999; Campbell et al., 2002). Long-term exposure to dietary Cd appeared to increase metabolic costs in Atlantic salmon, but O_2 consumption rates and activity were not monitored in this study (Berntssen and Lundebye, 2001).

In our study, we have compared the responses of juvenile tambaqui exposed via the diet to equal concentrations of Cu and Cd for 30 days, with endpoints chosen in light of previous studies on temperate species summarized above. Equal mass concentrations of Cu and Cd (500 μg/g dry weight) were chosen based on requirements for a database on which new Brazilian regulations for dietary metals in the aquatic environment, as well as metals in food for human consumption, could be founded for environmental regulatory purposes. Our first goal was to assess the tissue-specific bioaccumulation patterns of the two metals separately. Second, we looked for possible effects on plasma ions, ionoregulatory enzymes in gut and gills, and gut transport *in vitro*. Third we evaluated several physiological endpoints indicative of possible metabolic effects, including whole-body measurements (food consumption, conversion efficiency, growth, oxygen consumption, nitrogenous waste excretion), hematological parameters, and selected plasma metabolites. Lastly, since hypoxia tolerance is so important ecologically for the tambaqui, we looked for possible impacts using hypoxic challenge tests of time to loss-of-equilibrium (Chapman et al., 1995) and critical oxygen tension (P_{crit}; Yeager and Ultsch, 1989), and responses in plasma metabolites. Our specific hypotheses were (i) that Na^+ regulation would be impacted by dietary Cu, whereas Ca^{2+} regulation would be impacted by dietary Cd; (ii) that tissue-specific bioaccumulation patterns would differ between the two metals tested; (iii) that growth disturbance by dietary Cd would be more severe than by dietary Cu, but that Cu would have a greater stimulatory effect on metabolic rate; and (iv) that as a consequence, dietary Cu would reduce hypoxia tolerance to a greater extent than dietary Cd.

2. Methods

2.1. Animal acclimation

Juvenile tambaqui (*Colossoma macropomum*) were purchased from a commercial aquaculture farm (Sítio dos Rodrigues, Km 35, Rod. AM-010, Brazil) and transferred to the Ecophysiology and Molecular Evolution Laboratory at INPA (LEEM-INPA). All animals were held in well-aerated 500-L tanks, supplied with flow-through filtered groundwater (approximate composition: Na^+ = 53 μM, Cl^- = 25 μM, Ca^{2+} = 5 μM, Mg^{2+} = 4.1 μM, K^+ = 15 μM, alkalinity = 0.14 mEquiv/L, DOC = 0.34 mg C/L, Cu = 6 μg/L; Cd = 1.5 μg/L, pH = 6.0 and temperature = 28 °C). Fish were held at room temperature (28 °C) with a natural photoperiod of approximately 12 h light: 12 h dark, fed daily to satiation with commercial pellets (Nutripiscis- Presence® AL 45,

São Paulo, SP, Brazil; 45% protein), and acclimated to these conditions for 21 days prior to the start of the experiments. At this time, the fish weighed approximately 140 g each. All procedures were performed in compliance with guidelines of the Ethics Committee on Animal Experiments of INPA under registration number 047/2012, and conformed to national animal care regulations.

2.2. Experimental design and diet preparation

The experimental diets (nominally 500 µg Cd/g or 500 µg Cu/g dry weight) were prepared following protocols described in Matsuo and Val (2007) and Klinck et al. (2009) in which the Nutripiscis- Presence® AL 45 commercial pellets were ground to a fine powder in a food processor. The concentrations were initially chosen to match that used by Matsuo and Val (2007) for Cd, and many other studies (see Discussion) for both Cd and Cu. The powder was then thoroughly mixed for 1 h in the food processor with a solution of either $Cd(NO_3)_2{\cdot}4H_2O$ in nanopure water (cadmium-contaminated diet) or $CuNO_3$ in nanopure water (copper-contaminated diet) to ensure complete distribution of metal throughout the food paste. Analytical grade salts were used (Fisher Scientific, Toronto, Canada). The control diet was mixed with nanopure water alone in a similar fashion. The food paste was passed through a commercial pasta maker, and the resulting strings dried in an oven at 60 °C for several days. The strings were then manually broken down into pellets, and stored at 4 °C in hermetically sealed containers. All the experimental diets used in this experiment were prepared all at one time, with a single batch for each treatment. The final measured Cd and Cu concentrations in the experimental diets were close to nominal levels (500 µg/g; Table 1). Note that the molecular weights of Cd and Cu differ, so the mean measured concentrations in the diets equate to 7.47 µmol Cu/g and 4.54 µmol Cd/g. The control diet contained negligible Cd (~2 µg/g), but appreciable Cu (~40 µg/g), reflecting the normal levels of this essential element in commercial pellets.

Fish were placed into three 1000-L tanks (width: 1.26 m, length: 1.89 m and height: 0.42 m; N = 120 fish per tank) supplied with flow-through INPA groundwater at ~10 L/min (composition as above). After a 1-week settling and training period on the new diet in these different tanks during which they were fed daily to satiation with control pellets, fish were netted out, individually weighed without anaesthetization, and then returned to a netted area inside of their respective tanks. Once all fish had been weighed, the net was removed. Starting the next day (day 0) each tank was fed, from weighed bags, with either control, copper or cadmium-contaminated diets once daily until the end of the 35-day experiment. The food was administered slowly, until satiation was achieved; the amount of food provided each day was noted, and food that was not consumed was removed from the tanks approximately 0.5 h after feeding. The average dry weight of an individual pellet had been predetermined. The weight of the uneaten food was estimated by counting the number of uneaten pellets, converting it to the calibrated dry weight, and subtracting this weight from the weight administered, in order to obtain the total amount of dry food consumed daily. The tanks were syphoned daily to remove faecal material. Care was taken so that throughout all different sampling days, all fish had been starved for the same amount of time (24 h). Throughout the entire duration of the experiment, water samples were taken from each tank daily just prior to siphoning (approximately 2–4 h post-feeding) to evaluate the extent of possible leaching of metals from the food pellets and faeces. For economy, only about one quarter of the total number of samples collected, chosen at random, were analyzed as the data were consistent. Mean measured water metal concentrations are reported in Table 1 and indicate that the normal background levels of Cu and Cd in INPA water were elevated by about 1.6-fold and 3.8-fold respectively; only the latter was significant.

Over the next month, fish were periodically removed for various experiments and measurements, only some of which are reported here, while a careful record of the number of fish in each tank was kept. On day 30, at which time the number of fish remaining in each tank was 44–59, all the fish in each tank were individually weighed again. The crude food conversion efficiency (CE) on a percentage basis was calculated from the ratio of the mean amount of weight gained per fish (over 30 days) to the mean cumulative food consumption per fish.

Table 1
Total metal concentration (µg/L) analyzed in water samples from experimental tanks where *C. macropomum* was exposed to control, copper and cadmium contaminated diet. Total metal concentration (µg/g food) analyzed in contaminated or control food. Data are means ± 1 SEM (n = 10 for water analyses and n = 2 for food analyses). Asterisks means significant difference from control through an unpaired, two-tailed *t*-test.

	Metal analyzed			
	Copper		Cadmium	
	Control	Copper	Control	Cadmium
Water metal concentration (µg/L)	7.6 ± 0.6	12.2 ± 2.1	1.8 ± 0.1	6.8 ± 1.2*
Food metal concentration (µg/g food)	40.2 ± 0.5	474.5 ± 18.1*	2.0 ± 0.1	510.3 ± 55.5*

2.3. Sampling and gastro-intestinal "gut-sac" preparations

At 10–14 days and 33–35 days, 8 fish were randomly chosen and removed individually from each experimental tank. Each fish was euthanized in a NaOH-neutralized solution of 0.5 g/L MS-222 (Syndel Laboratories, Vancouver, B.C., Canada). The fish was weighed and its fork length was measured, to allow calculation of Fulton's condition factor ($100\times$ [weight/length^3]). A 1-mL blood sample was taken immediately from the caudal vein into a syringe treated with lithium heparin (Sigma-Aldrich, St. Louis, MO, USA) and processed for determinations of hemoglobin concentration and hematocrit. Hemoglobin was measured spectrophotometrically at 540 nm (triplicate 10-µL whole-blood samples mixed with 2 mL of Drabkin's reagent, Sigma-Aldrich, St. Louis, MO, USA) and hematocrit by centrifugation of triplicate samples at 1000 g for 10 min in sodium-heparinized microhematocrit capillaries (Fisherbrand, Fisher Scientific). The remaining blood was centrifuged at 13000 g for 2 min and the red blood cells and plasma were separately flash-frozen in liquid N_2 for later analysis of ions, metabolites and metal bioaccumulation. Given the duration of the procedures that followed sampling, sampling lasted from 3 to 5 days. Care was taken so the same number of fish from all three treatments was processed in a single day. There was no significant effect of sampling time on any of the parameters measured, at either 10–14 days, or 33–35 days by one-way ANOVAS.

The entire gastrointestinal tract (GIT) was excised, and its length from pyloric sphincter to anus was measured, to allow calculation of the gut length/fork length ratio. The intestine was then processed for the preparation of "gut sacs" (as described below). Following that, samples of gills, liver, stomach, anterior, mid and posterior intestine, kidney, white muscle and brain were dissected out and quickly frozen in liquid N_2 for later analyses of metal bioaccumulation, and for some tissues, enzyme activities. Before freezing, gut tissue samples were gently scraped in order to remove any remaining chyme, and then thoroughly rinsed with Cortland saline (Wolf, 1963) and lightly blotted. In some instances, the tissues were too small to be partitioned for the multiple analyses performed, therefore for some tissues the sample size for bioaccumulation measurements ranges from 3 to 8. All tissues were kept in −80 °C prior to the analysis.

In vitro fluid transport rates were determined in the anterior, mid, and posterior sections of the intestine as a proxy for ion transport capacity. In identical preparations, Pelster et al. (2015) showed that fluid

absorption in the tambaqui is isosmotic, such that fluid absorption reflects net Na^+ and Cl^- absorption. All procedures were the same as those used by Pelster et al. (2015) so will not be detailed here. In brief, each section of the intestine was made into a sac filled with a modified Cortland saline, incubated in a similar isosmotic saline for 3 h, and the rate of fluid absorption was measured by the change in weight, factored by the measured surface area of the sac preparation and time:

$$\text{Fluid transport rate} = [W_i - W_f]/SA * t \quad (1)$$

where W_i and W_f are the initial and final weight (mg) respectively, SA is the total surface area of the sac (cm^2) determined as described by Grosell and Jensen (1999), and t is time (h).

2.4. Metal, metabolite, ion, and cortisol measurements

Frozen tissues were thawed, blotted dry, weighed (wet weight) and digested with 4N nitric acid (HNO_3 trace metal grade; Fisher Scientific) in sealed vials for 48 h at 68 °C. Diet samples were similarly digested. Water samples (1% HNO_3) as well as aliquots of the acid digests were taken for measurements of Cu and Cd by graphite furnace atomic absorption spectroscopy (Perkin-Elmer AAnalyst 800 AA187 spectrophotometer, Norwalk CT, USA). A metal reference solution (PE N9300224, Calibration Solution, Perkin Elmer) was used to create internal standard curves. In addition, a certified reference material (DORM-2 (Dogfish Muscle), NRC-CNRC, Ottawa, Canada) was used to demonstrate that metal recovery was within 85–100% of certified values, with a coefficient of variation of 2% for Cu and 6% for Cd. The limit of detection for Cu was 1 µg/L and that for Cd was 0.1 µg/L; none of the digests were below these values.

Plasma Na^+ and K^+ concentrations were measured with a 910 Digital Flame Photometer (Analyzer – Instrumentação Analítica, São Paulo, SP, Brazil), plasma Ca^{2+} was determined by atomic absorption spectroscopy (Perkin-Elmer AAnalyst 800 AA187), and plasma Cl^- concentration was assayed colorimetrically using the method described by Zall et al. (1956). Certified standards (Radiometer, Copenhagen, Denmark) were used for calibration. Plasma concentrations of total ammonia (Raichem assay, Cliniqa Corporation, San Marcos, CA, USA), glucose (Infinity™ Glucose Hexokinase Liquid Stable Reagent, ThermoFisher Scientific, Burlington, ON, Canada), ethanol (Megazyme, Bray, County Wicklow, Ireland), and cortisol (ICN Pharmaceuticals ELISA assay, Costa Mesa, CA, USA), were measured using commercial kits, according to the manufacturers' instructions. Plasma lactate was assayed using a commercial meter (Lactate Pro, Arkray Inc., Kyoto, Japan) calibrated with sodium lactate standards.

2.5. Enzyme analyses

Na^+/K^+-ATPase and v-type H^+-ATPase activities in tissues were measured following protocols similar to those described in McCormick et al. (1989) and Lin and Randall (1993) respectively. Gill, anterior intestine, and posterior intestine samples were thawed and homogenized (1:10 w/v) in ice-cold buffer solution containing 50 mM imidazole, 125 mM sucrose and 5 mM EGTA (pH 7.3). Samples were centrifuged (5000*g*, 4 °C, 3 min), then supernatants were collected and kept on ice throughout the analysis. In both methods, the difference in the amount of adenosine diphosphate (ADP) produced by the samples when assayed in a control reaction (no inhibitor added) and in an inhibited reaction was measured. Ouabain (Sigma–Aldrich) and N-ethylmaleimide (NEM) with sodium azide (both Sigma–Aldrich) were used as inhibitors of Na^+/K^+-ATPase and v-type H^+-ATPase, respectively. Enzyme activity was calculated by the difference between control and inhibited reactions. Absorbance (340 nm) from the reaction mixture was evaluated at 10 s intervals over 5 min at 20 °C using a microplate reader (SpectraMAX Plus; Molecular Devices, Menlo Park, CA, USA). Enzyme activity was expressed as µmol ADP/mg protein/h. Protein concentration in the homogenates was measured using the Bradford reagent (Sigma-Aldrich) against a bovine serum albumin (BSA) standard.

2.6. Oxygen consumption and hypoxia tolerance experiments

At 18–20 days of exposure to control or metal-contaminated diet, oxygen consumption rates (MO_2, µmol/kg/h) were measured by closed-system respirometry in 8 fish randomly selected from each treatment. Fish were removed from the experimental tanks and allowed to acclimate overnight (12 h) in individual respirometry chambers (5 L), covered with dark plastic and supplied with flow-through INPA groundwater. At the start of the experiment, water flow was interrupted and chambers were sealed to prevent oxygen diffusion from the air, and initial PO_2 was recorded with a handheld oxygen meter (Accumet AP84, Fisher Scientific, Ottawa, ON, Canada); the probe was inserted through a snug hole in the lid which was otherwise sealed with a rubber stopper. PO_2 was recorded every 10 min as fish were consuming the remaining O_2 in the chambers, until a minimum PO_2 of 7.5 Torr was reached. On average, the trials lasted 330 min. At this point, the fish were quickly weighed, tagged with a surgical silk stitch in the dorsal fin to avoid re-use in future tests, then returned to flowing water to recover. The PO_2 versus time data for each fish were fitted by successive overlapping 3-point linear regressions in Excel, and MO_2 was calculated over successive 5 Torr intervals:

$$MO_2 = [\Delta PO_2 \times \alpha O_2 \times V]/(t \times m) \quad (2)$$

where ΔPO_2 is the change in PO_2 (Torr), αO_2 is the solubility constant (µmol/Torr) for O_2 in water at 28 °C (Boutilier et al., 1984), t is the time interval (h), and m is the mass of the fish (kg). For each fish, the calculated MO_2 was plotted against water PO_2 and the inflection point at which MO_2 transitioned from being independent to being dependent on PO_2 (i.e. P_{crit}) was calculated using the BASIC program designed by Yeager and Ultsch (1989). Note that closed system respirometry was used; a recent review (Rogers et al., 2016) found no consistent difference in P_{crit} determined on the same species by open *versus* closed-system respirometry but concluded there is still the need for more studies comparing the two systems. The lack of difference was directly confirmed in a recent experimental study on goldfish (Regan and Richards, 2017). A blank (respirometer with no fish in it) was performed every day where trials we run, and oxygen consumption due to bacterial activity in the water was negligible.

Additionally, during the experiment described above, water samples (5 mL) were taken every 20 min for ammonia and urea determinations. Immediately after sampling, the same amount of water at the same approximate PO_2 was inserted back into the chamber, resulting in an approximate 0.001 fold dilution of the media. Ammonia and urea were measured colorimetrically according to Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. In practice, it was only possible to reliably determine changes in ammonia and urea-N concentrations over longer periods (> 1 h), so rates of ammonia-N (J_{amm}; µmol N/kg/h) and urea-N (J_{urea}; µmol N/kg/h) flux were calculated over three PO_2 intervals (150–60 Torr, 60–30 Torr, and < 30 Torr):

$$J_x = [X_f - X_i] \times V/(t \times m) \quad (3)$$

where X_f and X_i are the final and initial water total ammonia-N or urea-N concentrations (µmol N), and the other parameters are as in Eq. (2). The total ammonia in the water at the end of the respirometry trials was never higher than 110 µmol/L.

The relative protein use as a fuel for aerobic metabolism was calculated over the same intervals according to Lauff and Wood (1996). Firstly, the nitrogen quotient (NQ) was calculated as:

$$NQ = J_{N\text{-}total}/MO_2 \quad (4)$$

where $J_{N\text{-}total}$ is the sum of $J_{Amm\text{-}N} + J_{Urea\text{-}N}$.

The percentage of metabolism fueled by the oxidation of protein was then determined by:

$$\% \text{ Protein} = NQ/0.27 \quad (5)$$

where 0.27 represents the maximum theoretical value when protein is the only fuel being metabolized (Van Den Thillart and Kesbeke, 1978).

In order to determine the hypoxia tolerance of control fish, and of animals fed a metal contaminated diet, at 20 days of exposure, 12 fish were randomly chosen from each experimental treatment and tagged for further identification, using a surgical silk stitch on selected fins. The 36 fish were pooled together in a ~800 L fiberglass tank, the water surface was sealed with a transparent plastic cover, and a recirculating water pump and 3 large gas diffusers were placed on the bottom of the tank. A subsurface net prevented air access. The tank was bubbled with nitrogen and PO_2 in the water was reduced at a rate of ~3 Torr/min, until a PO_2 of ~7.5 Torr was reached. After that, the water PO_2 was monitored continuously to ensure uniformity throughout the entire tank, and maintained at 6–9 Torr by gentle bubbling with either N_2 or air, which also ensured mixing. Fish were allowed to swim freely until they could no longer sustain an upright position in the water column. Once an individual fish lost equilibrium, the time to loss of equilibrium (LOE) was noted, the fish was quickly removed without disturbance to other fish and euthanized by MS-222 overdose, and a 1-mL blood sample was taken. As the fish were being removed from tank, the total fish number changed. We cannot rule out any possible extraneous effects caused by changes in biomass on time to LOE, or on the physiology of these fish. Euthanasia, blood sampling, and plasma processing, storage, and analyses were performed as described above. Samples were analyzed for glucose, total ammonia, ethanol, cortisol, and lactate for comparison to similarly sampled normoxic fish in the three dietary treatments.

2.7. Blood P_{50} and Bohr coefficient calculation

In order to investigate the origin of the greater hypoxia tolerance displayed by tambaqui fed a Cd-contaminated diet, blood samples were taken from 6 Cd-exposed fish and 6 control fish randomly chosen from the experimental tanks at ~20 days of exposure. The oxygen tension at which hemoglobin is 50% saturated with oxygen (P_{50}) was measured in whole blood at pH 7.8 and at pH 7.2, and 28 °C, using a Hemox Analyzer (TSC Scientific corporation, New Hope, PA, USA). Bohr coefficients for each blood sample were calculated using the following equation:

$$\text{Bohr coefficient} = -[\log P_{50} \text{ at pH } 7.8 - \log P_{50} \text{ at pH } 7.2]/[7.8–7.2] \quad (6)$$

2.8. Statistical analysis

In general, data have been expressed as means ± 1 SEM (N = number of animals). Mean values were compared through either one-way or two-way analysis of variance (ANOVA) as appropriate, followed by post-hoc Tukey's test. Unpaired Student's *t*-test was used for single comparisons. In all cases, mean values were considered significantly different when $p < .05$. ANOVA assumptions (data normality and homogeneity of variances) were checked, and if not achieved, data were transformed using a square root transformation. Details on statistical tests used and their results are provided in specific Figure and Table captions.

3. Results

3.1. Cu and Cd effects on food consumption and weight gain

There were no mortalities associated with any of the three diets, so all physiological effects were sublethal. Although all three groups of fish were eating similar amounts at the start of the experiment, fish fed a Cd-contaminated diet ate progressively less over time. By 30 days, Cd-fed fish had consumed 77% and 70% of what fish fed control or Cu-contaminated diets had consumed respectively (Table 2). Although Cu-fed fish started the experiment slightly smaller in mass, by day 30, all three groups exhibited very similar weights, with increases of about 51 g, 70 g, and 45 g per fish for the control, Cu-fed, and Cd-fed treatments respectively (Table 2). Therefore, the calculated crude conversion efficiency from 0 to 30 days of exposure was 58% in the Cu-fed fish, 54% in the Cd-fed fish, and 47% in the controls (tank based measurements–Table 2). Additional body metrics on a small subsample of each treatment were determined using fish sacrificed for gut transport measurements (Supplementary Table S1). At 10–14 days, the ratio of gut length to body fork length was significantly higher in the Cd-fed fish than in the control fish, but the Cu-fed fish do not seem to have been affected. Also at 10–14 days, Fulton's condition factor was actually higher in the Cd treatment than in the controls. Neither of these differences persisted at 33-35 days.

Table 2
Cumulative food consumptions (CFC), body weights (BW), and the resulting calculated crude conversion efficiencies (CE = 100 * (weight gain/food consumed)) of fish fed control, copper and cadmium contaminated diets over 30 days. Body weight (grams) was measured at the start (0) and at 30 days of exposure. Data are means ± 1 SEM (n = 120 at day 0, n = 44–59 at day 30) for BW, and a single measured value (n = 1 tank) for CFC and calculated CE.

	Cumulative food consumption (g/fish)				Body weight (g)		Conversion efficiency (%)
	Days of exposure				Days of exposure		Days of exposure
	0	10	20	30	0	30	0–30
Control	0	44	75	109	147.5 ± 5.4	198.3 ± 7.9	46.6
Copper	0	50	85	120	127.9 ± 5.6	197.4 ± 6.7	57.9
Cadmium	0	43	71	84	144.8 ± 5.1	190.2 ± 8.6	54.0

3.2. Cu and Cd effects on ionoregulatory function

Plasma concentrations of Na^+ (Fig. 1A), Cl^- (Fig. 1B), Ca^{2+} (Fig. 1C), and K^+ (Fig. 1D) were typical of freshwater teleosts, and exhibited no changes associated with the two metal-amended diets, and no changes over time. Na^+,K^+-ATPase and v-type H^+-ATPase activities in gills, anterior intestine, and posterior intestine were also unaffected by the Cu-feeding and Cd-feeding (Table 5). The activities of these enzymes in the posterior intestine were measured at both 10–14 d and 33–35 d, and were not significantly different.

In vitro fluid transport rates (μL/cm^2/h) in gut sac preparations were measured at 10–14 d and 33–35 d as a general indicator of intestinal ion transport (Table 3). In all treatments, fluid transport rates in the anterior portion of the intestine tended to be greatest (up to 55 μL/cm^2/h), while those in the posterior intestine tended to be the lowest (Table 3). Exposure to either Cu- or Cd-contaminated diets had no significant effect on *in vitro* fluid transport rates, and while there were no significant differences between sample times, two-way ANOVA identified a significant overall effect of time in the anterior intestine (Table 3).

3.3. Bioaccumulation of Cu and Cd

Both dietary Cu and dietary Cd led to bioaccumulation in tissues, and that was affected by time of exposure. Note that data have been plotted in molar units (nmol/g).

Fig. 2 shows patterns in those tissues which exhibited generally high levels of bioaccumulation. Burdens of both metals in the gills (Fig. 2A)

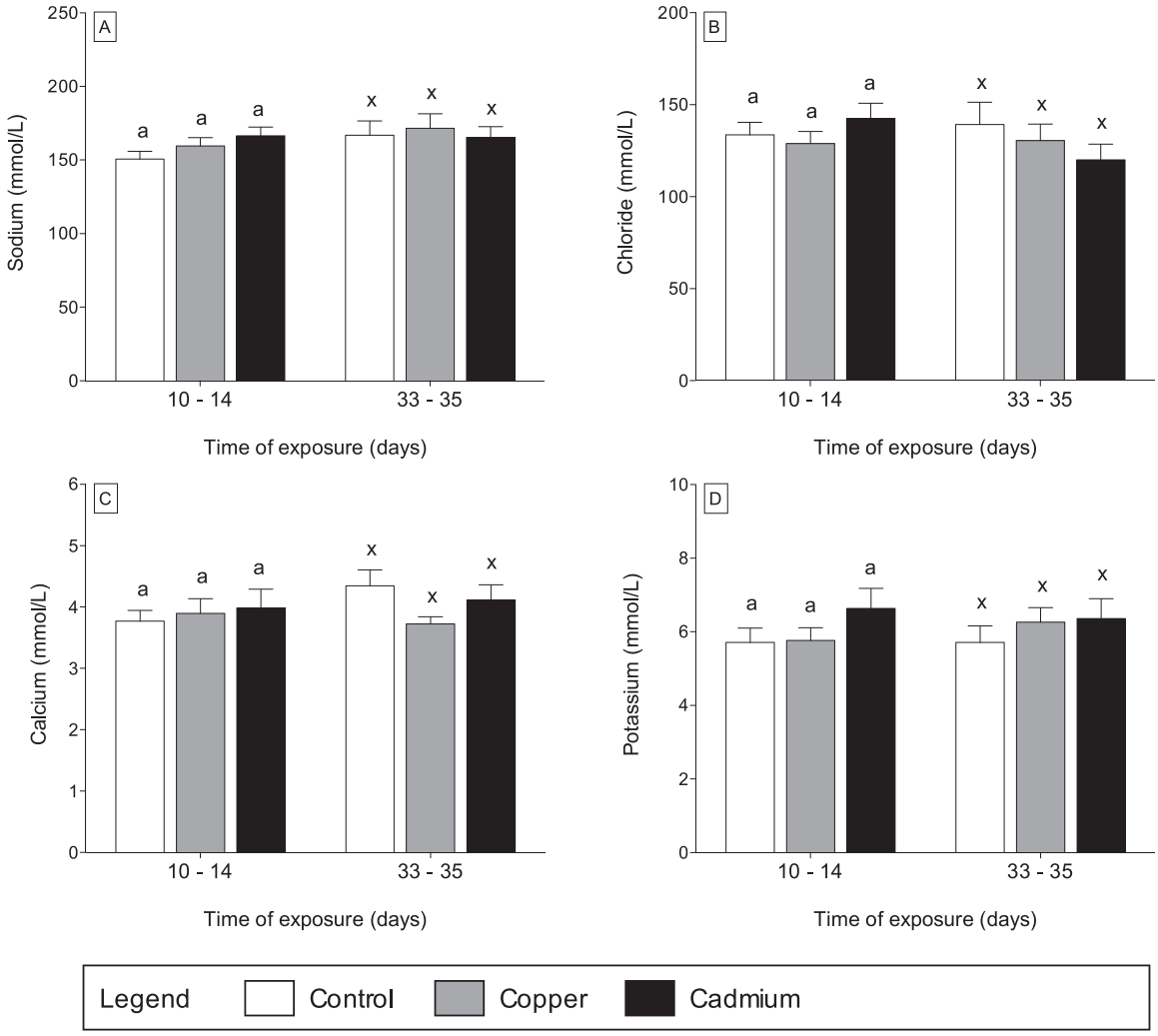


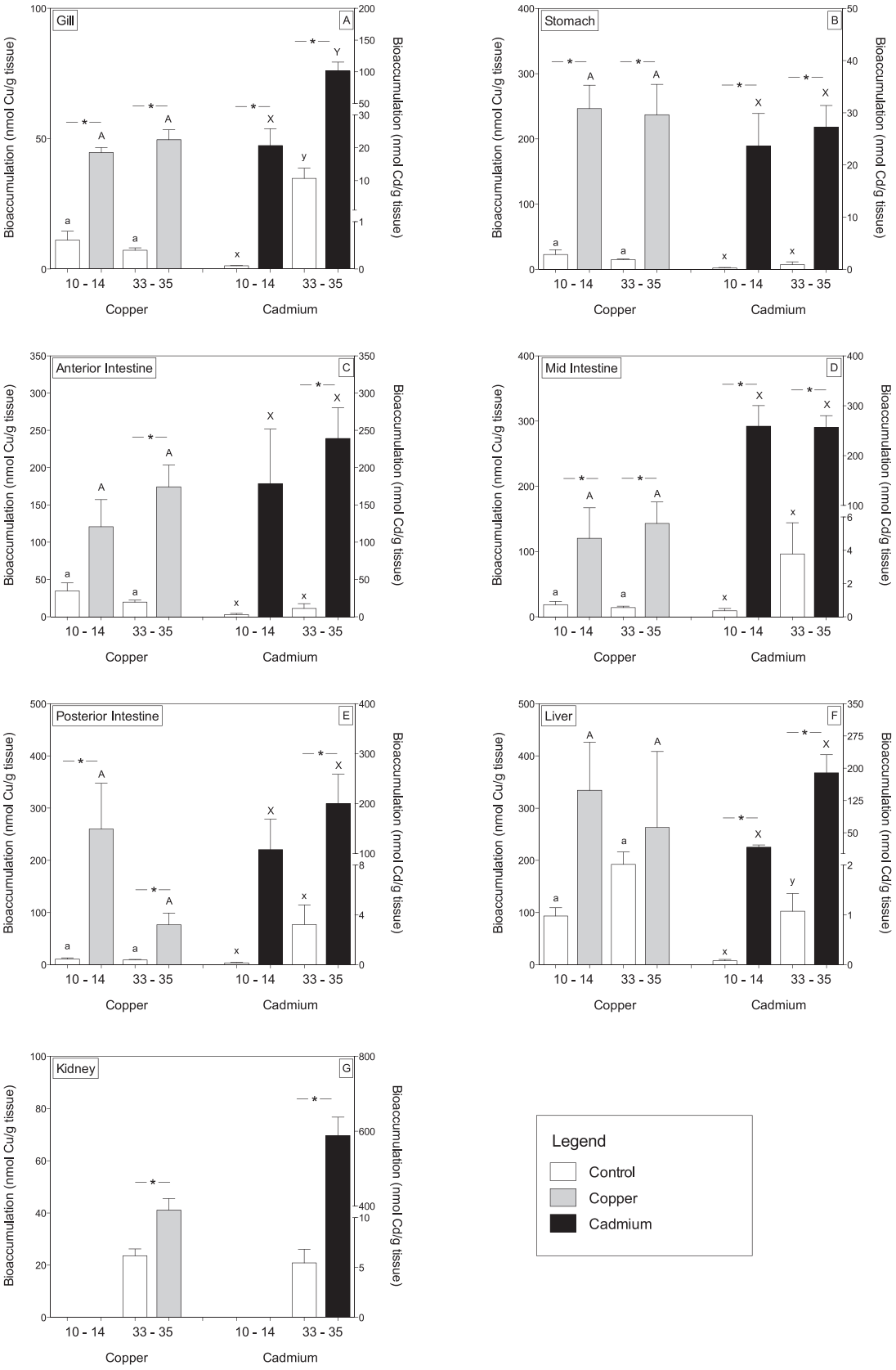
Fig. 1. Plasma concentrations of (A) sodium, (B) chloride, (C) calcium, and (D) potassium in *C. macropomum* at 10–14 and 33–35 days of exposure to control (white bars), copper (grey bars) and cadmium (black bars) contaminated diets. Data are means ± 1 SEM (n = 7–8). Means sharing the same lower case letters are not significantly different across treatments within the same sampling time (10–14 days). Means sharing same upper case letters are not significantly different across treatments within the same sampling time (33–35 days). Two-way ANOVA was performed for each ion, and no overall effect of diet or time were identified, as well as no interaction.

Table 3
in vitro fluid transport rates (μL/cm^2/h) in the anterior, mid and posterior intestine of *C. macropomum* at 10–14 and 33–35 days of exposure to control, copper and cadmium contaminated diets. Data are means ± 1 SEM (n = 7–8). [Two-way ANOVA results: (Anterior) interaction: p = .876, diet: p = .146, time p = .006; (Mid) interaction: p = .202, diet: p = .493, time: p = .891; (Posterior) interaction: 0.519, diet: p = .047; time: p = .094].

	Days of Exposure	In vitro fluid transport rates (μL/cm^2/h)		
		Control	Copper	Cadmium
Anterior Intestine	10–14	24.82 ± 6.04	33.76 ± 5.77	26.99 ± 7.16
	33–35	37.54 ± 4.95	52.65 ± 7.09	41.25 ± 6.79
Mid Intestine	10–14	33.23 ± 6.65	27.06 ± 5.93	27.46 ± 9.61
	33–35	23.28 ± 6.23	38.93 ± 4.72	23.44 ± 4.28
Posterior Intestine	10–14	18.22 ± 4.77	21.19 ± 5.87	35.24 ± 8.78
	33–35	12.33 ± 4.23	19.33 ± 3.99	21.51 ± 3.40

were generally lower than in gastrointestinal tissues or liver (Figs. 2B, C, D, E, F). While accumulation of Cu in gill tissue was regulated over time, the accumulation of Cd in the gills was significantly higher at 33–35 d of exposure than at 10–14 days. Amongst the four sections of the gut, the stomach (Fig. 2B) and the posterior intestine (Fig. 2E) accumulated the most Cu, again with evidence of regulation as Cu concentrations were stable or declined between the two sampling times, with similar patterns in the anterior (Fig. 2C) and mid-intestine (Fig. 2D). In all four sections of the gut, Cd accumulated significantly to levels that were approximately stable over time of exposure. The Cd burden in the stomach was dramatically smaller than in the other three sections (Fig. 2C–E). In the liver (Fig. 2F), background levels of the essential element Cu were high in the control treatment, and increased over time, and further hepatic bioaccumulation associated with dietary Cu was only moderate. Control background levels of Cd were very low, although significantly different at the two times, whereas tambaqui fed dietary Cd exhibited marked hepatic Cd bioaccumulation which increased greatly over time (Fig. 2F). Amongst all the tissues analyzed, Cd bioaccumulated to the highest level in the kidney (approximately 600 nmol Cd/g tissue). Cu accumulation in the kidney was at the same level as in tissues of the GI, and significant (Fig. 2G).

Table 4 displays patterns for those tissues which showed generally low levels of bioaccumulation. Cu levels in white muscle, red blood cells and blood plasma were low (< 5 nmol/g tissue) relative to all other compartments sampled, and exhibited no bioaccumulation relative to control values (Table 4), though these control levels increased over time in muscle and red blood cells. Background Cd concentrations



(caption on next page)

Fig. 2. Bioaccumulation of copper (nmol Cu/g wet tissue plotted on left y axis) and cadmium (nmol Cd/g wet tissue plotted on right y axis) in (A) gill, (B) stomach, (C) anterior intestine, (D) mid intestine, (E) posterior intestine, (F) liver and (G) kidney of *C. macropomum* at 10–14 and 33–35 days of exposure to control (white), copper (gray) and cadmium (black bars) contaminated diet. Data are means ± 1 SEM (n = 3–8). Different lower case letters indicate significant differences between fish fed a control diet at two different sampling times. Different upper case letters indicate significant differences between fish fed a contaminated diet at two different sampling times. Asterisks indicate significant differences between fish fed control and metal contaminated diets at the same sampling time. Copper and cadmium-contaminated diets were not compared with each other. [Two-way ANOVA results: (Gill-Cu) interaction: p = .134 diet: p < .001, time: p = .876; (Gill-Cd) interaction: p = .004 diet: p < .0001, time: p < .0001; (Stomach-Cu) interaction: p = .980, diet: p < .0001, time: p = .783; (Stomach-Cd) interaction: p = .692, diet: p < .001, time: p = .565; (AI-Cu) interaction: p = .253, diet: p = .0006, time: p = .515; (AI-Cd) interaction: p = .573, diet: p = .0003, time: p = .458; (MI-Cu) interaction: p = .6388, diet: p = .0004, time: p = .7567; (MI-Cd) interaction: p = .889, diet: p < 0.0001, time: p = .989; (PI-Cu) interaction: p = .1074, diet: p = .007, time: p = .100; (PI-Cd) interaction: p = .3718, diet: p = .003, time: p = .2964; (Liver-Cu) interaction: p = .3847, diet: p = .1174, time: p = .885; (Liver-Cd) interaction: p < .001, diet: p < .0001, time: p < 0001; no data available for kidney control, Cu and Cd at 10–14 days in panel G].

in these same compartments were extremely low, but in all three there was small but significant bioaccumulation which increased over time, particularly in the red blood cells (Table 4). Note that Cd concentrations in the red blood cells were many-fold higher than those in the plasma (Table 4) by 33–35 d. Background Cu levels were higher in the brain than in those of the other tissues in Table 4, and comparable to those in the tissues of Fig. 2. However, there was again no evidence of Cu bioaccumulation as a result of dietary loading, apart from a very small increase in the plasma at 33–35 d. The brain accumulated Cd as early as 10–14 d, and the amount accumulated increased significantly at 33–35 d, indicating that Cd is capable of crossing the blood-brain barrier (Table 4).

3.4. Cu and Cd effects on plasma metabolites and cortisol

Plasma glucose concentrations were similar amongst the three treatments at 10–14 d (Fig. 3A). The glucose levels in the plasma of control fish did not change over time (Fig. 3A). Plasma ammonia levels were also similar amongst the three groups at the first sampling time, but declined significantly in all treatments at the second sampling time; at 33–35 d, fish fed a Cu-contaminated diet had the highest ammonia levels in the plasma (Fig. 3B). Ethanol (Fig. 3C) was detected in the plasma of all three groups. At 10–14 d, there were no significant differences among treatments, but by 33–35 d, ethanol concentrations were higher in the Cu-fed fish than in the other two treatments (Fig. 3C). In contrast, cortisol levels were similar amongst all three treatments at both 10–14 d and 33–35 d of exposure (Fig. 3D). Plasma lactate concentrations were similar amongst the three groups at both sampling times, but tended to be lower at 33–35 d, a difference which was significant only in the control treatment (Fig. 3E).

3.5. Cu and Cd effects on respirometric parameters and indicators of hypoxia tolerance

Respirometric parameters were measured in all three groups at 18–20 d of exposure. Routine rates of oxygen consumption (MO_2) under normoxia did not differ significantly between the two metal-fed groups and the controls (Fig. 4A). As water oxygen tension declined, tambaqui in all treatments were able to regulate a fairly constant MO_2 down to about 55 Torr (Fig. 5). However thereafter, fish fed a Cd-contaminated diet exhibited a significantly lower P_{crit} (30 Torr) when compared to controls (41 Torr), while the P_{crit} (40 Torr) measured for Cu-fed fish was similar to that in the controls (Figs.4B and 5).

In order to follow up this interesting observation of possibly greater hypoxia tolerance in tambaqui as a result of Cd-feeding, a time to loss of equilibrium (LOE) experiment under severe hypoxia (5% air saturation = 7.5 Torr) was performed on an additional 12 fish randomly selected from each treatment tank at 20 days of exposure. While control fish were able to maintain equilibrium in the water column for only about 5 h, animals fed a Cd-contaminated diet maintained equilibrium for more than twice as long (12.7 h), a highly significant difference (Fig. 4C). In turn, Cu-fed fish showed an intermediate tolerance of about 7 h (Fig. 4C).

In order to further understand the enhanced hypoxia tolerance displayed by fish fed a Cd-contaminated diet, several blood O_2 transport parameters were measured on controls and Cd-fed fish (Fig. 6). No significant differences were seen in the blood hematocrits and hemoglobin concentrations between control and Cd-fed fish (Fig. 6A,B). As shown in Fig. 6C, *Colossoma macropomum* have a very low P_{50} (about 8 Torr at pH 7.8) allowing them a high hypoxia tolerance, but the measured P_{50} and calculated Bohr coefficient were not different between control and Cd-fed fish (Fig. 6C,D).

Ammonia efflux rates (Fig. 7A) were similar in the three treatments under normoxic conditions (150–60 Torr). However, in moderate

Table 4
Bioaccumulation of copper in muscle, red blood cells, plasma, and brain of *C. macropomum* at 10–14 and 33–35 days of exposure to control, copper and cadmium contaminated diets. Data are means ± 1 SEM (n = 3–8). Different lower case letters indicate significant differences between fish fed a control diet at two different sampling times. Different upper case letters indicate significant differences between fish fed a contaminated diet at two different sampling times. Asterisks indicate significant differences between fish fed control and metal contaminated diets at the same sampling time. Copper and cadmium-contaminated diets were not compared with each other. [Two-way ANOVA results: (Muscle-Cu) interaction: p = .169 diet: p = .094, time: p < .001; (Muscle-Cd) interaction: p = .0006 diet: p = .002, time: p = .001; (RBC-Cu) interaction: p = .976 diet: p = .889, time: p = .006; (RBC-Cd) interaction: p = .004 diet: p = .006, time: p = .002; (Plasma-Cu) interaction: p = .077 diet: p = .923, time: p = .1649; (Plasma-Cd) interaction: p = .036 diet: p < .001, time: p = .300; (Brain-Cu) interaction: p = .066 diet: p = .1379, time: p = .861; (Brain-Cd) interaction: p = .002 diet: p = .003, time: p = .006].

	Days of Exposure	Copper (nmol Cu/g tissue)		Cadmium (nmol Cd/g tissue)	
		Control	Cu-fed	Control	Cd-fed
Muscle	10–14	1.28 ± 0.04^{a}	1.33 ± 0.13^{A}	0.03 ± 0.02^{x}	0.04 ± 0.01^{X}
	33–35	3.67 ± 0.18^{b}	4.19 ± 0.22^{B}	0.02 ± 0.00^{x}	$0.37 \pm 0.08^{Y*}$
Red Blood Cells	10–14	1.08 ± 0.10^{a}	1.14 ± 0.09^{A}	0.01 ± 0.01^{x}	$0.42 \pm 0.04^{X*}$
	33–35	2.39 ± 0.55^{b}	2.43 ± 0.36^{B}	0.11 ± 0.06^{x}	$3.43 \pm 0.59^{Y*}$
Plasma	10–14	3.16 ± 0.54^{a}	2.54 ± 0.35^{A}	0.01 ± 0.00^{x}	$0.06 \pm 0.01^{X*}$
	33–35	3.02 ± 0.22^{a}	3.71 ± 0.16^{A}	0.00 ± 0.00^{x}	$0.08 \pm 0.00^{Y*}$
Brain	10–14	25.57 ± 3.70^{a}	27.31 ± 4.35^{A}	0.08 ± 0.03^{x}	$0.22 \pm 0.04^{X*}$
	33–35	34.85 ± 6.67^{a}	19.57 ± 1.70^{A}	0.02 ± 0.01^{y}	$1.14 \pm 0.47^{Y*}$

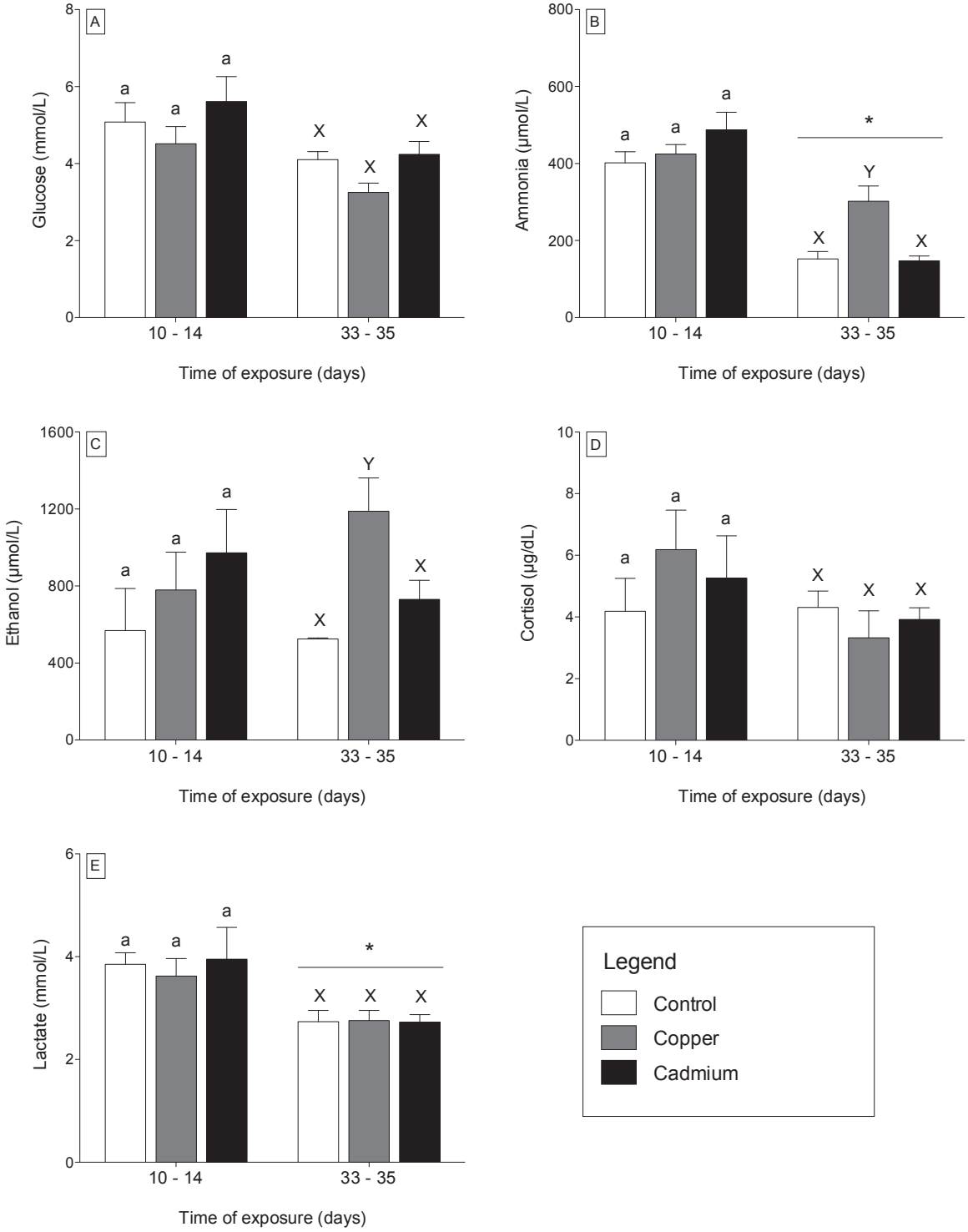


Fig. 3. Plasma concentrations of (A) glucose, (B) ammonia, (C) ethanol, (D) cortisol, and (E) lactate in *C. macropomum* at 10–14 and 33–35 days of exposure to control (white bars), copper (grey bars) and cadmium (black bars) contaminated diet. Data are means ± 1 SEM (n = 7–8). Means sharing the same lower case letters are not significantly different across treatments within the same sampling time (10–14 days). Means sharing the same upper case letters are not significantly different across treatments within the same sampling time (33–35 days). Asterisks indicate significant differences within a treatment between the two sampling times. [Two-way ANOVA results: (Glucose) diet: p = .05; time: p = .001; interaction: p = .890. (Ammonia) diet: p = .027; time: p < .001; interaction: p = .004. (Ethanol) diet: p = .041; time: p = .765; interaction: p = .160. (Cortisol) diet: p = .873; time: p = .099; interaction: p = .330. (Lactate) diet: p = .907; time: p = .004; interaction: p = .866].

hypoxia (60–30 Torr), both Cu- and Cd-fed fish exhibited significantly lower efflux rates of ammonia, when compared to controls, and also relative to their rates in normoxia. When fish were exposed to more severe levels of hypoxia (30–10 Torr) ammonia efflux rates were persistently lower in Cu and Cd-fed fish, while control fish did not significantly change their ammonia efflux rates under all three levels of O_2 in the water (Fig. 7A). Urea efflux rates were 5–10% of ammonia efflux rates under normoxia and were not affected by either dietary

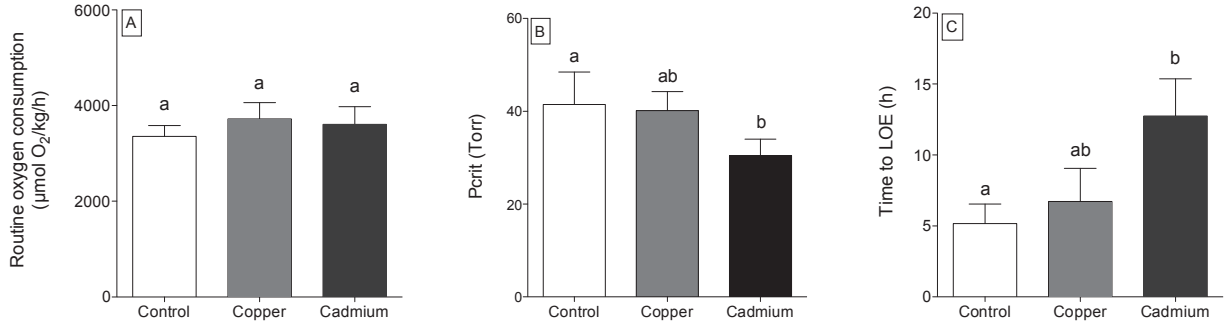


Fig. 4. Routine oxygen consumption rate (A), calculated P_{crit} (B) and time to loss of equilibrium (C) in *C. macropomum* at 18–20 days of exposure to control (white bars), copper (grey bars) and cadmium (black bars) contaminated diets. Data are means ± 1 SEM (n = 7–8 for panels A and B, n = 12 for panel C). Means sharing the same lower case letter are not significantly different from each other (independent one-way ANOVAs).

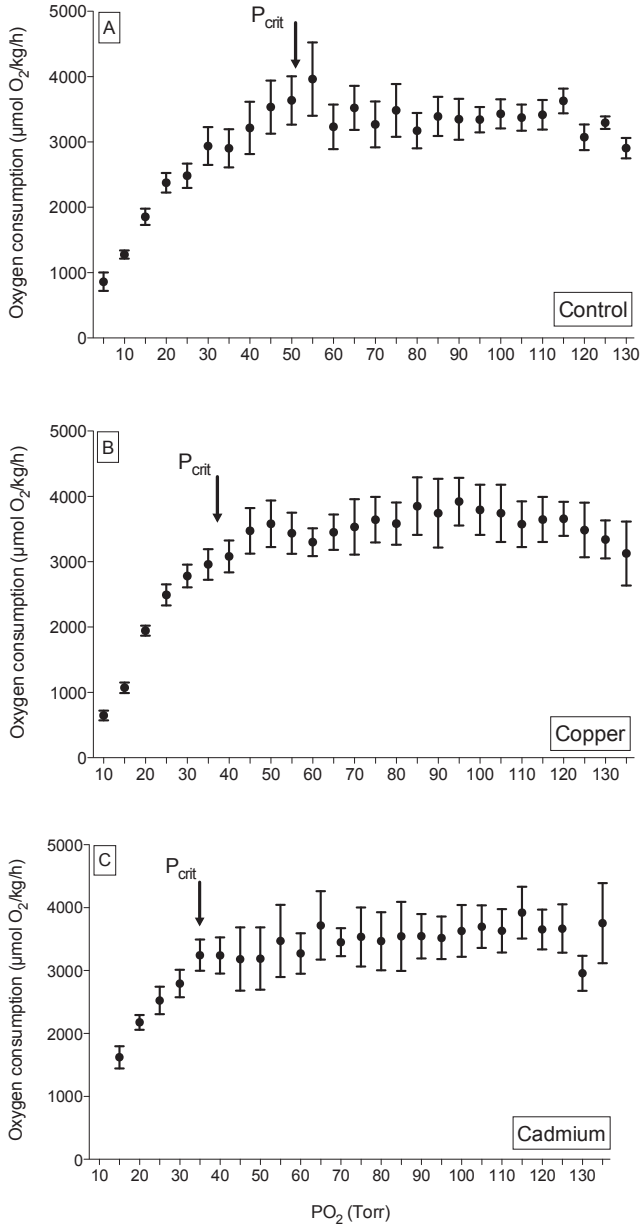


Fig. 5. Oxygen consumption over a range of PO_2 values and P_{crit} in *C. macropomum* at 18–20 days of exposure to (A) control, (B) copper and (C) cadmium contaminated diets. The mean P_{crit} values, calculated based on the averages for the individual fish in each treatment, are indicated with arrows. Data are means ± 1 SEM (n = 7–8).

treatment or by the level of hypoxia (Fig. 7B). Additionally, the calculated nitrogen quotients (NQ) were relatively high, indicating a 75–100% reliance on protein to fuel aerobic metabolism, and did not change over the different levels of O_2 tested for control and Cu-fed fish (Fig. 7C). The NQ of Cd-fed fish in moderate and severe hypoxia was lower than in normoxia, indicating a lower usage of protein (~60%) fueling aerobic metabolism (Fig. 7C).

In the loss of equilibrium test, as soon as each animal had turned over, a blood sample was taken and metabolite concentrations were measured in the plasma (Fig. 8). After the loss of equilibrium, both control and Cu-fed fish had significantly elevated levels of glucose by about 70% in the plasma, while Cd-fed fish did not show any increase relative to normoxic levels (Fig. 8A). Conversely, ammonia levels in the plasma did not change after exposure to hypoxia in any of the different dietary treatments (Fig. 8B). Plasma ethanol in hypoxia was reduced in metal-fed fish in comparison to controls, and while control fish exhibited approximately 50% increase with hypoxia exposure relative to normoxia levels, fish fed either of the metal contaminated diets displayed the opposite, where ethanol levels decreased by 30–40% (Fig. 8C). Additionally, all three groups of fish showed 8–10-fold elevations in plasma cortisol (Fig. 8D) and 2.5-fold elevations in plasma lactate (Fig. 8E) after exposure to hypoxia in comparison to normoxic levels (Fig. 3).

4. Discussion

4.1. Overview

In our study, fish were fed a metal spiked commercial diet for up to 35 days, where the aimed concentrations of both metals (500 μg metal/g food) were readily achieved (Table 1). Uneaten food was removed from the experimental tanks within 0.5 h after feeding, and faeces were syphoned daily to ensure that metal leaching to the water was minimal. Nevertheless, mean Cd levels in the water were significantly elevated (Table 1). The 96h-LC50 for this species is 38.5 mg/L (Salazar-Lugo et al., 2013), while in our experiment, the water Cd concentration was never higher than 10 μg/L, a difference of more than three orders of magnitude. Nevertheless, acute exposure of tambaqui to 10 μg Cd/L in this same water quality has been shown to inhibit waterborne Ca^{2+} uptake by 51% (Matsuo et al., 2005). Therefore, we cannot eliminate the possibility that waterborne exposure made a contribution to some of the observed effects of the Cd-contaminated diet (further addressed below). Although some reviews (*e.g.* Dallinger et al., 1987; Meyer et al., 2005; Grosell, 2012; McGeer et al., 2012; DeForest and Meyer, 2015) comparing experimental methodologies suggest different dietborne toxicity results between studies performed using spiked commercial feed versus contaminated live diet (mostly in terms of greater metal bioavailability in the latter), our results are valuable in terms of establishing a database of dietborne metal toxicity for tropical species.

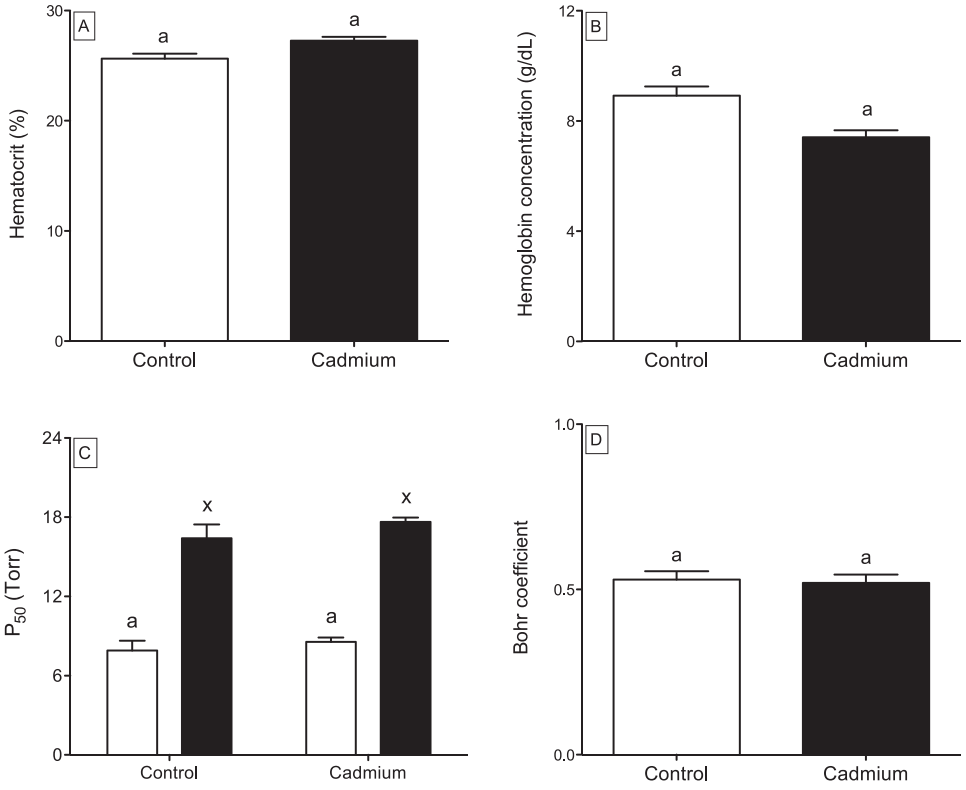


Fig. 6. (A) Hematocrit, (B) hemoglobin concentration, (C) whole-blood P_{50} values measured at pH 7.8 (white bars) and 7.2 (black bars), and (D) associated Bohr coefficients in *C. macropomum* at 20 days of exposure to control (white bars), and cadmium (gray bars) contaminated diets. Data are means ± 1 SEM (n = 5–8). Means sharing the same lower case letters are not significantly different from each other (unpaired Student *t*-tests).

Due to logistic restrictions, the sampling dates where some physiological parameters were measured (day 18–20: RMR and hypoxia tolerance; plasma metabolites) did not match the dates where fish were sampled for bioaccumulation (day 10–14 and 32–35). Those dates were chosen to be approximately halfway through the experiments, but nonetheless, care should be taken when comparing bioaccumulation data with physiological indicators of metabolic homeostasis. Another caveat of our study was the utilization of a single tank to house fish fed each of the different diets (total = 3 tanks). The experimental design utilized in our study can be defined as under-replicated according to Tincani et al. (2017), meaning that our experimental media (tanks: n = 1) were not replicated, while our experimental units (fish: n = 6–8

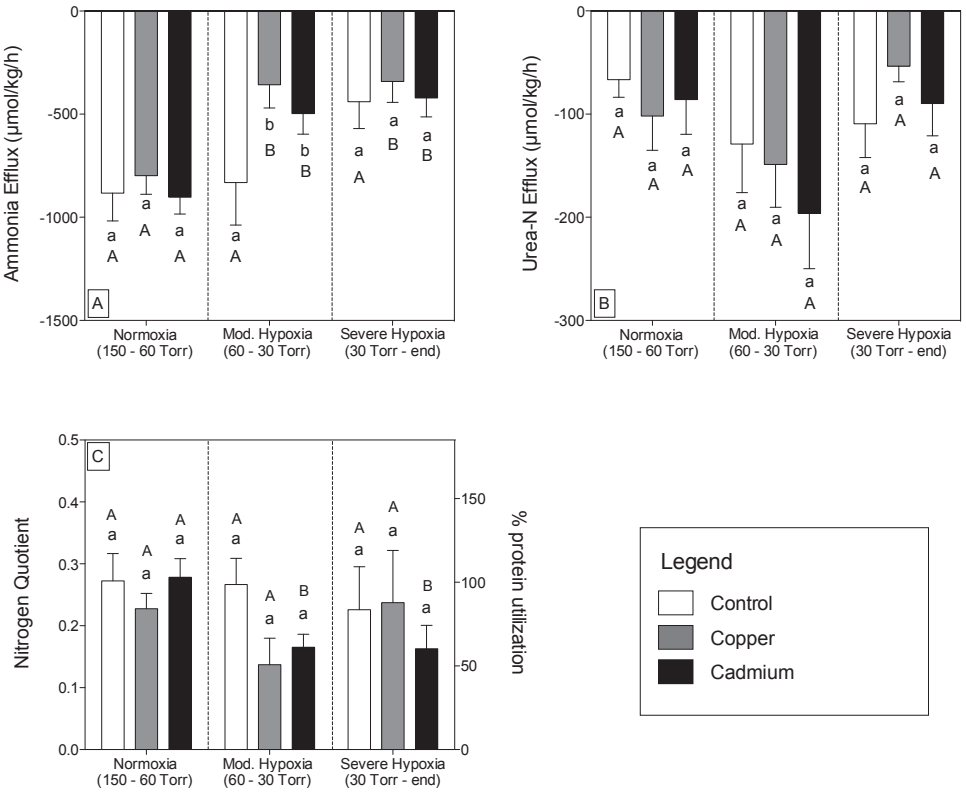


Fig. 7. Rates of (A) ammonia, and (B) urea-N efflux, and (C) calculated nitrogen quotients in *C. macropomum* under different oxygen tensions, measured at 18–20 days of exposure to control, copper, and cadmium contaminated diets. Data are means ± 1 SEM (n = 5–8). Lower case letters indicate significant differences between diet treatments at the same oxygen tension. Upper case letters indicate significant differences between the same diet treatments at different oxygen tensions. Means sharing the same letters are not significantly different from each other. [Two-way ANOVA results: (Ammonia) interaction: p = .432, diet: p = .095, oxygen: p < .001; (Urea) interaction: p = .656, diet: p = .720, oxygen: p = .029; (NQ) interaction: p = .428, diet: p = .237, oxygen: p = .012].

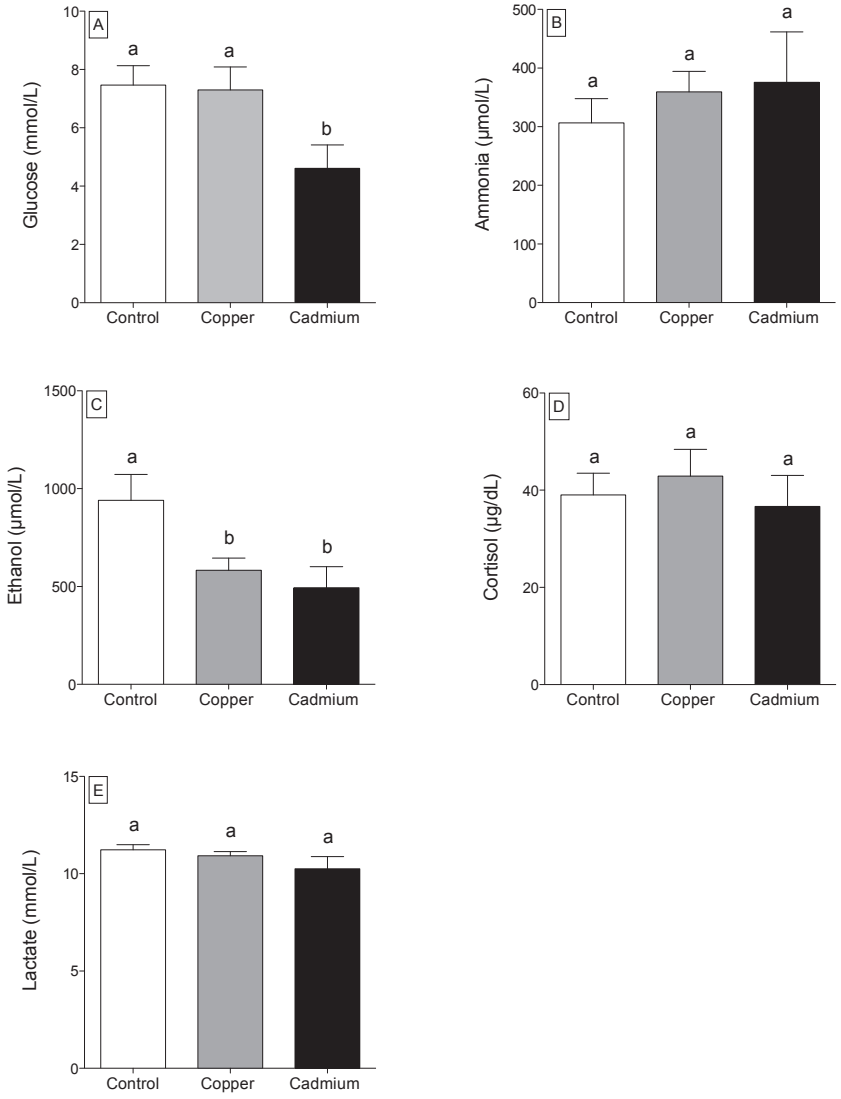


Fig. 8. The effect of severe hypoxia exposure (7.6 Torr − in the time to LOE test) on plasma concentrations of (A) glucose, (B) ammonia, (C) ethanol, (D) cortisol, and (E) lactate in *C. macropomum* at 20 days of exposure to control (white), copper (grey) and cadmium (black bars) contaminated diets. Data are means ± 1 SEM (n = 12). Means sharing the same lower case letters are not significantly different across diets (One-way ANOVA).

Table 5
Na^+/K^+-ATPase and H^+-ATPase activities in tissues of *C. macropomum* at 10–14 and 33–35 days of exposure to control, copper and cadmium contaminated diets. Data are means ± 1 SEM (n = 4–6). Means were not significantly different from each other within the same tissue when different dietary treatments at the same sampling time were compared (independent one-way ANOVA) or for the posterior intestine where the effects of both time and diet, and their interaction were not significant by independent two-way ANOVA. Different tissues were not compared against each other.

	Days of Exposure	Enzyme activity					
		Na^+/K^+-ATPase (µmol ADP/mg protein/h)			H^+-ATPase (µmol ADP/mg protein/h)		
		Control	Copper	Cadmium	Control	Copper	Cadmium
Gill	10–14	0.83 ± 0.15	0.98 ± 0.18	1.08 ± 0.19	1.33 ± 0.34	1.28 ± 0.24	1.18 ± 0.37
Anterior Intestine	10–14	2.61 ± 0.82	3.78 ± 0.70	2.03 ± 0.72	2.36 ± 0.81	2.41 ± 0.56	1.93 ± 0.73
Posterior	10–14	9.79 ± 3.45	7.92 ± 1.42	9.62 ± 1.66	1.41 ± 0.44	0.73 ± 0.16	1.09 ± 0.37
Intestine	33–35	7.77 ± 0.70	7.78 ± 1.09	6.75 ± 1.16	0.67 ± 0.29	0.87 ± 0.30	1.16 ± 0.31

for most measurements) were sufficiently replicated. Towards the end of the experiment, the number of fish per tank (biomass) changed differentially in the tanks, and we cannot rule out that the change in social setting could have resulted in different responses in some of the physiological parameters measured here.

We had hypothesized initially that exposure to dietary Cu would impact whole-body Na^+ regulation, while dietary Cd would impact whole-body Ca^{2+} regulation, as already seen in temperate fish species. That was not the case for either of the exposed groups, where plasma concentration of ions (Na^+, Cl^-, Ca^{2+} and K^+) were unaffected by the metal-contaminated diet and activities of important ionoregulatory enzymes in gills and intestine (Na^+/K^+-ATPase and v-type H^+-ATPase) also did not differ between control and metal-fed fish (Table 5). Our results support our second hypothesis that the patterns of tissue

bioaccumulation would differ between the two metals tested. Indeed the tissues that exhibited the highest metal burdens in response to the Cu-spiked diet were not the same as the ones most impacted in this manner by the Cd-spiked diet. Detailed results will be discussed below. Thirdly, we had hypothesized that dietary Cd would impact growth to a greater extent than dietary Cu. There were no differences in weight between the two metal contaminated diets, although our results indicated that different physiological strategies were probably employed in order to maintain body weight. Additionally, our results did not support our hypothesis that Cu would have a greater stimulatory effect on metabolic rate, since there were no differences in routine metabolic rate between control and either of the metal-fed fish. Lastly, we hypothesized that fish fed a Cu-contaminated diet would show an even more compromised hypoxia tolerance than fish fed with Cd-contaminated diet. This hypothesis was not supported. As measured by both the time to LOE and P_{crit} (widely used methods to determine hypoxia tolerance), surprisingly, control fish showed the lowest hypoxia tolerance, followed by Cu-fed fish. The fish that were fed a Cd-contaminated diet were the most hypoxia tolerant, with more than double the time to LOE than control fish, and this greater tolerance of hypoxia was reflected in several physiological parameters. We cannot disregard the fact that the doses of metal, which were the same on a mass basis, differed on a molar basis, and that this may have contributed to some of the differences observed. However it is notable that most of the marked responses occurred with Cd, which was presented at a molar dose only 60% that of Cu.

4.2. Whole animal effects

One of the primary observed effects of the metal contaminated diet was a decrease in appetite in Cd-fed fish. While fish that were fed a Cu-contaminated diet were eating similar daily amounts of food to the controls, Cd-fed fish ate progressively less during the experiment (Table 2). However there were no apparent changes in weight gain or food conversion efficiency, though statistical tests could not be done. This suggests that Cd could have been acting to suppress metabolism, and/or to directly affect hormones that regulate appetite and satiation in the brain. Alternately or additionally, food palatability may have been affected by Cd. Similar behaviour was observed when rainbow trout were exposed to sub-lethal waterborne levels of Cd for 35 days (McGeer et al., 2000). As these were in the same range as the waterborne Cd concentrations measured in the present study, these effects could have resulted from the waterborne component. In future studies, in addition to measuring hormones likely to affect anabolism or catabolism, it would also be valuable to measure changes in proximate composition (protein, lipid, glycogen content) in tissues of tambaqui.

4.3. Lack of ionoregulatory effects

Plasma concentrations of major ions (Na^+, Cl^-, Ca^{2+} and K^+; Fig. 1) were similar to previous values that we have measured in tambaqui obtained from the same source and held in the same water quality at INPA in Manaus (Wood et al., 1998; Duarte et al., 2010), but Cl^-, Ca^{2+} and K^+ concentrations were all higher than reported by Tavares-Dias and Moraes (2010) in tambaqui obtained from a farm in São Paulo and held in water of unstated ion composition. Presumably difference in animal history or water quality account for these differences.

Contrary to our initial hypothesis, neither of the metal-contaminated diets had any effects on the ionoregulatory status of the tambaqui as measured by the concentration of ions in the plasma. Since one of the most prominent toxic effects of waterborne Cu is the impairment of Na^+ regulation (Grosell, 2012), we had hypothesized that dietary Cu would affect plasma Na^+ concentration. Nevertheless, the lack of effects on plasma Na^+ concentration is consistent with a previous study on tambaqui showing low sensitivity of Na^+ regulation to waterborne Cu in this species (Matsuo et al., 2005), and more importantly with previous dietary Cu studies on the freshwater rainbow trout (Clearwater et al., 2000; Kamunde et al., 2001). Similarly, one of the most prominent toxic effects of waterborne Cd is the impairment of Ca^{2+} regulation (McGeer et al., 2012), and the tambaqui is very sensitive to this effect (Matsuo et al., 2005), so we had hypothesized that dietary Cd would affect plasma Ca^{2+} concentration. Indeed this did not occur, even though waterborne Cd concentration (Table 1) was also elevated to a level that might be expected to inhibit Ca^{2+} uptake in an acute exposure (Matsuo et al., 2005). Interestingly, the lack of observed effect is consistent with a previous study on trout fed with the same concentration of dietary Cd (Franklin et al., 2005). However, Pratap et al. (1989) reported a transient hypocalcemia and hypermagnesia in the Mozambique tilapia fed a diet contaminated with a two-fold higher Cd concentration than used in the present study. However, as early as 4 days of exposure, plasma ion levels had returned to normal. Our first sampling point at 10 days of exposure was beyond the reported time for effects in plasma (Pratap et al., 1989), suggesting that we may have overlooked short term deficits in Ca^{2+} regulation. Furthermore, we did not see any effects of either of the metal contaminated diets on the activity of key ionoregulatory enzymes (Na^+/K^+-ATPase and v-type H^+-ATPase) in the gills and two sections of the intestine (Table 5). Similar results were reported by Hoyle et al. (2007) on the African walking catfish exposed to a three-fold higher dietary Cu concentration, who found only a moderate and transient decrease of Na^+/K^+-ATPase activity in the posterior intestine. Our data on fluid transport rate across three sections of the intestine (Table 3) agrees with the recent study by Pelster et al. (2015) where the anterior section seems to be the region with slightly higher fluid transport activity, while there were no differences between the other sections. There were no effects of the Cu and Cd-contaminated diets on the fluid transport rates of the three sections of the intestine (a proxy for NaCl transport, Pelster et al., 2015), indicating a lack of overall effect on salt and water transport in the intestine of tambaqui. In summary, our study did not identify any effects of metal contaminated diets on the classical ionoregulatory endpoints often reported as impaired by waterborne exposure to metals.

4.4. Metal bioaccumulation

In comparison with the waterborne exposure route, far less is known about bioaccumulation in dietborne exposure studies. In our study we have done an extensive analysis of metal accumulation in eleven selected tissues in an attempt to establish tissue accumulation patterns for this species. The results will be discussed in terms of metal burden as well as exposure time. The gastrointestinal tract (GIT) is the initial metal uptake route in dietborne exposures. The GIT in tambaqui, an omnivore, is morphologically segmented into 4 sections: the stomach, anterior intestine, mid-intestine and the posterior intestine. Dietborne metals circulate from the GIT to the liver via the portal system before reaching other tissues (Campbell et al., 2002).

In our study, the accumulation of metals in the GIT varied greatly between metals, with the stomach being the GIT section that accumulated the most Cu (Fig. 2B), while in Cd-fed fish, it was the tissue that accumulated the least, whereas levels of both metals were fairly uniform in the other three sections. Surprisingly, the accumulation levels of both metals in the GIT tissues did not change between the two sampling times (approximately 20 days apart), in any of the four compartments, indicating either that some saturation of the metal storage capacity had been reached, or that the tissue has some capacity of regulating metal burden. In the freshwater rainbow trout, Cu can be taken up by both the gastric and the intestinal portions of the GIT (Nadella et al., 2006, Nadella et al., 2011), where it is dependent on the Na^+ concentration in the lumen, and the anterior region seems to be capable of transporting it at the highest rates (Nadella et al., 2006; Grosell, 2012). Cd uptake in the gut occurs in partial association with Ca^{2+} transport pathways, with marked differences amongst the

different regions (Klinck et al., 2009; Klinck and Wood, 2011; Klinck and Wood, 2013). It is important to point out that metal burdens in section-specific tissues of the GIT do not necessarily indicate the relative transport rates into the systemic tissues. For example, in the rainbow trout (unlike the tambaqui), Cu burdens in the four parts of the digestive tract were fairly uniform, but as noted above, Cu uptake rates were highest in the anterior intestine. Conversely, in trout Cd burdens in the stomach were lower than in the intestinal sections (as in the tambaqui), yet the stomach was the quantitatively most important section for uptake of dietary Cd (Wood et al., 2006; Klinck et al., 2009). Future studies should focus on the cellular and molecular details of Cu and Cd transport in the GIT of the tambaqui, and on the relative transport rates in the different sections.

Overall, the present data agree with previous studies on other species that used comparable or greater concentrations of Cd or Cu in the diet. The two organs outside of the GIT that accumulate the most metal during dietborne exposures are the liver and kidney (*e.g.* Harrison and Klaverkamp, 1989; Szebedinszky et al., 2001; Chowdhury et al., 2003; Kjoss et al., 2006), and this is likely due to these organs being important for metal detoxification. In a study investigating the elimination of Cd in the common carp, Cinier et al. (1999) concluded that the translocation from other tissues might explain the increase in liver Cd concentration. Additionally, Harrison and Klaverkamp (1989) found that Cd is more likely accumulated from dietary exposure rather than waterborne exposure. Cd concentrations accumulated in the tissues of tambaqui were relatively high when compared to values reported for rainbow trout (Handy, 1993 [1000 μg Cd/g dry food]; Szebedinszky et al., 2001 [1500 μg Cd/g dry food]; Baldisserotto et al., 2005 [300 μg Cd/g dry food]), and tambaqui (Matsuo and Val, 2007 [500 μg Cd/g dry food]). Muscle Cd burden was similar than the values reported by Matsuo and Val (2007). In the liver, Cd concentration was nearly ten times higher at 30 days of exposure when compared to 15 days, but the accumulation of Cu did not change with time (Fig. 2F). Quantitatively, the liver was the tissue that accumulated the highest levels of Cu, a pattern previously shown for the rainbow trout (Kamunde et al., 2002; Kjoss et al., 2006). Kamunde et al. (2002) reported that the liver bioaccumulation accounted for 60% of total body Cu accumulated of juvenile rainbow trout exposed to dietary Cu.

Although both metals accumulated in the muscle at only very low concentrations (Table 4), there was a significant increase from the first to the second sampling time. The rather negligible metal accumulations in the muscle is important due to the economic value of tambaqui as a highly prized species for human consumption. The metal accumulation in the plasma was also very low (Table 4), and seemed to follow the same temporal patterns as in the muscle. It is thought that Cd is transported in the bloodstream as free Cd ion or bound to detoxifying organic molecules such as metallothionein (MT) and glutathione (Zalups and Ahmad, 2003; McGeer et al., 2012). Exposure to dietary Cd has been shown previously to increase Cd burden in the RBCs (Chowdhury et al., 2004), which could be a result of MT synthesis in the RBCs. In our study, Cd accumulated at much higher levels in the RBC than in the plasma (Table 4), while the opposite was seen for Cu, which was regulated over time in both compartments while Cd was not. This pattern of regulation for Cu has been observed previously for the rainbow trout (Grosell et al., 1997), and it could be due to the essential nature of Cu as micronutrient and cofactor of many enzymes. Similarly, Cu was well-regulated in the brain of the tambaqui. It is known that Cd usually does not bioaccumulate at high levels in the brain of trout (Szebedinszky et al., 2001; McGeer et al., 2012). However, the small accumulated Cd levels observed in the tambaqui brain (Table 4) shows that Cd is capable of crossing the blood brain barrier, in contrast to the situation in trout.

4.5. Physiological parameters and hypoxia tolerance

Severe hypoxia is a phenomenon that can occur seasonally or even daily in some water bodies of the Amazon, so fish which inhabit those regions have evolved several strategies in order to cope with it (Val and Almeida-Val, 1995; Val, 1999). *Colossoma macropomum* has a remarkable ability to withstand hypoxia, utilizing behavioural, morphological, physiological and biochemical mechanisms in order to survive hypoxia in the wild (Saint-Paul, 1984; Val and Almeida-Val, 1995; Sundin et al., 2000). We had hypothesized that fish exposed to a metal contaminated diet would have shown a compromised hypoxia tolerance, but this did not occur, and indeed Cd-treated fish showed a greater hypoxia tolerance. We looked at a suite of metabolites in the plasma at two sampling periods (Fig. 3). In normoxia, glucose was decreased in Cu-fed fish at the later sampling point, perhaps indicating an interference of Cu with carbohydrate metabolism (Fig. 3A). However, glucose is not the only substrate utilized in order to fuel the aerobic metabolism, and in fact tambaqui rely primarily on protein and amino acid breakdown (Wood et al., 2017). Additionally, glucose is a sensitive marker for stress in fish, as its presence in the plasma can respond to both catecholamines and cortisol release in bloodstream (Iwama, 1998). When fish were exposed to hypoxia for several hours, control and Cu-fed fish upregulated glucose levels in the plasma, while Cd-fed fish did not (Fig. 8A), suggesting that the latter were less stressed by the hypoxia exposure. Significant similar increases in plasma cortisol during hypoxia were seen in all three groups, so differential cortisol mobilization does not explain the lack of plasma glucose mobilization in the Cd-group, and it could perhaps be due to a lack of catecholamine mobilization in this treatment. Additionally, catecholamine release could be more effective than cortisol in glucose mobilization in Amazonian tambaqui.

When the oxygen concentration in the blood becomes scarce, fish can rely on glycolysis to maintain energy production (Richards, 2011). In normoxia, there were no differences in plasma lactate levels between control and metal fed fish (Fig. 3E), but after exposure to severe hypoxia, all three groups showed a dramatic increase in plasma lactate (by approximately 2.5 fold), a response commonly seen in fish exposed to hypoxia (Almeida-Val and Farias, 1996). To date, only a few fish species have been shown to produce ethanol as an end product of anaerobic metabolism in an attempt to avoid the deleterious effects of lactate accumulation (goldfish: Shoubridge and Hochachka, 1980; crucian carp: Johnston and Bernard, 1983; desert pupfish: Heuton et al., 2015). In normoxia there were no clear differences in plasma ethanol between control and metal-fed fish (Fig. 3C), but surprisingly, when fish were exposed to severe hypoxia, both metal-fed groups significantly decreased plasma ethanol, compared to control fish (Fig. 8C). At this point, we cannot determine if this occurred because the metal-fed fish produced less ethanol during hypoxia, or they were more effective in excreting it, as ethanol appearance in the water was not measured. In fact, we can only be sure that tambaqui has the metabolic capacity to synthesize ethanol, but whether this is a beneficial adaptation to hypoxia and the mechanisms involved remains an interesting and exciting avenue for research.

In the wild, stressors will often happen in combination. Therefore, one of our goals was to evaluate how chronic exposure to a metal contaminated diet would affect the fish's ability to deal with hypoxia. The routine oxygen consumption (RMR) did not change with metal exposure (Fig. 4A), suggesting that the whole body aerobic metabolism was not being affected, different from what had been reported in previous studies with temperate species (McGeer et al., 2000; Couture and Kumar, 2003). The critical oxygen tension (P_{crit}), that is, the PO_2 at which the fish is no longer able to regulate oxygen consumption independent of environmental PO_2, is a widely used metric of hypoxia tolerance, indicating the capacity to regulate oxygen extraction from the environment (Yeager and Ultsch, 1989; Mandic et al., 2009; Rogers et al., 2016). The time to loss of equilibrium (LOE) test (Chapman et al., 1995) assesses the fish's ability to sustain an upright position in the water column in hypoxic conditions. A longer time is indicative of high hypoxia tolerance. The fact that the P_{crit} of Cd-fed fish was significantly lower than in control fish (Fig. 4B, Fig. 5) suggests an improved ability

to take up oxygen at decreasing PO_2s, though reduced metabolic demand may also be involved. That, along with a time to LOE twice as high as in control fish (Fig. 4C) evidences the remarkable hypoxia tolerance of Cd-fed fish. Glucose in the plasma remained unchanged (Fig. 8A) while ethanol was downregulated (Fig. 8C) after Cd-fed fish were exposed to severe hypoxia in comparison with normoxia levels, indicating that these two metabolites might play important roles in the hypoxia tolerance of these fish.

Most studies on the interaction of metal exposure and hypoxia show a compromised hypoxia tolerance with metal exposure (*e.g. Cyprinus carpio:* Hattink et al., 2005; *Fundulus heteroclitus*: Ransberry et al., 2016; *Pimephales promelas*: Bennett et al., 1995). We believe this is the first study to show a direct *in vivo* improvement of hypoxia tolerance in Cd-exposed fish. In light of the bioaccumulation of Cd (and not Cu) in tambaqui red blood cells (Table 4), we investigated the blood oxygen affinity (P_{50}, Fig. 6C), its responsiveness to blood pH (Bohr coefficient; Fig. 6D) and two metrics of blood oxygen capacity (hemoglobin and hematocrit; Fig. 6A, B). None of the measured blood parameters explained the increased hypoxia tolerance, so we suspect that a modification at the tissue metabolism level is responsible. Notably, the NQ of Cd-fed fish was also significantly lower during moderate and severe hypoxia (Fig. 7C). Tambaqui are unusual in having a very high NQ, indicating that protein/amino acids are the primary fuel burned in aerobic metabolism (Pelster et al., 2015; Wood et al., 2017). The reduction in protein oxidation is yet more evidence of a metabolic adjustment in the Cd-fed fish. In a study by Onukwufor et al. (2014), isolated mitochondria of rainbow trout treated with low concentrations of Cd showed an attenuation of the proton leak caused by cycles of hypoxia and reoxygenation. It is believed that proton leak at the mitochondrial level is the main source of reactive oxygen species production. Therefore, we speculate that mitochondrial aerobic metabolism may be benefiting from mild doses of Cd, allowing maintenance of ATP production down to lower O_2 tensions (Onukwufor et al., 2014). This should be evaluated in future *in vitro* studies on the respiration of isolated mitochondria from control and Cd-treated fish. Finally we note that waterborne Cd did not appear to have metabolic effects in trout (McGeer et al., 2000), so despite the elevation of waterborne Cd, the metabolic effects of Cd seen in the present study were likely due to the dietary Cd component.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2018.03.021.

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