Kidney activity increases in copper exposed goldfish (**Carassius auratus auratus**)

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Kidney activity increases in copper exposed goldfish (*Carassius auratus auratus*)

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Abstract

In the present study, the effect of copper was examined in the common goldfish (*Carassius auratus auratus*). Fish were fasted and exposed to either a high (0.84 µM), a low (0.34 µM) or a control copper concentration (0.05 µM) for 1 and 7 days. Swimming performance was not affected by either fasting or copper exposure. Food deprivation alone had no effect on ionoregulation, but low plasma osmolality levels and plasma Na^+ were noticed in fasted fish exposed to Cu for 7 days. Both gill Na^+/K^+-ATPase and H^+-ATPase activities were undisturbed, while both kidney ATPase activities were up-regulated when challenged with the high Cu levels. Up-regulated kidney ATPase activities likely acted as compensatory strategy to enhance Na^+ reabsorption. However, this up-regulation was not sufficient to restore Na^+ to control levels in the highest exposure group.

Key words: Shubunkin, ionoregulation, toxicity, swimming performance, osmolality, heavy metal, ATPase

1. Introduction

Copper is an essential nutrient for all living organisms and has numerous functions in cellular biochemistry (Burke and Handy, 2005). Increased use of heavy metals in anthropogenic activities during the last decades led to increased metal levels in aquatic environments worldwide, especially in mining and industrialised areas (Yang and Rose, 2003). The average copper level in lake and river water is 0.16 µM, but in contaminated water the concentrations can rise above 15.74 µM (ATSDR, 2004). In Flanders, as an example of an industrialised area, the average norm for environmental quality of surface waters is 0.11 µM dissolved copper, or 0.77 µM total copper. The goal to decrease copper concentrations in 2010 with a minimal of 75% compared to 1985, was not reached but between 2000 and 2010, average copper concentrations in surface waters have successfully decreased with 78% (MIRA, 2010). However, due to historic pollution, the norm for ground water quality of 1.57 µM is still occasionally exceeded (VMM, 2013). Besides industry, agriculture and aquaculture can also significantly contribute to surface water copper concentrations. Currently, increasing copper concentrations are often seen in aquaculture (Vutukuru et al., 2006) and they potentially can cause problems. Even sublethal concentrations of toxic substances can induce biochemical, physiological, morphological and genetic changes in aquatic organisms, depending on fish size and water composition (Wood, 2001). Furthermore, different fish species suffer in a varying degree from pollution. In the case of copper, gibel carp (*Carassius auratus gibelio)* appeared considerably less sensitive to aqueous Cu than common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). Based on LC50 values (96h), Cu was three times more toxic for rainbow trout (LC50: 3.3 µM) than for common carp (LC50: 10.4 µM), and seven times more toxic than for gibel carp (LC50: 22.0 µM) (De Boeck et al., 2004). It was suggested that the genus *Carassius* has a relative higher tolerance to copper compared to other freshwater species (De Boeck et al., 2004; Schjolden et al., 2007; Eyckmans et al., 2011).

Waterborne copper affects the gills of fish, the main location for gas and ion exchange, and causes mucus production, cell swelling and lifting of the epithelium (Wood, 2001). The gills are in continuous contact with the external environment and are thus a primary target for waterborne pollutants (Pandey et al., 2008). Fast copper accumulation in this organ precedes accumulation in other organs, such as liver, kidney and muscle (Grosell and Wood, 2002). This accumulation can lead to a number of adverse effects in gill tissue such as a disruption of the active uptake mechanisms for Na^+ and Cl^- (mainly through an inhibition of Na^+/K^+-ATPase activity), an increase in gill permeability, and oxidative stress (Eyckmans et al., 2010, 2011). Therefore, it has been suggested that the sodium turnover rate determines the sensitivity to lethal copper exposure (Grosell et al., 2002).

Additionally, copper is believed to inhibit ammonia excretion (Beaumont et al., 1995a,b, 2000a,b, 2003), by which increased ammonia levels can lead to reduced swimming capacity by depolarising muscle cells (McKenzie et al., 2003). A possible explanation for this is that copper may act to disrupt the enzymes (e.g. carbonic anhydrase) and /or transporters such as Na^+/K^+ ATPase involved the Na^+/NH_4^+ exchange mechanism (Zimmer et al., 2012). Another possibility is that copper can inhibit the bidirectional transport of ammonia by binding to peptide residues, suggesting Rh glycoproteins as potential targets for Cu toxicity (Lim et al., 2015). A recent study suggests that Rhcg-a transcript level declined following Cu exposure which might account for Cu induced ammonia efflux inhibition (Sinha et al., 2016) supporting the former. In a comparative study, it was seen that common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*) reacted to sublethal copper exposure (1µM) with an immediate decrease in swimming speed, while rainbow trout (*Oncorynchus mykiss*) displayed a delayed response, and clear increases in plasma and muscle ammonia were observed (De Boeck et al., 2006).

A wide range of toxicological responses to copper has been reported in several tissues (gills, liver, intestine and muscle) and plasma for various species (De Boeck et al., 2004, 2006, 2010; Ebrahimpour et al., 2011). However, not much is known about the effect of Cu toxicity at sublethal level on the kidney as secondary ionoregulatory organ. The present research was conducted to investigate the ionoregulatory responses in both gill and kidney of fish exposed to sublethal Cu concentrations. We chose to work with shubunkin (*Carassius auratus auratus)*, a goldfish variety, since the gibel carp, another *Carassius auratus* subspecies, showed the clearest increase in kidney Cu accumulation in contrast with common carp and rainbow trout (De Boeck et al., 2006). *Carassius auratus auratus* is the most common ornamental fish species worldwide and is thought to be the domesticated form of gibel carp, which is an invasive species in the waters of the Benelux. Furthermore, this species also provides an excellent research model to understand the response to environmental challenges (e.g. Lushchak, 2001; Sinha et al., 2012; Kong, et al., 2013).

In the present study, goldfish were exposed to either a high (0.84 µM) or a low (0.34 µM) sublethal copper concentration for 1 day or 7 days. The exposure doses were based on an earlier study (De Boeck et al., 1995), where these doses immediately decreased MO_2 in common carp, but allowed recovery at the lower exposure level. To reduce the effects of feeding on ammonia accumulation and excretion, experimental fish were fasted during the exposure, in parallel with fasted control fish. Our first aim was to investigate the effect of copper exposure on swimming performance. We hypothesised that, due to a decreased ammonia excretion, swimming capacity would be reduced at the high exposure concentration. A second aim was to examine the resulting plasma osmolality and Na^+ levels of fish exposed to different Cu levels. We expected to observe reduced plasma Na^+ levels, a reduced plasma osmolality and an increased plasma ammonia concentration. Finally, a third goal was to compare sublethal Cu effects on ionoregulatory responses in the gill and kidney of goldfish, by measuring Na^+/K^{+-}ATPase and

H^+-ATPase activity. An initial reduction of gill ATPase followed by a recovery was expected. We further hypothesised that kidney ATPase might be activated to compensate for the potential reduction in ionoregulatory capacities at the gills. Finally, it was expected that fasting would not have any significant effects on the measured parameters, as earlier studies in our lab showed that one week of fasting only had minor effects on goldfish (Liew et al., 2012, 2013).

2. Materials and methods

2.1. Fish maintenance

Goldfish, *Carassius auratus auratus*, of the shubunkin colour variety were obtained from a local fish supplier (Aqua Hobby, Heist op den Berg, Belgium). The fish were kept in the aquaria facilities of the laboratory of 'Systemic Physiological and Ecotoxicological Research' at the University of Antwerp with softened Antwerp City tap water (17°C, pH 8.2 ± 0.4, water hardness 297 ± 11 $mg.L^{-1}$ CaCO3, 33 $mg.L^{-1}$ Na, 44 $mg.L^{-1}$ Cl, 60 $mg.L^{-1}$ Ca, 7.1 $mg.L^{-1}$ Mg). Water quality was checked daily by using Standard Tetra Test Kits (Visocolor, Machery-Nagel, Germany) and values remained <0.1 $mg.L^{-1}$ of NH_3/NH_4^+; <0.03 $mg.L^{-1}$ of NO_2^- and <25 $mg.L^{-1}$ of NO_3^-. Water was filtered through biological filters containing wadding, lava stones (0.8-16.0 mm) and activated carbon (charcoal). About 80% of the water was replaced twice a week. Fish were fed at 2% body weight (BW) with commercial pellets ('Hikari Staple', Kyorin Food Ind. Ltd., Japan) twice a day.

One day before the experiment, 56 fish with a body mass of 12.18 ± 0.41 g (mean ± S.D.) were transferred from their maintenance tanks into a temperature-controlled room set at 17°C with a photoperiod of 14L:10D. Fish were randomly distributed into seven 50-60 L glass aquaria, filled with 40 L well aerated water, with a density of 8 fish per aquarium. A bio-filter, filled with lava stones and wadding, ensured the water quality and aeration. Black plastic shielding

minimized visual disturbance. Water quality was monitored as mentioned above and about 80% of the water was statically replaced every two days.

2.2. Experimental Design

The experimental setup consisted of 3 control groups: 1 fed control group and 1 fasted control group at day 1 and 1 fasted control group at day 7. Four exposed groups of goldfish were exposed to either 0.34 µM copper (LCu) or 0.84 µM copper (HCu) for 1 day or for 7 days and fish were fasted during the exposure. A copper sulphate solution ($CuSO_4.5H_2O$ RPL, Leuven, Belgium) was added to the water of the experimental groups. After each water replacement the water was again spiked with copper stock solution. Copper concentrations (measured by High Resolution Inductively Coupled Plasma Mass Spectrometer ICP-MS, Element XR, Thermofisher Scientific, Bremen, Germany) were monitored daily and were on average 0.05 ± 0.01 µM (control), 0.34 ± 0.02 µM (LCu) and 0.84 ± 0.19 µM (HCu).

2.3. Critical swimming speed, U_{crit}

After 1 and 7 days, U_{crit} of both exposed and control groups was determined. Eight fish from the same group were placed 14h prior to the experiment in individual separate Blazka-style swimming respirometers with a known volume (≈ 4.1 L). During this acclimatization period, fish were oriented using a water velocity of 10 $cm.s^{-1}$. The head tank provided a continuous flow of air-saturated water through each flume at a rate of 4 $L.min^{-1}$. Water had the same copper concentration as during the exposure period. The total content of the recirculation system was approximately 450 L. After the acclimatization period, water velocity was increased with 5 $cm.s^{-1}$ every 20 minutes, until fish fatigued. Fatigue was determined by the fact that fish could no longer maintain position against the current and were swept and held against the mesh screen. Then, speed was briefly lowered to allow fish to restart swimming. Fish were considered

totally fatigued when they were swept downstream for the second time within the same 20 min interval (Tudorache et al., 2007). At this point, the performance test was terminated. U_{crit} was calculated as $U_{crit} = U_i + [U_{ii}(T_i/T_{ii})]$, where U_i is referred as the highest velocity sustained for the whole interval, while U_{ii} is the velocity increment (5 $cm.s^{-1}$), T_i is the time elapsed at fatigue velocity and T_{ii} is the interval time (20 min) (Brett, 1964; De Boeck et al., 2006). The absolute values ($cm.s^{-1}$) were converted to relative swimming speeds in body lengths per second ($BL.s^{-1}$) by factoring the absolute values with body length (fork length). The biometric parameters body mass and fork length did not significantly differ between groups (Table2).

2.4. Sampling procedure

Immediately after the swimming challenge, fish were anaesthetized with 0.5 $g.L^{-1}$ ethyl-3-aminobenzoate methanesulfonic acid (MS-222, Acros 197 Chemicals, Geel, Belgium) neutralised with NaOH (Merck Eurolab nv/sa, Leuven, Belgium). Fish were blotted dry, measured and weighed. Physical parameters were measured individually and the condition factor was calculated as $K = (BW/BL^3) \times 100$. Before decapitation, a blood sample was taken from the caudal blood vessel using a heparinised syringe (heparin from Sigma-Aldrich, co, St. Louis, 200 USA). Blood was immediately centrifuged for 1 minutes at 13,200 rpm at 4°C. Plasma was carefully pipetted into cryogenic vials and frozen in liquid N_2. Subsequently, fish were killed by severing their spinal cord prior to organ sampling. Gills, liver, kidney and white muscle tissues were excised on ice. The whole liver mass was recorded and the hepatosomatic index was calculated as HSI = (LM/BW) x 100, where LM is referred as liver mass. Collected tissues were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

2.5. Biochemical analysis

Plasma osmolality was determined by using The AdvancedTM Micro Osmometer (Model 3300, Advanced Instruments, USA). The diacetyl monoxime assay (Price and Harrison, 1988) was used to measure plasma urea. For measuring plasma ammonia concentrations, a commercial Enzymatic Kit (R-Biopharm AG, Darmstadt, Germany) was used. Plasma Na^+ concentrations were analysed using an Electrolyte Analyser 9180 (AVL Scientific corporation, GA, USA).

To determine muscle water content (MWC), muscle tissue was defrosted, weighed and dried in the oven at 60°C for 48 h. Then the tissue was transferred into a desiccator for 1 h, allowing it reaching room temperature without absorbing moisture, and weighed again. MWC (%) was determined as the percentage of weight loss. Na^+/K^+-ATPase activity in gill and kidney samples were measured using the McCormick method (1993), while the method of Lin and Randall (1993) was used to measure H^+-ATPase activity in gill and kidney samples. Tissues were homogenized with ice cooled 4:1 SEI/SEID buffer solution (150mM sucrose; 10mM EDTA; 50mM imidazole/SEI with 0.1% sodium deoxycholate) and centrifuged (5000 g; 4°C; 1 min). Duplicate 10 µl homogenates were pipetted into a 96-well microplate in four series. 200 µl of mixture assay-A was added to the first series (400U lactate dehydrogenase; 500U pyruvate kinase; 2.8 mM phosphoenolpyruvate; 0.7 mM ATP; 0.22 mM NADH; 50 mM imidazole) and 200 µl of mixture assay-B was added to the second series (mixture assay-A with 0.4 mM ouabain) for Na^+/K^+-ATPase activity measurement. For measurement of H^+-ATPase activity, 200 µl of mixture assay-C was added to the third series (mixture assay-B with 500 mM NaN3) and 200 µl of mixture assay-D was added to the fourth series (mixture assay-C with 100 mM NEN). The enzyme activities were measured kinetically with a plate reader (ELX808IU Bio-Tek Instruments Inc. VT, USA) at 340 nm for 30 min at 30 s intervals. An ADP standard curve was used to calculate ATPase activity by subtracting oxidation rate of NADH to NAD in the absence (assay A,C) or in the presence (assay B,D) of ouabain for Na^+/K^+-ATPase activity and

NEN for H^+-ATPase. Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard, at 595 nm (US Biochemical, Cleveland, OH, USA).

2.6. Statistical analysis

All data are presented as mean values ± standard error (SEM). Data were analysed with the statistical program 'R', version 3.1.2, with a 5% level of significance. Normality was checked by the Shapiro–Wilk test. The Bartlett test was used to verify the homogeneity of variances. If the requirements for ANOVA were not fulfilled, a log-transformation or (reciprocal) square root transformation of data was applied. Overall, the main effects of copper and exposure time and their interaction were analysed by a multivariate analysis of variance (MANOVA) (Table 1). Subsequently, Bonferroni's multiple comparisons test was used to determine the differences between groups. Data were compared to the control and the corresponding fasted non exposed group. The Bonferroni correction was performed with the statistical program 'GraphPad Prism' (GraphPad Software Inc., CA, USA).

3. Results

Overall, only copper treatment had a main significant effect ($P<0.001$).

3.1. Hepatosomatic index, K-factor, MWC, U_{crit}

Neither copper exposure, nor exposure time had a significant effect on K-factor, MWC and U_{crit} (Tables 1, 2 and 3). Fasting decreased the hepatosomatic index after 7 days ($P<0.05$) compared to the fed control. The interaction between treatment and time only had a significant effect on MWC ($P<0.05$) (Table 1).

241 *3.2. Biochemical analyses*

242 *3.2.1. Plasma values*

243 Plasma urea and plasma ammonia were not significantly affected by copper treatment nor by
244 exposure time (Table 1, Figs 1, 2). Fish exposed for 7 days to low copper concentration had
245 about 10% lower plasma osmolality compared to fed (P<0.05) and fasted controls (P<0.01)
246 (Fig.3). Plasma Na^+ concentration of fish exposed to LCu for 7 days and to HCu for both 1 day
247 and 7 days, were respectively 10.9% (P<0.05), 10.7% (P<0.05) and 15.1% (P<0.001) lower
248 compared to the fed control group (Fig.4), while fasted control fish did not show a significant
249 difference with the fed control. Exposure time had a significant effect on osmolality, while
250 treatment had a significant effect on both osmolality and plasma Na^+ concentration (Table1).

251

252 *3.2.2. Enzyme activity*

253 No significant difference was observed for Na^+/K^+-ATPase activity and H^+-ATPase activity in
254 gills (Fig.5A,B).

255 Copper increased Na^+/K^+-ATPase activity in kidney of copper exposed fish (Fig.5C). Kidney
256 Na^+/K^+-ATPase activity after 1 day of exposure of the HCu group was respectively 113.6%
257 (P<0.01) and 110.1% (P<0.001) higher than of fed and fasted control fish. Copper also induced
258 a significant increase in kidney H^+-ATPase activity of fish after 1 day exposure to the highest
259 concentration. The enzyme activity was 164.2% (P<0.001) and 165.7% (P<0.001) higher
260 compared to respectively the fed and fasted control group (Fig.5D).

261 Treatment had a significant effect on Na^+/K^+-ATPase and H^+-ATPase activity in kidney, but
262 not in gills. The interaction between treatment and exposure period also had a significant effect
263 on Na^+/K^+-ATPase and H^+-ATPase activity in kidney (Table1).

264

265 **Discussion**

We studied the effect of sublethal Cu exposure, using copper concentrations similar to those found in surface waters. Our study confirms that even at low concentrations the osmoregulation was disturbed. After copper exposure, plasma osmolality and plasma Na^+ concentrations were reduced, while both kidney ATPase activities were upregulated. Both gill Na^+/K^+-ATPase and H^+-ATPase activities were undisturbed. Copper exposure was relatively short, therefore almost no significant effects were observed on HSI or K-factor. In contrast to our original hypothesis, no significant effect on U_{crit} was observed.

Copper exposure is known to inhibit sodium influx by inhibiting the Na^+/K^+-ATPase, resulting in reduced plasma Na^+ levels, by nonspecific binding to thiol groups on the subunits of the transporter and also by binding to the Mg^{2+} binding site (Laurén and McDonald, 1987; Li et al., 1998), plus competitive inhibition at the apical Na^+-channel (Grosell and Wood, 2002; Pyle and Wood, 2008). In line with this, the reduced plasma osmolality (7 days) and Na^+ levels (1 and 7 days) in our study indicate a disruption of the iono- and osmoregulatory capacity. Since water content of the tissues remained unaffected, the osmoregulatory disturbance was limited. Eyckmans and co-workers (2010), who used a similar copper concentration of 0.8 µM, found a short lived decreasing gill Na^+/K^+-ATPase activity at 12h of exposure in gibel carp followed by decreasing plasma sodium concentrations starting only from 3 days onward, while effects in rainbow trout were more prominent in the first days of exposure. In the present study, gill Na^+/K^+-ATPase in goldfish remained largely undisturbed, although an early short lived disturbance (e.g. 12h) might have been missed. Nevertheless, plasma Na^+ was reduced, in accordance with the effects seen in gibel carp (Eyckmans et al., 2010). However, other mechanisms caused by Cu exposure might alter Na^+ fluxes as well such as increases of sodium loss caused by a displacement of calcium by copper in the tight junctions, leading to changes in permeability (Laurén and McDonald, 1985; Handy et al., 2002; Niyogi et al., 2006). Another possible cause of reduced Na^+ uptake could be inhibition of H^+-ATPase activities. In the apical

membrane, H^+-ATPase activities play an important role in Na^+ uptake, as Na^+ uptake through the apical Na^+ channel (ENaC) is thought to be coupled to electrogenic proton extrusion generating an electrical gradient (Evans et al., 2005). However, the numerical reduction in gill H^+-ATPase activity at 7 days HCu showed not to be significant.

Our study showed that kidney ATPase activities were activated under Cu exposure. This presumably is to counteract reduced Na^+ uptake at the gills by increasing Na^+ reabsorption from the urine. It was observed before that in rainbow trout exposure to both long (28d) and short (3d) term acclimation to low levels of waterborne Cu (20 $\mu g.L^{-1}$ ~ 0.31 $\mu mol.L^{-1}$), led to a 40-48% reduction in renal Na^+ losses (Grosell et al, 1998). This occurred through a combination of reduced urine flow rate and urinary Na^+ content, indicating increased reabsorption of Na^+ and adaptation of tubular function within the first 3 days of Cu exposure. Na^+/K^+-ATPase plays a pivotal role in renal Na^+ reabsorption, therefore the increased Na+/K+-ATPase activity suggests a comparable tendency for reduced urinary Na^+ losses under Cu exposure in goldfish. Interestingly, a similar pattern was observed for both kidney Na^+/K^+-ATPase and H^+-ATPase activity, with increased ATPase activities for both transporters at the high copper level after 1 day. Although kidney ATPase activities likely acted as compensatory strategy to enhance Na^+ reabsorption, this was not sufficient to restore Na^+ to control levels in the highest exposure group. Overall, plasma Na^+ levels of fasted groups were slightly lower than those of fed fish, but this was not reflected in plasma osmolality or ATPase activities.

No statistically significant treatment effect on U_{crit} was observed which was in contrast with our original hypothesis. In an earlier study on rainbow trout, common and gibel carp exposed to 1 µM of Cu (De Boeck et al., 2006) both trout and common carp showed extended reductions in U_{crit} up to 7 days while in gibel carp a limited reduction in U_{crit} was seen only within the first 12 hours after copper exposure. After 24 hours of exposure, the gibel carp had almost recovered and no further reduction in swimming performance was observed, similar to what we noticed

in goldfish in the present study. Reductions in swimming capacity under Cu exposure are believed to be attributed to increased ammonia levels, with closer correlations to plasma ammonia rather than muscle ammonia, as it is the distribution of ammonia between intra- and extracellular compartments that causes depolarisation of the muscle cells (Beaumont et al., 1995a,b, 2000a,b; McKenzie et al., 2003, De Boeck et al., 2006). However, in the present study plasma ammonia and plasma urea were not significantly increased, which likely explains why swimming capacity remained unaffected.

Cu levels in aquatic environments can run as high as 15 µM. Our exposure concentrations are much lower, albeit above the average concentration of 0.16 µM. Overall, we conclude that at these ecologically relevant concentrations osmoregulation in the relatively robust goldfish is still disturbed. Although increased renal Na^+/K^+-ATPase and H^+-ATPase activities likely supported increased renal Na^+ reabsorption in goldfish in an attempt to counteract ion loss, this upregulation did not suffice to restore plasma Na^+ or osmolality within the first week of exposure.

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464

Tables

Table1: Effect of treatment (exposure to low (0.34 µM) or high (0.84 µM) copper concentrations) and exposure time (1 or 7 days) and their interaction on physiological parameters of goldfish.

	Treatment		Time		Treatment x time	
	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value
HSI	3.619	**0.021**	1.697	0.200	0.509	0.678
K-factor	0.172	0.915	1.512	0.226	0.308	0.819
MWC	0.356	0.785	0.022	0.884	2.955	**0.044**
Ucrit	0.768	0.519	0.253	0.618	0.632	0.559
Plasma urea	0.591	0.625	0.052	0.821	0.807	0.498
Plasma ammonia	0.822	0.489	0.649	0.425	1.523	0.223
Osmolality	9.113	**0.000**	4.523	**0.040**	1.090	0.364
Sodium	10.096	**0.000**	1.010	0.321	0.877	0.461
Na+/K+-ATPase gill	2.364	0.085	1.160	0.288	2.424	0.080
H+ATPase gill	1.363	0.268	1.613	0.211	0.937	0.432
Na+/K+-ATPase kidney	6.420	**0.001**	0.230	0.634	3.229	**0.032**
H+ATPase kidney	23.442	**0.000**	0.009	0.926	4.262	**0.011**

Table2: Biometric data of goldfish exposed to low (LCu, 0.34 µM) or high (HCu, 0.84 µM) copper concentrations. Values are mean ± SEM. Asterisks denote significant differences ($P < 0.05$) compared to the fed control (*). No significant differences were found compared to the fasted control.

Treatments		Body mass (g)	Fork length (cm)	K-factor	HSI
Fed control		12.54 ± 1.63	8.7 ± 0.4	1.88 ± 0.16	5.49 ± 0.29
Fasted control	1 day	12.41 ± 0.78	8.5 ± 0.2	2.02 ± 0.15	3.20 ± 0.60
	7 days	10.85 ± 1.05	8.6 ± 0.2	1.69 ± 0.08	2.22 ± 0.30 *
LCu	1 day	13.28 ± 1.08	8.0 ± 0.7	2.05 ± 0.15	5.14 ± 1.49
	7 days	12.05 ± 0.87	8.8 ±0.2	1.75 ± 0.06	2.75 ± 0.49
HCu	1 day	13.30 ± 0.87	8.7 ±0.3	2.05 ± 0.13	4.12 ± 0.94
	7 days	10.88 ± 1.08	8.3 ± 0.3	1.92 ± 0.15	2.24 ± 0.24

Table3: MWC and U_{crit} of goldfish exposed to low (LCu, 0.34 µM) or high (HCu, 0.84 µM) copper concentrations. Values are mean ± SEM. No significant differences were found.

Treatments		Ucrit (BL/s)	MWC (%)
Fed control		5.92 ± 0.52	34.35 ± 1.60
Fasted control	1 day	6.77 ± 0.27	33.66 ± 0.83
	7 days	6.42 ± 0.53	34.17 ± 1.82
LCu	1 day	5.57 ± 0.62	30.26 ± 1.64
	7 days	6.09 ± 0.40	34.55 ± 1.08
HCu	1 day	5.98 ± 0.37	33.97 ± 1.56
	7 days	6.80 ± 0.44	31.86 ± 1.70

Figures

Fig1. Plasma urea accumulation of goldfish exposed to low (LCu, 0.34 µM) or high (HCu, 0.84 µM) copper concentrations. Values are mean ± SEM. No significant differences were found.

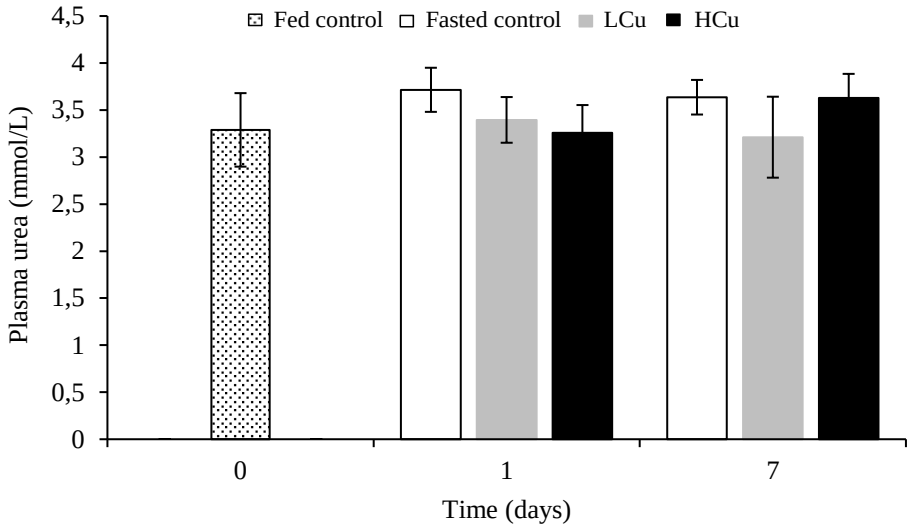
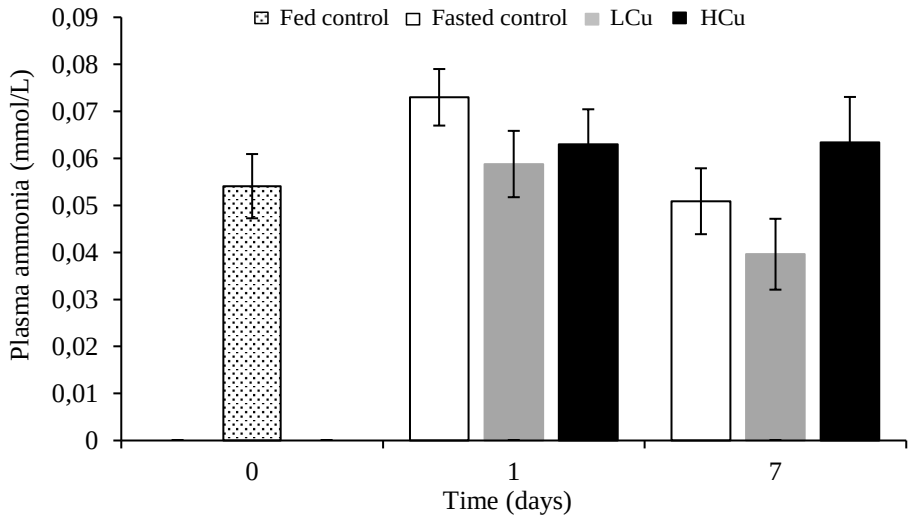


Fig2. Plasma ammonia accumulation of goldfish exposed to low (LCu, 0.34 µM) or high (HCu, 0.84 µM) copper concentrations. Values are mean ± SEM. No significant differences were found.



509

510

511 **Fig3.** Osmolality of goldfish exposed to low (LCu, 0.34 µM) or high (HCu, 0.84 µM) copper
512 concentrations. Values are mean ± SEM. Different symbols denote significant differences (P <
513 0.05) between fed control (*) or fasted control (†).

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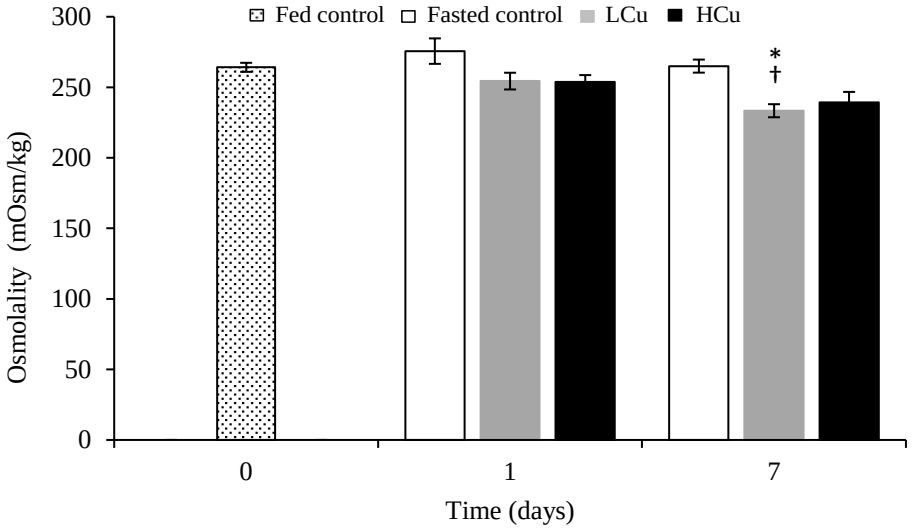

516

517

518 **Fig4.** Plasma sodium of goldfish exposed to low (LCu, 0.34 µM) or high (HCu, 0.84 µM)
519 copper concentrations. Values are mean ± SEM. Asterisks denote significant differences (P <

520 0.05) compared to the fed control (*). No significant differences were found compared to the
521 fasted control.
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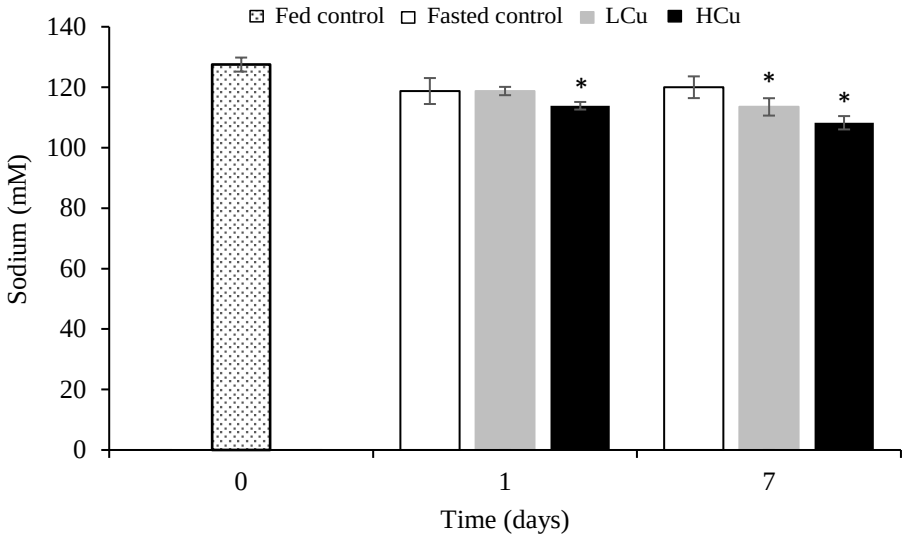
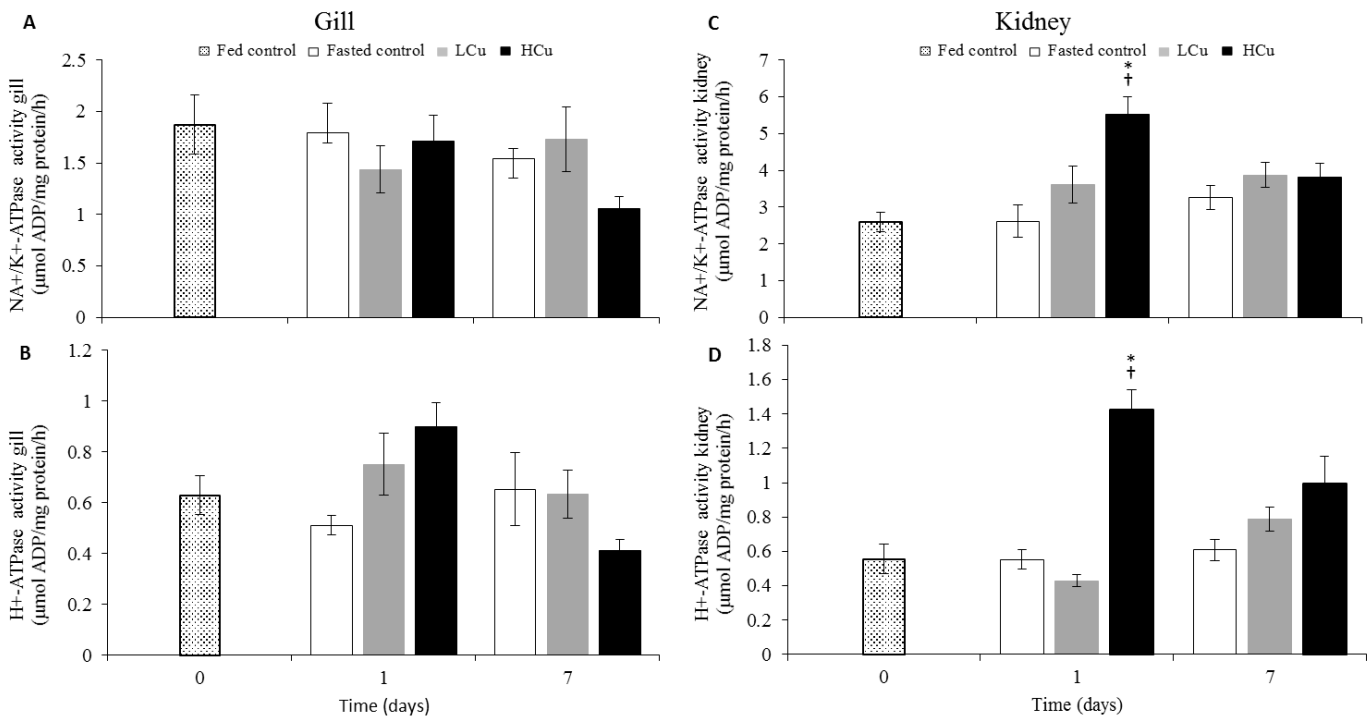


524

525 **Fig5.** Enzyme activity; Na^+/K^+-ATPase in gill (A), H^+-ATPase in gill (B), Na^+/K^+-ATPase in
526 kidney (C), H^+-ATPase in kidney (D) of goldfish exposed to low (LCu, 0.34 µM) or high (HCu,
527 0.84 µM) copper concentrations. Values are mean ± SEM. Different symbols denote significant
528 differences ($P < 0.05$) between fed control (*) or fasted control (†).
529
530


A
Gill
Fed control
Fasted control
LCu
HCu
NA+/K+-ATPase activity gill (µmol ADP/mg protein/h)
B
H+-ATPase activity gill (µmol ADP/mg protein/h)
Time (days)
C
Kidney
NA+/K+-ATPase activity kidney (µmol ADP/mg protein/h)
D
H+-ATPase activity kidney (µmol ADP/mg protein/h)
Time (days)

531