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Tissue-Specific Metabolic Responses of *Cyprinus flammans* to Copper

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Abstract Copper (Cu) contamination is serious in China, with ≤2.76 mg/L in some waters. Exposure to Cu causes a high toxicity to the aquatic organisms and subsequent ecological risk. To understand fish responses to Cu exposure, we analyzed the metabonomic changes in multiple tissues (gill, liver, and muscle) of *Cyprinus flammans* using an nuclear magnetic resonance-based metabonomic technique. Our results showed that metabolic alterations are dose-dependent. No significant metabolic alterations in three tissues of fish are caused by 0.25 mg/L Cu. However, 1.53 mg/L Cu caused changes of energy-related metabolites and amino acids, which we suggest are due to enhanced metabolic acidosis in gill and muscle, decreased tricarboxylic acid cycle activity in muscle, increased gluconeogenesis from amino acids in liver, and improved glycogenesis in liver and muscle. The Cori cycle between liver and muscle is concurrently triggered. Furthermore, high concentration of Cu resulted in the alteration of choline metabolism such that we hypothesize that Cu induces membrane damage and detoxification of $CuSO_4$ in gill as well as altered osmoregulation in all three tissues. Choline-*O*-sulfate in gill may be used as a biomarker to provide an early warning of Cu exposure in *C. flammans*. Moreover, Cu exposure caused alterations of nucleoside and nucleotide metabolism in both gill and muscle. These findings provide a new insight into the metabolic effects of Cu exposure on *C. flammans* and highlight the value of metabonomics in the study of metabolic metal disturbance in fish.

Copper sulfate ($CuSO_4$) is one of the most widely used algicides and herbicides in aquaculture systems for the control of algal blooms and infestations of aquatic macrophyte (Effler et al. 1980). The introduction of Cu into aquatic ecosystems is increasing as a result of industrial and agricultural practices. As a top consumer of Cu in the world, China is facing incredibly heavy Cu contamination. The largest dissolved concentration of Cu in southern Bohai Sea is 2755 μg/L, which is far greater than concentrations measured in general heavily contaminated rivers. Although Cu is an essential trace element, which serves as a cofactor for a variety of metalloenzymes (Harris 2000), high levels are acutely toxic for aquatic organisms. For instance, exposure of fish to 0.25–4.0 mg/L $CuSO_4$ concentrations in the water can lead to gill lesions, including epithelial hyperplasia, subepithelial edema, and curling of secondary lamellae, as well as mucus and chloride cell damage (Karan et al. 1998).

Conventional biochemical studies have shown that the toxicity of Cu is related to changed activities of enzymes and ionoregulatory disturbances. After a 14-day exposure to 0.5–4.0 mg/L of $CuSO_4$, an increase was observed in the activities of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase in the blood serum and gills of *Cyprinus carpio* (Karan et al. 1998). In addition in *C. carpio*, 1.9 μM of Cu exposure induces decreasing Na^+-K^+-ATPase activity (De Boeck et al. 2001). Furthermore, proteomics research indicates that Cu induces increased expression of phenylalanyl-transfer RNA synthetase, transcription factor

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protein, and a cold-inducible RNA-binding protein (Eyckmans et al. 2012). Such toxic effects of Cu exposure appear to be related to the generation of oxidative stress. This is because the cytoskeleton, considered as an early target of reactive oxygen species, is disrupted in the hepatocytes and gills of *C. carpio* after Cu exposure (Nawaz et al. 2005; Eyckmans et al. 2012). As a result, Cu exposure causes alterations in metabolic processes such as ammonia accumulation (Hashemi et al. 2008; Kunwar et al. 2009), increased level of glucose (Laurén and McDonald 1985), and altered levels of adenosine triphosphate, adenosine diphosphate, phosphocreatine, and inorganic phosphate (Heath 1991; De Boeck et al. 1997). Despite these metabolic data on Cu toxicity, clarity is still needed on the systematical investigation on the other Cu-induced metabolic alterations in fish.

Metabonomics is of great value for toxicological studies, particularly for the identification of potential biomarkers of contaminant exposure (Lindon et al. 2000; Coen et al. 2003). The widespread application of nuclear magnetic resonance (NMR)-based metabonomics has provided a comprehensive understanding of the impacts of toxicants on aquatic organisms including mussels (Wu and Wang 2010, 2011), abalone (Shofer et al. 1997; Martello et al. 1998); (Tracey et al. 2010), and marine fish (van Scoy et al. 2010; Samuelsson et al. 2006). NMR enables the rapid and reproducible detection of metabolite, and is rich in structural information (Zhang et al. 2011). The method has provided important information on mechanisms of toxicity in organisms and on candidate biomarkers.

In the present work, we investigated the effects of acute Cu treatment on metabolism in the multiple tissues of fish, including gill, liver, and muscle, using NMR spectroscopy coupled with multivariate data analysis. The objectives were to obtain information on metabolic responses of multiple tissues of fish to acute Cu exposure and to further our understanding of Cu toxicity at the systems level.

Materials and Methods

Chemicals

Analytical-grade acetonitrile, $CuSO_4 \cdot 5H_2O$, NaCl, $K_2HPO_4 \cdot 3H_2O$, and $NaH_2PO_4 \cdot 2H_2O$ were all purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Deuterated water (99.9 %) and sodium 3-trimethylsilyl (2,2,3,3-d_4) propionate (TSP) were purchased from Cambridge Isotope Laboratories (Miami, Florida, USA). Na^+-K^+ buffer [K_2HPO_4-NaH_2PO_4, 0.1 M (pH 7.4)], containing 0.31 mM of TSP was prepared in D_2O (Xiao et al. 2009). The extract solution for intracellular metabolites was prepared by mixing equal volumes of acetonitrile and H_2O.

Animals and Toxicity Testing

All female *Cyprinus flammans* (length 8 ± 0.5 cm, weight 12 ± 2.5 g) were purchased from TianSheng Market in Ningbo, China and acclimated in groups of eight in dechlorinated tap water, in an aerated glass tank, under a 12-h light and 12-h dark cycle, at room temperature. Fish were fed with fish feed (Sanyuan, Beijing Sanyou Chuangmei Feed Technology Co., LTD, Beijing, China) at a ratio of 2 % of body wt/days. In preliminary acute toxicity tests, fish were exposed to a range of Cu concentrations for 96 h. Mortality of fish was recorded after 96 h, and the lethal concentration (LC)-50 value (2.07 mg/L of Cu) was calculated using the method of Litchfield and Wilcoxon (1949). Thus, three exposure doses of Cu (0.25, 0.89, and 1.53 mg/L) were used in our experiment according to the 96-h LC50 value.

Cu Exposure and Sample Preparation

After acclimation to the holding conditions for 2 weeks, 32 fish were randomly divided into four groups of eight: (1) untreated controls and fish subjected to (2) 0.25 mg/L, (3) 0.89 mg/L, and (4) 1.53 mg/L of Cu for 24 h. No death and disease symptoms were observed in fish during the 24-h Cu-exposure period. We recorded the dissolved oxygen (DO) of the water during the experiment. Subsequently, all fish were dissected, and samples of gill, liver, and muscle tissues were immediately collected, frozen in liquid nitrogen, and stored at −80 °C for later NMR analysis.

Cu Analysis of Tissue Samples

Tissues samples were analyzed for Cu by atomic absorption spectrometry (UNICAM-919, UK) according to the methods described in *Methods in Clinical Chemistry* (Alcock 1987). Three replicates were prepared from each fish in the Cu-treated and control groups.

Extraction of Metabolites for ^{1}H-NMR Spectroscopy

Metabolites were extracted from gill, liver, and muscle tissues. Briefly, metabolites of gill tissue (approximately 150 mg) were extracted twice with 600 μL of 50 % aqueous acetonitrile using a hand-held grinder in an ice bath (Ningbo Scientz Biotechnology, Ningbo, China) and an ultrasonic cell disruptor (First European Instrument Manufacturing, Ningbo, China). After 20-minute centrifugation at 16,000*g* at 4 °C, the combined supernatants were lyophilized after removing acetonitrile in vacuo. Each of the aqueous gill extracts was separately reconstituted into 600 μL of Na^+-K^+ buffer. After centrifugation, 550 μL of supernatant of each extract was then pipetted into 5-mm NMR tubes (Norell, ST500-7; Norell, Inc., Landisville, NJ). The same protocol was applied to extract the

metabolites of liver (approximately 60 mg) and muscle (approximately 150 mg) samples.

NMR Spectroscopic Analysis

^{1}H-NMR spectra of the tissue extracts from four groups were acquired using a Bruker Avance III 400-MHz spectrometer equipped with an inverse detection probe (Bruker Biospin GmbH, Rheinstetten, Germany) operating at 400.13 MHz for proton at 298 K. A standard, water-suppressed, one-dimensional NMR spectrum was recorded using the first increment of a NOESY pulse sequence (recycle delay—90°–t_1–90°–t_m–90°—acquisition) with relaxation delay of 2 s, t_1 of 3 μs, and a mixing time t_m of 100 ms. Typically, 90° pulse length was approximately 10 μs, and 128 transients were collected into 32-k data points for each spectrum with a spectral width of 20 ppm. All ^{1}H-NMR spectra were centered at the water resonance. Spectra were apodized through multiplication with an exponential decay corresponding to 1-Hz line broadening before Fourier transformation (FT) and then manually phased, baseline corrected, and calibrated to the TSP at δ 0.00.

To facilitate NMR-signal assignments, a range of two-dimensional (2D) NMR spectra were acquired for selected samples and processed as described in Dai et al. (2010) including ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{1}H total correlation spectroscopy (TOCSY), ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC), and ^{1}H–^{13}C heteronuclear multiple bond correlation spectra (HMBC). For COSY and TOCSY experiments, 128 transients/increment and 256 increments were collected into 2048 data points with a spectral width of 12.0 ppm in both dimensions. In TOCSY, MLEV-17 was used as a spin-lock scheme, and the mixing time was set to 80 ms. HSQC and HMBC spectra were recorded using the gradient-selected sequence. In HSQC, composite pulse broad decoupling (globally alternating optimized rectangular pulses) was employed on ^{13}C during the acquisition period, and 4096 data points with 400 scans/increment and 256 increments were acquired with a spectral width of 12.0 ppm in the ^{1}H dimension and 220 ppm in the ^{13}C dimension. In HMBC, the spectral width was set to 12.0 ppm in the ^{1}H dimension and 250 ppm in the ^{13}C dimension, and 400 transients were collected into 4096 data points for each of 256 increments. The data were zero-filled into 4096 data points in the evolution dimensions and a sine or a shifted sinebell-squared function provided in TopSpin (Bruker Biospin GmbH, Rheinstetten, Germany) was applied to the free induction decay prior to FT.

Data Analysis

The spectral regions δ 8.5–0.7 from gill, δ 8.9–0.6 from liver, and δ 8.8–0.6 from muscle were integrated into regions with equal widths of 0.005 ppm (2 Hz). Regions distorted by imperfect water suppression were omitted: δ 5.20–3.96 for liver and δ 5.15–4.60 for gill and muscle. Each bucketed region was then normalized to the total sum of the spectral integrals to compensate for the intersample differences in sample volume/concentration.

The resulting NMR data were imported into the SIMCA-P$^+$ software (version 11.5, Umetrics, Umeå, Sweden) for multivariate data analysis. Initially, principal component analysis (PCA) was performed on the NMR data scaled to unit variance to generate an overview and find potential outliers. Orthogonal projection to latent structure with discriminant analysis (OPLS-DA) was subsequently performed using NMR data (scaled to unit variance) as the *X*-matirx and class information (*i.e.*, Cu dose in this study) as the *Y*-matirx to identify metabolites having statistically significant intergroup differences. After calculated with a 7-fold cross-validation (CV), all of OPLS-DA models were further validated by CV-ANOVA approach with $p < 0.05$ as significant level (Eriksson et al. 2008). To facilitate interpretation of the results, loadings that indicated altered metabolites induced by Cu were back-transformed and plotted with color-coded correlation coefficients (r) of the metabolites responsible for the intergroup differentiation (e.g., Fig. 2). The coefficient plots were generated with a Matlab script (http://www.mathworks.com/, MathWorks, Natick, MA) with some modifications. The r value indicates the significance of the metabolites contribution to intergroup separation with a hot color (i.e., red) being more significant (positive/negative) than a cold color (i.e., blue). The absolute value of r, $|r| = 0.666$, was used as the cutoff value for statistical significance for the metabolite changes based on the discrimination significance ($p < 0.05$) of the Pearson's product–moment correlation coefficient in this study.

Results

Concentration of Cu in Tissue Samples

Cu concentrations in gill, liver, and muscle samples are listed in Table 1. After 24 h of exposure to Cu, accumulation of Cu was greater in gill than in liver, which was in turn greater than in muscle tissue. Moreover, Cu treatment increased the accumulation of Cu in all three tissues relative to the controls. The greater concentration of Cu was used, the more accumulation of Cu was observed in these three tissues.

^{1}H-NMR Spectroscopic Analysis of Tissue Extracts

Representative ^{1}H NMR spectra of gill, liver, and muscle extracts obtained from control and Cu-treated fishes are shown in Fig. 1. The resonances were assigned to specific metabolites according to publically available published (Fan 1996;

Table 1 Residual concentration of Cu in gill, liver, and muscle of *C. flammans* after Cu exposure

Cu exposure (mg/L)	Residual concentration of Cu (mg/kg)[a]		
	Gill	Liver	Muscle
0	80.8 ± 11.4	21.3 ± 2.7	1.9 ± 0.3
0.25	161.6 ± 5.6[b]	32.6 ± 1.6[b]	2.8 ± 0.2[b]
0.89	189.4 ± 20.8[b,c]	39.8 ± 3.4[b,c]	3.6 ± 0.7[b,c]
1.53	204.9 ± 22.5[b,c,d]	47.9 ± 8.9[b,c,d]	4.0 ± 0.9[b,c]

[a] The average concentration and SD (Mean ± SD) were obtained from three parallel samples

[b] Significant compared with untreated control

[c] Significant compared with 0.25 mg/L Cu-treated group

[d] Significant compared with untreated control 0.89 mg/L Cu-treated group

Fan and Lane 2008) and in-house databases and further confirmed with extensive 2D NMR analysis with both ^{1}H and ^{13}C data listed in Table 2. A total of 27, 27, and 20 metabolites were identified in gill, liver, and muscle extracts, respectively. ^{1}H-NMR spectra of aqueous gill, liver, and muscle extracts contained peaks from amino acids, organic acids, nucleotides, glucose, glycogen, dimethylamine, and choline. However, some metabolites were only detected in specific tissues. For example, aspartate, arginine, and adenine were only observed in gill extracts, whereas dimethylamine, phosphorylcholine, and formate were only detected in liver extracts. Visual inspection of these spectra showed different changes in the distributions of metabolites among the tissues. For example, relative to the control tissues, 1.53 mg/L Cu treatment caused greater acetate and choline-*O*-sulfate levels in gill, greater taurine and glucose levels in liver, and greater adenosine monophosphate (AMP) level in muscle.

Cu-Induced Metabolite Alterations in Three Tissues

More detailed information concerning Cu-induced metabolic alterations in ill, liver, and muscle tissues was obtained from multivariate data analysis of the NMR profiles. As an unsupervised projection method, PCA can provide an overview of the multivariate data and show "clustering," trends, and possible outliers. In this study, PCA of normalized NMR data from extracts of gill, liver and muscle was performed and showed a clear separation between control groups and two greater concentrations of Cu-treated groups except for 0.25 mg/L Cu-treated groups (Supplemental Information Fig. S1). Further analysis using OPLS-DA [Fig. 2 ([left)] indicated significantly altered metabolites between the tissues obtained from Cu-treated fish and the corresponding controls with the quality of such models assured by the Q^2 values and p values obtained from CV-ANOVA [$p < 0.05$; Fig. 2 (left)]. All of three OPLS-DA models contructed from NMR data of 0.25 mg/L Cu-treated tissues and corresponding controls did not pass the rigorous test of CV-ANOVA (data not shown). The metabolites with statistically significant contributions to the differentiation between control and Cu-treated groups are displayed in the corresponding OPLS-DA coefficient plots [Fig. 2 (right)]. Their corresponding correlation coefficients are listed in Table S1. A summary of altered metabolites associated with three tissues and Cu concentration is shown in Fig. 3.

A number of metabolites were found to vary with Cu exposure. Relative to the untreated controls, gills obtained from 0.89 mg/L Cu-treated fish presented relatively greater amounts of acetate but lower amounts of citrate, taurine, methionine, and histidine (Fig. 2a). Gill samples from 1.53 mg/L Cu-treated fish contained greater levels of acetate, choline-*O*-sulfate, choline, glucose, glycogen, adenosine, and formate, as well as lower levels of alanine, methionine, histidine, and adenine (Fig. 2b) with respect to control samples.

The same OPLS-DA strategy was further applied to the liver metabolic profiles of the controls and Cu-treated fish. Livers obtained from 0.89 mg/L Cu-treated fish contained greater levels of lactate, taurine, glucose, glycogen, and AMP and lower levels of a range of amino acids levels including isoleucine, leucine, valine, alanine, lysine, tyrosine, histidine, and phenylalanine (Fig. 2c). Similar changes of metabolites in liver were induced by 1.53 mg/L if Cu except for lactate and AMP (Fig. 2d).

Metabolic profiles of muscle were also markedly altered after Cu exposure as illustrated in the OPLS-DA coefficient plots (Fig. 2e, f). Both 0.89 and 1.53 mg/L of Cu led to a marked accumulation in levels of lactate, acetate, glycogen, and AMP accompanied by a significant depletion in levels of citrate and adenosine in muscle (Fig. 2e, f). The accumulation of alanine, taurine, glycine, and creatine was only observed in the muscle extracts from 1.53 mg/L Cu-treated fish (Fig. 2f).

Discussion

The Cu concentrations of 96-h LC50 values in *C. carpio* are variable (4 to 160,000 μg/L) depending water hardness, temperature, and size of the animals used (De Boeck et al. 2004). In our study, the Cu concentration of the 96-h LC50

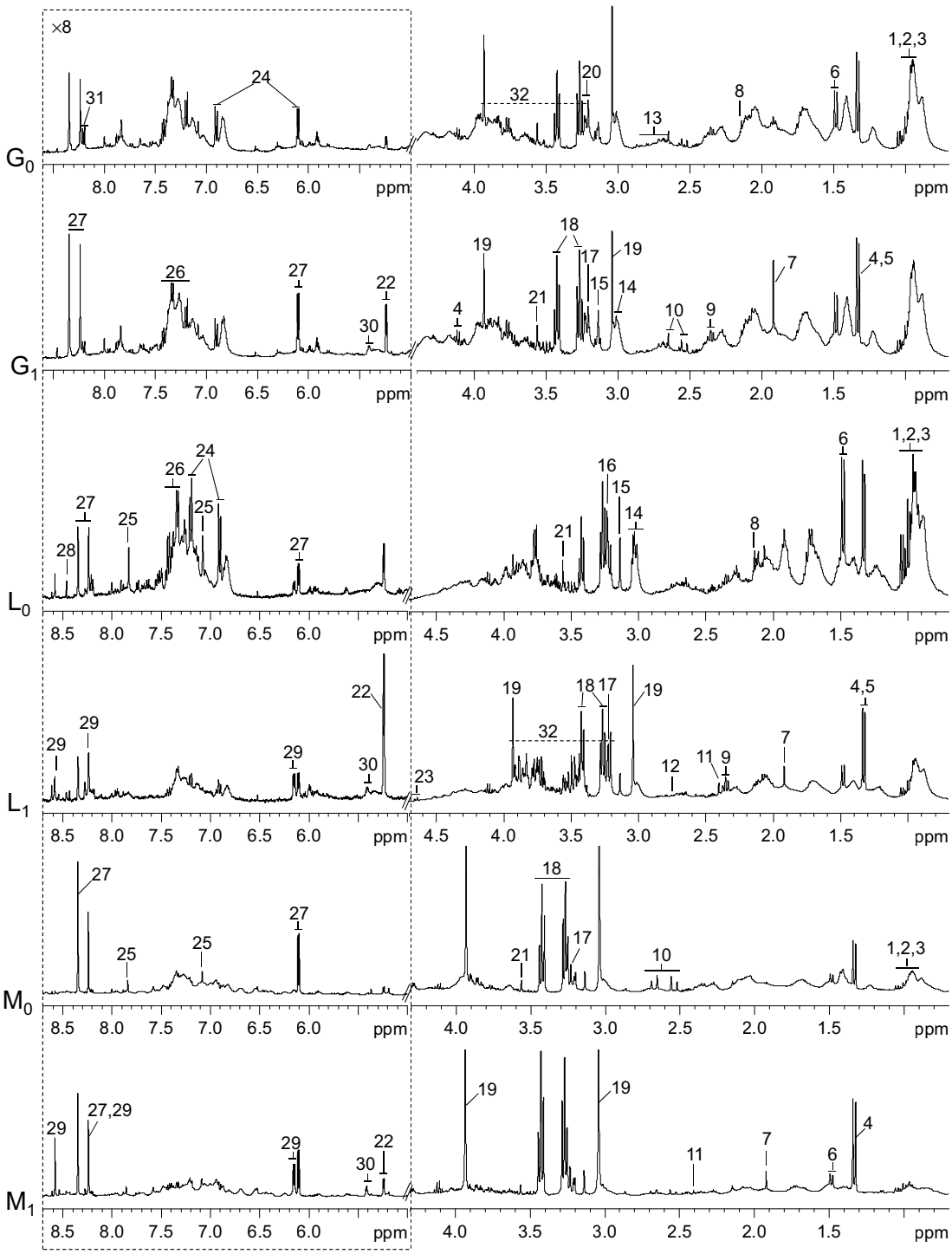

Fig. 1 Typical 400-MHz ^{1}H NMR spectra of gill, liver, and muscle extracts from control and Cu-treated *C. flammans*. G_0 control gill, G_1 gill obtained from 1.53 mg/L Cu-treated fish, L_0 control liver, L_1 liver obtained from 1.53 mg/L Cu-treated fish, M_0 control muscle, M_1 muscle obtained from 1.53 mg/L Cu-treated fish. The *dotted regions* are expanded eight times. Resonance assignments are listed in Table 2

value in *C. flammans* was 2.07 mg/L, and water hardness was 180 mg/L as $CaCO_3$. Normalizing our data to a hardness of 50 mg/L $CaCO_3$ using the conversion formula (LC50 value at 50 mg/L $= e^{\ln(LC50)-0.9422 \times (\ln(hardness)-\ln(50))}$) gives 96-h LC50 values of 0.619 mg/L for *C. flammans*. In De Boeck et al. article (2004), 96-h LC50 values of Cu concentrations were 40 μg/L for rainbow trout, 125 μg/L for common carp, and 265 μg/L for gibel carp at a hardness of 50 mg/L $CaCO_3$.

Table 2 ^{1}H- and ^{13}C-NMR data and assignments of the metabolites in gill, liver, and muscle extracts of *C. flammans*

Key	Metabolites	Moieties	δ^{1} H (ppm) and multiplicity[a]	δ^{13} C (ppm)	Sample[b]
1	Isoleucine	αCH, βCH, γCH_2, $\gamma' CH_3$, δCH_3	3.67(d), 1.98(m), 1.26(m), 1.45(m), 1.01(d), 0.94(t)	62.4, 38.4, 27.3, 17.7, 13.8	G, L,M
2	Leucine	αCH, βCH_2, γCH, δCH_3, $\delta' CH_3$	3.73(#[c]), 1.73(#), 1.67(#), 0.98(d), 0.96(d)	62.4, 42.7, 27.0, 24.7, 23.8	G, L, M
3	Valine	αCH, βCH, γCH_3, $\gamma' CH_3$	3.62(d), 2.27(m), 1.04(d), 0.99(d)	62.9, 31.7, 20.9, 19.6	G, L, M
4	Lactate	αCH, βCH_3, COOH	4.11(q), 1.33(d)	71.5, 20.2, 185.5	G, L, M
5	Threonine	αCH, βCH, γCH_3	3.58(d), 4.26(m), 1.33(d)	68.7, 63.3, 22.9	G, L
6	Alanine	αCH, βCH_3, COOH	3.79(q), 1.48(d)	53.4, 19.1, 178.2	G, L, M
7	Acetate	CH_3, COOH	1.92(s)	26.1, 184.4	G, L, M
8	Methionine	αCH, βCH_2, γCH_2, S-CH_3	3.85(#), 2.15(m), 2.65(t), 2.14(s)	56.9, 32.8, 31.9, 16.9	G, L
9	Glutamate	σCO, αCH, βCH_2, γCH_2, COOH	3.77(m), 2.12(m), 2.05(m), 2.36(dt)	184.2, 57.1, 29.8, 36.2, 177.7	G, L
10	Citrate	αCH_2, βCH_2, COOH	2.67(d), 2.54(d)	78.2, 48.0, 181.4	G, M
11	Succinate	CH_2	2.41(s)	36.9	L, M
12	Dimethylamine	CH_3	2.75(s)	41.2	L
13	Aspartate	αCH, βCH_2, γCOOH	3.92(dd), 2.80(dd), 2.70(dd)	56.6, 40.1, 180.8	G
14	Lysine	αCH, βCH_2, γCH_2, σCH_2, εCH_2	3.76(#), 1.92(m), 1.48(d), 1.73(#), 3.03(t)	56.9, 32.6, 24.4, 29.3, 41.9	G, L
15	Choline-*O*-sulfate	σCH_2, βCH_2, N-CH_3	4.33(#), 3.68(#), 3.14(s)	68.9, 55.7	G, L
16	Phosphorylcholine	σCH_2, βCH_2, N-CH_3	4.07(d), 3.53 (d), 3.21(s)	70.4, 56.7	L
17	Choline	αCH_2, βCH_2, N-CH_3	4.26(#), 3.58(d), 3.23(s)	70.2, 56.8	G, L, M
18	Taurine	CH_2NH_2, CH_2SO_3	3.43(t), 3.27(t)	38.2, 50.6	G, L, M
19	Creatine	CH_2, CH_3	3.93(s), 3.04(s)	56.7, 40.0	G, L, M
20	Arginine	εC, αCH, βCH_2, γCH_2, δCH_2	3.76(#), 1.92(m), 1.70(#), 3.25(t)	160.0, 57.3, 31.6, 28.6, 43.4	G
21	Glycine	αCH_2 COOH	3.56(s)	44.4, 175.6	G, L, M
22	α-Glucose	C_1H, C_2H, C_4H, C_5H, C_6H	5.24(d), 3.54(d), 3.83(#), 3.74(d)	95.0, 74.4, 74.5	G, L, M
23	β-Glucose	C_1H, C_2H, C_3H	4.65(d), 3.25(t), 3.50(#)	98.6, 77.1, 78.7	G, L, M
24	Tyrosine	Ring$C_{2,6}H$, Ring$C_{3,5}H$, RingC_4	7.2(d), 6.90 (d)	133.6, 118.8, 157.5	G, L
25	Histidine	C_1H, C_2H, C_3, C_4H, C_5H, COOH	7.88(d), 7.09(d), 3.15(dd), 3.25(dd), 4.00(dd)	139.0, 119.6, 133.9, 30.8, 57.6, 176.9	L, M
26	Phenylalanine	Ring-CH	7.38(m), 7.33(q), 7.43(t)	130.5, 132.1, 131.9	G, L
27	Adenosine	C_8H, C_6, C_4, C_2H, $C_{1'}H$, $C_{2'}H$, $C_{3'}H$, $C_{4'}$, $C_{5'}H$	8.34(s), 8.24(s), 6.11(d), 4.78(#), 4.45(#), 4.28(#), 3.86(#)	143.1,162.3,151.0, 149.3, 76.9	G, L, M
28	Formate	CH	8.46(s)	174.1	L
29	Adenosine monophosphate	C_2H, C_4,C_5,C_6, C_8H, $C_{1'}H$, $C_{2'}H$, $C_{3'}H$, $C_{4'}H$, $C_{5'}H$	8.23(s), 8.59(s), 6.15(d), 4.81(#), 4.52(m), 4.38(d), 4.02(s)	152.5, 151.8, 126.1, 161.4, 155.9, 90.2, 77.8, 73.6, 87.8, 66.4	M
30	Glycogen	1-CH	5.37–5.43		G, L, M
31	Adenine	C_2H, C_6H	8.21(s), 8.19(s)	144.6, 144.8	G
32	Glucose and amino acids	αCH resonances	3.3–3.9		G, L, M

[a] Multiplicity: *s* singlet, *d* doublet, *t* triplet, *q* quartet, *dd* doublet of doublets, *dt* doublet of triples, *m* multiplet

[b] *G* gill, *L* liver, *M* muscle

#[c] The signals or multiplicities were not determined

Considering that fish used in De Boeck's study were much smaller (<1 g) than those in our experiments (approximately 12 g), the Cu concentrations were not very high in our study.

The aim of the present study was to investigate the metabolic consequences of acute Cu exposure in a holistic manner by using an NMR-based metabonomic strategy. Although comparatively high Cu concentrations were used

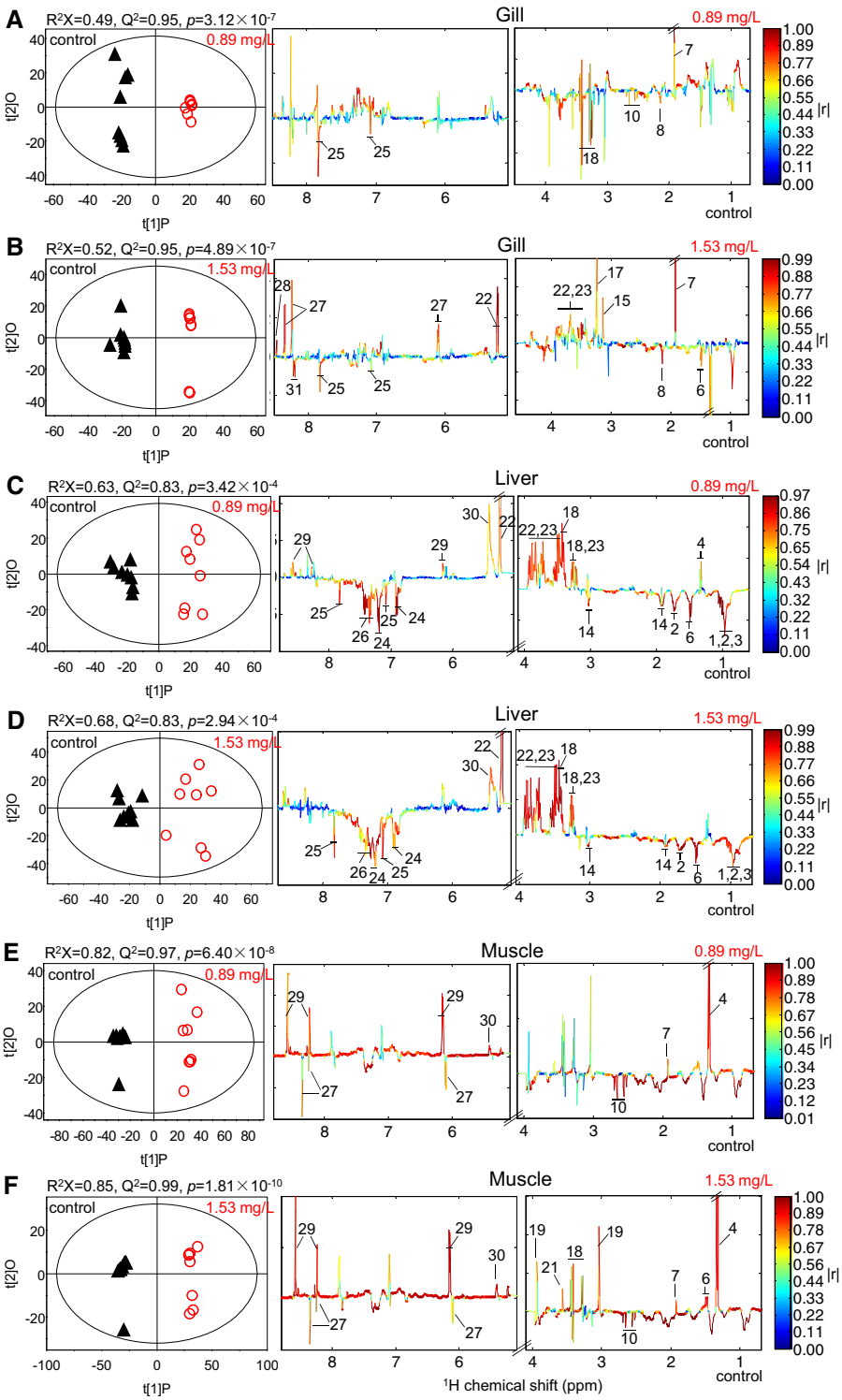

Fig. 2 OPLS-DA scores (*right*) and coefficient-coded loading plots (*left*) for the models discriminating the control (*triangles*) and Cu-treated tissues (*circles*) of *C. flammans*. **a** Gill for fish treated with 0.89 mg/L Cu. **b** Gill from fish treated with 1.53 mg/L Cu. **c** Liver from fish treated with 89 mg/L Cu. **d** Liver from fish treated with 1.53 mg/L of Cu. **e** Muscle from fish treated with 0.89 mg/L Cu. **f** Muscle from fish treated with 1.53 mg/L Cu (see Table 2 for metabolite key)

in the study, no significant metabonomic changes were observed in gill, liver, and muscle of *C. flammans* after 24-h exposure to 0.25 mg/L of Cu. Therefore, the NMR metabonomic profiles of *C. flammans* would be not expected when Cu concentration is low. However, two greater concentrations of Cu induced significant changes of the metabonome of three tissues. These metabonomic changes indicated that Cu exposure caused comprehensive metabolic alterations in gill, liver, and muscle tissues involving many related metabolic pathways (Fig. 4).

Fish gill is often considered as the primary target organ of Cu toxicity (Laurén and McDonald 1985; Boitel and

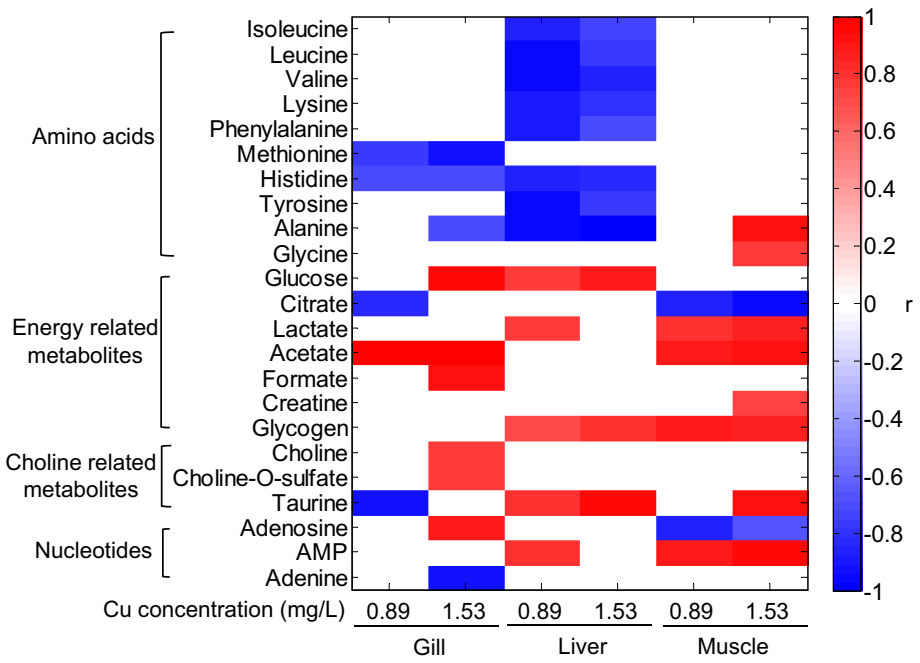

Fig. 3 Dynamic alternations of key metabolites in the gill, liver, and muscle of fish in response to Cu exposure. The *color* indicates a correction coefficient as scaled on the right-hand side. The warm color (e.g., *red*) denotes an increase in the level of metabolites in the tissues obtained from 0.89 and 1.53 mg/L Cu-treated fish against the respective controls, and the cold color (e.g., *blue*) indicates a decrease (Color figure online)

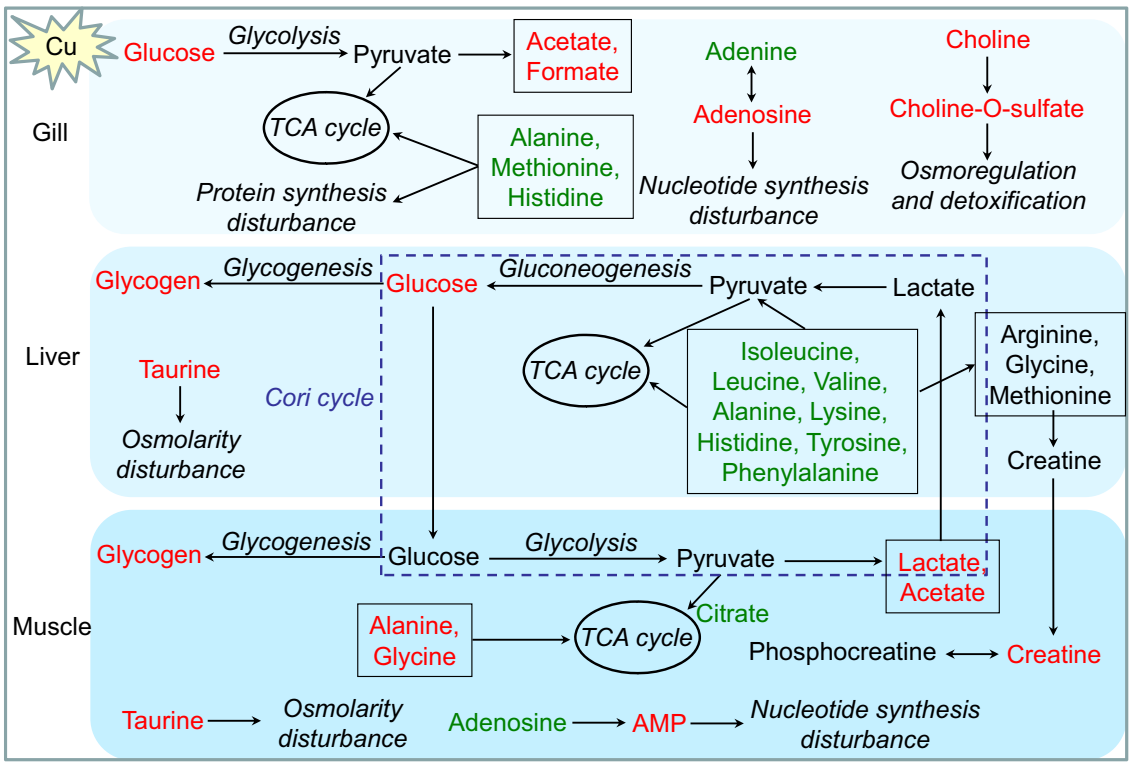

Fig. 4 Summarized changes of metabolic pathways in gill, liver, and muscle of *C. flammans* associated with Cu exposure. Metabolites are shown in *red* when their level is significantly greater in the tissues obtained from Cu-treated fish ($p < 0.05$), in *blue* when it is lower, and in *black* when it is unchanged (Color figure online)

Truchot 1989); hence, the function of the gill could be compromised by Cu exposure, which is what we found in this study. Some evidence reported that gill cannot work as an efficient respiratory organ after Cu exposure (Mallat 1985). A marked increase in the level of choline induced by 1.53 mg/L of Cu is highly likely to be consistent with

this observation because choline is essential for the structural integrity of cell membranes (Klein 2000). The increased accumulation of choline may therefore reflect damage to the membrane of gill epithelium. Similar stress-induced choline accumulation has been reported in plasma of common fish (Seiichi et al. 2008). Moreover, we observed a decrease in DO concentration from 8.49 to 5.55 mg/dm^3 in water after a 24-h Cu exposure (data not shown). These observations may consequently lead to internal hypoxia, decreased respiration rate, and inhibited activity of mitochondrial enzymes in fish (Couture and Kumar 2003; Boitel and Truchot 1989; De Boeck et al. 2007). This notion is consistent with the observed increase in the acetate and formate levels because these two metabolites are known as anaerobic metabolites. In general, Cu is also an osmoregulatory toxicant to fish (De Boeck et al. 2010), and hence it is anticipated that Cu exposure would cause a disturbance in osmoregulation. In fact, gill is not only involved in osmoregulation but also in detoxification of $CuSO_4$, which might be manifested by the increased level of choline-*O*-sulfate in the fish exposed to 1.53 mg/L of $CuSO_4$ (Fig. 2b). This is because choline-*O*-sulfate can exhibit an osmoprotective property comparable with glycine betaine, a common osmolyte (Rhodes and Hanson 1993). Oxygen is required in the first step of choline oxidation to glycine betaine. Alternatively, under hypoxic conditions, choline can be metabolized to produce choline-*O*-sulfate instead of glycine betaine. Such an osmoprotective property of choline-*O*-sulfate has been observed in diverse members of the Plumbaginaceae and fungi (Hanson et al. 1991, 1994; Park and Gander 1998). Furthermore, previous reports have suggested that choline-*O*-sulfate accumulation serves in the detoxification of sulfate by the conjugation of sulfate with choline (Rivoal and Hanson 1994). Therefore, choline-*O*-sulfate may not only replace glycine betaine as an osmoregulatory solute, it may also act to detoxify sulfate derived from $CuSO_4$. Choline-*O*-sulfate in gill may be used as a biomarker to provide an early warning of Cu exposure in *C. flammans*. In addition, we observed a marked decrease in the levels of some amino acids—including alanine, methionine, and histidine—as well as significant changes in the levels of nucleoside such as adenine and adenosine. These observations suggest that Cu exposure causes a slight disturbance in protein and nucleotide synthesis (Fig. 4). The metabolic alteration in gill occurs in a dose-dependent fashion (Figs. 2, 3).

Fish liver is another organ responsible for the detoxification of metals. Although there is a lower level of Cu accumulation in liver than in gill (Table 1), there were metabolic alterations in the extracts of livers from fish exposed to two greater concentrations of Cu. The most prominent metabolic effect of Cu on liver is the depleted levels of a range of amino acids including isoleucine, leucine, valine, alanine, lysine, histidine, tyrosine, and phenylalanine (Fig. 2). Moreover, a concurrent increase in levels of glucose and glycogen was observed in liver of Cu-exposed fish. Because liver is the primary organ for amino acid metabolism (Brosnan 2003), our observation suggests that Cu exposure stimulates the gluconeogenesis pathway from amino acids. Intriguingly, we also noted a significant accumulation of glycogen and lactate in Cu-exposed muscle (Fig. 2) indicating a Cori cycle between liver and muscle (dashed blue box, Fig. 4). Cori cycle occurs when oxygen supply is insufficient in the muscle. Energy must be released through anaerobic lactate fermentation. Instead of accumulating inside the muscle cells, lactate moves to liver and is converted to glucose through gluconeogenesis. Glucose is then supplied to muscles through the bloodstream and is metabolized back to lactate. Hence, Cu exposure causes acute hypoxia leading to metabolic acidosis in muscle. To prevent lactic acidosis in muscle, the Cori cycle is used to handle the excess lactate shifting the metabolic burden from muscle to liver. However, gluconeogenesis from lactate and amino acids seems to produce the excess glucose, which is stored as glycogen through glycogenesis in liver and muscle. A previous study reported that Cu exposure induces increased plasma glucose in rainbow trout (Laurén and McDonald 1985). This change further increases the accumulation of glycogen by way of gluconeogenesis (Jobling 1994). In fact, increasing glycogen level has been observed in liver of fish exposed to low concentrations of Cu (0.8 μM) (De Boeck et al. 1997). An increase in the glycogen level not only indicates a greater gluconeogenic capacity, it is also is a consequence of a defense mechanism against hypoxia due to Cu exposure (De Boeck et al. 1997). However, sublethal Cu leads to glycogen depletion in gibel and crucian fish (De Boeck et al. 2006, 2010). The conflicting consequences likely result from the different fish species, exposure time, and/or Cu concentration. Furthermore, we found a marked increase in levels of taurine in liver of *C. flammans* exposed to 0.89 and 1.53 mg/L of Cu (Fig. 2). Taurine is an important osmolyte in bacteria, plants, and animals (Graham and Wilkinson 1992; Holmes et al. 2006; Carr et al. 1996; Fiess et al. 2007). Presumably, it also plays an osmoregulatory role in Cu-exposed *C. flammans*. Such increase of taurine has also been observed in liver and muscle tissues of tilapia subjected to salinity and temperature stress (Fiess et al. 2007).

Fish muscle contained the lowest level of Cu compared with the other tissues as shown in Table 1. However, a significant metabolite change was still observed in Cu-exposed muscle. In addition to the increased glycogen and lactate involved in the Cori cycle, we also observed highly accumulated acetate and depleted citrate in muscle of Cu-exposed fish (Fig. 2). These observations further support that Cu exposure results in acute hypoxia leading to metabolic acidosis in the muscle. This occurs because citrate is an intermediate

in the tricarboxylic acid (TCA) cycle. The decrease in the level of citrate suggests that Cu expose causes a decreased TCA cycle. Evidence of inhibition of the TCA cycle after Cu exposure was also observed in yellow perch where the activity of citrate synthase in muscle was inhibited (Couture and Kumar 2003). Moreover, the increase in the level of acetate suggests that anaerobic fermentation is enhanced. In fact, this change and associated metabolic acidosis has been observed in the muscle of crucian fish during acute hypoxia (Lardon et al. 2012). The increased acetate level may also have been a manifestation of acute hypoxia of muscle; notably, acetate has been used as a biomarker of anoxic conditions in mussels (Kluytmans et al. 1975). In addition, we observed a significant increase of creatine. Creatine is synthesized in liver and kidney from arginine, glycine, and methionine. It is then transported in the blood for use by muscles. In muscle cells, creatine can be conversed to phosphocreatine (PCr) as an energy reservoir. When the muscle consumes adenosine triphosphate (ATP) rapidly, PCr is hydrolyzed for the regeneration of ATP in situ. The increase of creatine suggests that Cu exposure causes increased energy demands of muscle. A previous study reported that 1.31 μM Cu exposure causes profound depletion of PCr levels in muscle of *C. carpio* (De Boeck et al. 1997), which indicates that energy demand is likely increased with the concentration of Cu. We also noted a significant alteration in levels of adenosine and AMP in muscle of fish exposed to two greater concentrations of Cu, which suggests that Cu exposure causes disturbance in nucleic acid metabolism (Fig. 4). In addition, a significant increase in the taurine level is likely related to a change of osmoregulation in fish muscle. The quantitative change of taurine induced by Cu, together with creatine, occurs in a dose-dependent fashion (Figs. 2, 3).

Conclusion

In this study, we observed the metabolic effects of Cu exposure, at different concentrations, on multiple tissues of *C. flammans*. Cu caused changes of energy-related metabolites and amino acids, which we suggest are due to enhanced metabolic acidosis in gill and muscle, decreased TCA activity in muscle, increased gluconeogenesis from amino acids in liver, and improved glycogenesis in liver and muscle. The Cori cycle between liver and muscle is concurrently triggered. Furthermore, Cu resulted in the alteration of choline metabolism, which we hypothesize that Cu induces membrane damage and detoxification of $CuSO_4$ in gill as well as altered osmoregulation in all three tissues. Choline-*O*-sulfate in gill may be used as a biomarker to provide an early warning of Cu exposure in *C. flammans*. Moreover, Cu exposure caused alterations of nucleoside and nucleotide metabolism in both gill and muscle. These findings provide comprehensive insight into the metabolic effects of high-concentration Cu exposure on *C. flammans* and highlight the value of metabonomics in the study of metabolic disturbance of metal in fish.

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References

Alcock NW (1987) Copper. In: Pesce AJ, Kaplan LA (eds) Methods in clinical chemistry. Mosby, Missouri, pp 527–538

Boitel F, Truchot JP (1989) Effects of sublethal and lethal copper levels on hemolymph acid-base balance and ion concentrations in the shore crab *Carcinus maenas* kept in undiluted sea water. Mar Biol 103(4):495–501

Brosnan JT (2003) Interorgan amino acid transport and its regulation. J Nutr 133(6):2068S–2072S

Carr WES, Netherton JC, Gleeson RA, Derby CD (1996) Stimulants of feeding behavior in fish: analyses of tissues of diverse marine organisms. Biol Bull 190(2):149–160

Coen M, Lenz EM, Nicholson JK, Wilson ID, Pognan F, Lindon JC (2003) An integrated metabonomic investigation of acetaminophen toxicity in the mouse using NMR spectroscopy. Chem Res Toxicol 16(3):295–303

Couture P, Kumar PR (2003) Impairment of metabolic capacities in copper and cadmium contaminated wild yellow perch (*Perca flavescens*). Aquat Toxicol 64(1):107–120

Dai H, Xiao CN, Liu HB, Tang HR (2010) Combined NMR and LC-MS analysis reveals the metabonomic changes in *Salvia miltiorrhiza* Bunge induced by water depletion. J Proteome Res 9(3):1460–1475

De Boeck G, Borger R, van der Linden A, Blust R (1997) Effects of sublethal copper exposure on muscle energy metabolism of common carp, measured by ^{31}P-nuclear magnetic resonance spectroscopy. Environ Toxicol Chem 16(4):676–684

De Boeck G, Vlaeminck A, Balm PHM, Lock RAC, De Wachter B, Blust R (2001) Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: interactions between Cu^{2+} and plasma cortisol increase. Environ Toxicol Chem 20(2):374–381

De Boeck G, Meeus W, De Coen W, Blust R (2004) Tissue-specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). Aquat Toxicol 70(3):179–188

De Boeck G, van der Ven K, Hattink J, Blust R (2006) Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. Aquat Toxicol 80(1):92–100

De Boeck G, van der Ven K, Meeus W, Blust R (2007) Sublethal copper exposure induces respiratory stress in common and gibel carp but not in rainbow trout. Comp Biochem Physiol C 144(4):380–390

De Boeck G, Smolders R, Blust R (2010) Copper toxicity in gibel carp *Carassius auratus gibelio*: importance of sodium and glycogen. Comp Biochem Physiol C 152(3):332–337

Effler SW, Litten S, Field SD, Tong-Ngork T, Hale F (1980) Whole lake responses to low level copper sulfate treatment. Water Res 14(10):1489–1499

Eriksson L, Trygg J, Wold S (2008) CV-ANOVA for significance testing of PLS and OPLS (R) models. J Chemom 22(11–12):594–600

Eyckmans M, Benoot D, Van Raemdonck GAA, Zegels G, Van Ostade XWM, Witters E et al (2012) Comparative proteomics of copper exposure and toxicity in rainbow trout, common carp and gibel carp. Comp Biochem Physiol Part D 7(2):220–232

Fan TWM (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. Prog Nucl Magn Reson Spectrosc 28:161–219

Fan TWM, Lane AN (2008) Structure-based profiling of metabolites and iotopomers by NMR. Prog Nucl Magn Reson Spectrosc 52:69–117

Fiess JC, Kunkel-Patterson A, Mathias L, Riley LG, Yancey PH, Hirano T et al (2007) Effects of environmental salinity and temperature on osmoregulatory ability, organic osmolytes, and plasma hormone profiles in the Mozambique tilapia (*Oreochromis mossambicus*). Comp Biochem Physiol A 146(2):252–264

Graham JE, Wilkinson BJ (1992) *Staphylococcus aureus* osmoregulation: roles for choline, glycine betaine, proline, and taurine. J Bacteriol 174(8):2711–2716

Hanson AD, Rathinasabapathi B, Chamberlin B, Gage DA (1991) Comparative physiological evidence that β-alanine betaine and choline-*O*-sulfate act as compatible osmolytes in halophytic *Limonium* species. Plant Physiol 97(3):1199–1205

Hanson AD, Rathinasabapathi B, Rivoal J, Burnet M, Dillon MO, Gage DA (1994) Osmoprotective compounds in the Plumbaginaceae: a natural experiment in metabolic engineering of stress tolerance. Proc Natl Acad Sci USA 91(1):306–310

Harris ED (2000) Cellular copper transport and metabolism. Annu Rev Nutr 20:291–310

Hashemi SM, Ghods S, Kolodgie FD (2008) A placebo controlled, dose-ranging, safety study of allogenic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. Eur Soc Cardiol 29(2):251–259

Heath AG (1991) Effect of water-borne copper on physiological responses of bluegill (*Lepomis macrochirus*) to acute hypoxic stress and subsequent recovery. Comp Biochem Physiol Part C 100(3):559–564

Holmes E, Tang H, Wang Y, Seger C (2006) The assessment of plant metabolite profiles by NMR-based methodologies. Planta Med 72(9):771–785

Jobling M (ed) (1994) Environmental tolerances and preferences. In: Fish bioenergetics. Chapman and Hall, London, UK, pp 207–294

Karan V, Vitorović S, Tutundzić V, Poleksić V (1998) Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery. Ecotoxicol Environ Saf 40(1–2):49–55

Klein J (2000) Membrane breakdown in acute and chronic neurodegeneration: focus on choline-containing phospholipids. J Neural Transmit 107(8–9):1027–1063

Kluytmans JHFM, Veenhof PR, De Zwaan A (1975) Anaerobic production of volatile fatty acids in the sea mussel *Mytilus edulis* (L.). J Comp Physiol 104(1):71–78

Kunwar PS, Tudorache C, Eyckmans M, Blust R, De Boeck G (2009) Influence of food ration, copper exposure and exercise on the energy metabolism of common carp (*Cyprinus carpio*). Comp Biochem Physiol C 149(1):113–119

Lardon I, Nilsson GE, Stecyk JAW, Vu TN, Laukens K, Dommisse R et al (2012) ^{1}H-NMR study of the metabolome of an exceptionally anoxia tolerant vertebrate, the crucian carp (*Carassius carassius*). Metabolomics 9(2):311–323

Laurén DJ, McDonald DG (1985) Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. J Comp Physiol B 155(5):635–644

Lindon JC, Nicholson JK, Holmes E, Everett JR (2000) Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. Concepts Magn Reson 12(5):289–320

Litchfield JT, Wilcoxon F (1949) A simplified method of evaluating dose-effect experiments. J Pharmacol 96(2):99–113

Mallat J (1985) Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can J Fish Aquat Sci 42(4):630–648

Martello LB, Tjeerdema RS, Smith WS, Kauten RJ, Crosby DG (1998) Influence of salinity on the actions of pentachlorophenol in Haliotis as measured by in vivo ^{31}P NMR spectroscopy. Aquat Toxicol 41(3):229–250

Nawaz M, Manzl C, Krumschnabel G (2005) *In vitro* toxicity of copper, cadmium, and chromium to isolated hepatocytes from carp, *Cyprinus carpio* L. Bull Environ Contam Toxicol 75(4):652–661

Park YI, Gander JE (1998) Choline derivatives involved in osmotolerance of *Penicillium fellutanum*. Appl Environ Microbiol 64(1):273–278

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in greater plants. Ann Rev Plant Physiol Plant Mol Biol 44:357–384

Rivoal J, Hanson AD (1994) Choline-*O*-sulfate biosynthesis in plants (identification and partial characterization of a salinity-inducible choline sulfotransferase from species of *Limonium* (Plumbaginaceae). Plant Physiol 106(3):1187–1193

Samuelsson LM, Förlin L, Karlsson G, Adolfsson-Erici M, Larsson DG (2006) Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. Aquat Toxicol 78(4):341–349

Seiichi U, Emiko K, Jiro K (2008) Super(1)H-NMR metabolomic evaluation on pollutants using fish plasma. Jpn J Environ Toxicol 11(2):63–67

Shofer SL, Willis JA, Tjeerdema RS (1997) Effects of hypoxia and toxicant exposure on arginine kinase function as measured by ^{31}P NMR magnetization transfer in living abalone. Comp Biochem Physiol C 117(3):283–289

Tracey BS, David AS, Lindy T, Karen GB, Louis EB, Arezue FBB et al (2010) Metabolomic analysis of Atlantic blue crab, *Callinectes sapidus,* hemolymph following oxidative stress. Metabolomics 6(2):250–262

Van Scoy AR, Yu Lin C, Anderson BS, Philips BM, Martin MJ, McCall J et al (2010) Metabolic responses produced by crude versus dispersed oil in Chinook salmon pre-smolts via NMR-based metabolomics. Ecotoxicol Environ Saf 73(5):710–717

Wu H, Wang W (2010) NMR-based metabolomic studies on the toxicological effects of cadmium and copper on green mussels *Perna viridis*. Aquat Toxicol 100(4):339–345

Wu H, Wang W (2011) Tissue-specific toxicological effects of cadmium in green mussels (*Perna viridis*): nuclear magnetic resonance-based metabolomics study. Environ Toxicol Chem 30(4):806–818

Xiao CN, Hao FH, Qin XR, Wang YL, Tang HR (2009) An optimized buffer system for NMR-based urinary metabonomics with effective pH control, chemical shift consistency and dilution minimization. Analyst 134(5):916–925

Zhang LB, Liu XL, You LP, Zhou D, Wang Q, Li F, Cong M et al (2011) Benzo(*a*)pyrene-induced metabolic responses in Manila clam *Ruditapes philippinarum* by proton nuclear magnetic resonance (^{1}H NMR) based metabolomics. Environ Toxicol Pharmacol 32(2):218–225