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Effect of Copper on Growth, Digestive and Antioxidant Enzyme Activities of Juvenile Qihe Crucian Carp, *Carassius carassius*, During Exposure and Recovery

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Abstract The toxicity of copper (Cu) on growth and activities of digestive and antioxidant enzymes in the hepatopancreas and intestine of juvenile Qihe crucian carp *Carassius carassius* was evaluated. The fish were exposed in different Cu solutions for 20 days, and the 0.60 mg/L group was then transferred to clean water to initiate a 20-day recovery period after Cu exposure. Results showed that all enzyme activities decreased significantly at high-concentration (0.30 and 0.60 mg/L) and long-time (20 days) Cu exposures and increased significantly at high-concentration (0.60 mg/L) and short-time Cu exposures (1 day). After the 20-day recovery period, all enzyme activities in the 0.60 mg/L group had recovered to control levels. High-concentration (0.60 mg/L) and long-time (20 days) Cu exposure markedly hindered the growth of fish, whereas the loss of fish growth can not be compensated for by a 20-day recovery period.

Keywords Copper exposure · Growth · Digestive enzyme · Antioxidant enzyme · Recovery · Qihe crucian carp

Pollution of water bodies by heavy metals has increased remarkably since the development of industry and agriculture in recent years, and has seriously threatened the survival of aquatic life. The Qihe crucian carp (*Carassius carassius*), a natural triploid gynogenetic freshwater fish, is only distributed in the Qihe River in Henan, China. The fish is renowned for its strong body, fast growth, delicious taste, abundant nutrients, and high market value, and is one of the key protected wild animals in Henan province of China. However, because $CuSO_4$ is often used as a therapeutic agent to control algae and some pathogens in fish ponds near the Qihe River, concentrations of Cu have increased recently, with concentrations as high as 83.7 μg/L in 2011 (Zhang and Yang 2012). This is about 8 times the water quality standard for fisheries of China ($Cu^{2+} \leq 0.01$ mg/L) (GB 1989). Cu is a non-biodegradable metal pollutant, and is toxic to many fish and aquatic invertebrate species at excessive levels in water (Mastin and Rodgers 2000). Cu pollution in Qihe River has seriously affected the physiology, growth and reproduction of Qihe crucian carp (Zhao and Xiao 2007). To protect water environments of Qihe River and this fish species, changes in the growth and biochemical properties of this fish during Cu exposure and post-exposure recovery should be carefully studied.

Fishes are known to be highly susceptible to environmental contamination, and changes in activity levels of various enzymes may serve as promising indicators of water pollution (Cengiz and Unlu 2006). Digestive enzymes (e.g., amylase, protease and lipase) play important roles in the nutrition and growth of animals. It has been hypothesized that toxic effects on digestive enzymes could reduce digestive efficiency and consequently affect the energy available for global metabolism (Dedourge-Geffard et al. 2009). Antioxidant enzymes are radical-scavenging enzymes that serve as the first line of defense against free radicals in organisms. Cu has reduction–oxidation potential and generates reactive oxygen species (ROS) which can cause oxidative stress and cellular damage (Vutukuru et al. 2006). Superoxide dismutase (SOD) and catalase (CAT) are two main antioxidant enzymes, and protect the cells against ROS damage. The effects of Cu on the activities of antioxidant

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enzymes in fish have been extensively investigated (Liu et al. 2010; Tang et al. 2013; Kong et al. 2013; Jiang et al. 2014). Some studies on the changes of the digestive enzyme activities in response to Cu in fish have also been reported (Kuz'mina et al. 2010; Tang et al. 2013). However, the actual toxic threshold differs quite widely in different genera (Das et al. 2004). To our knowledge, no studies have been reported on the effects of Cu exposure to Qihe crucian carp. Furthermore, it is yet unclear if the damage caused by Cu is able to be repaired sufficiently that growth and enzyme activities parameters return to levels comparable with pre-exposure levels. Thus, this study investigated the changes of body weight and length, and the activities of protease, amylase, SOD and CAT enzymes in the hepatopancreas and intestine of Qihe crucian carp during Cu exposure and post-exposure recovery.

Materials and Methods

Healthy Qihe crucian carp were obtained from a farming pond in Henan Normal University (Xinxiang, China). The body weights and lengths were 40.43 g ± 2.15 g and 10.36 cm ± 1.05 cm, respectively. Fish were acclimated for 14 d in 100 L tanks containing dechlorinated tap water (pH = 7.9 ± 0.2, hardness = 55.2 ± 10.3 mg/L $CaCO_3$). Hardness and pH were measured every 2 days and did not vary from that of tap water during the experiment. The experiment was conducted at 25 ± 1°C, with a 12 h light/dark photoperiod under continuous aeration provided by air pumps. Dissolved oxygen was monitored daily and never dropped below 65 % saturation. The fish were fed with commercial pellets at 2.0 % body weight. Pellet remains and fecal matter were removed daily by suction throughout the experiment.

The 96 h LC_{50} value (3.05 mg/L) of Cu for Qihe crucian carp was determined by acute toxicity testing. Based on this value, the sublethal Cu concentrations selected for this study were 0 (control), 0.03, 0.06, 0.15, 0.30, and 0.60 mg/L (respectively corresponding to 0 %, 1 %, 2 %, 5 %, 10 %, and 20 % of the 96 h LC_{50}). Cu solutions were prepared using analytical-grade $CuSO_4 \cdot 5H_2O$ from Chemical Reagent Company of China ($CuSO_4 \cdot 5H_2O$ ≥98 % purity). Test concentrations were triplicated and the solutions were renewed every 24 h (static renewal). The nominal Cu concentrations were confirmed using inductively coupled plasma optical emission spectrometry (ICP-OES, Optima-2000 DV, Perkin Elmer, Shelton, Ct, USA) prior to exposure (day 0) and on the 7th and 14th days of exposure (Table 1). Accuracy of element analysis was checked by standard reference material from the Center for Standard Reference of China, Beijing, CN. The detection limit of Cu by this method was 0.30 μg/L.

To investigate the effects of different concentrations of Cu on Qihe crucian carp, 90 fish were randomly distributed into six groups, and then exposed to control and five Cu concentrations (0.03, 0.06, 0.15, 0.30, and 0.60 mg/L) respectively. Each group had 15 fish which were divided into three replicate glass tanks (60 × 50 × 40 cm^3) with 5 fish each. After a 20-day exposure, all fish were measured for body weight and length to determine growth, and then 9 fish among them (3 fish/replicate) were dissected for biochemical analyses. To evaluate the responses of Qihe crucian carp to the high Cu exposure (0.60 mg/L) and post-exposure recovery, 270 fish were randomly distributed into two groups, and then exposed to a control and the 0.60 mg/L Cu concentration. Each group had 135 fish which were assigned to three replicate plastic tanks (150 × 100 × 60 cm^3) with 45 fish each. On the first, 5th, 10th, 15th and 20th days of exposure, 15 fish from each group (5 fish/replicate) were randomly selected and measured for body weight and length, and then 9 fish among them (3 fish/replicate) were dissected. At the end of the 20-day exposure, 60 fish remained in each group. Then, Cu solutions in the tanks of the 0.60 mg/L group were replaced with clean water, and the remaining fish in the 0.60 mg/L group were used as the recovery group to conduct the recovery experiment. On the 5th, 10th, 15th and 20th days of recovery, as described above, 15 fish from each group (5 fish/replicate) were randomly collected and measured for body weight and length, and then 9 fish among them (3 fish/replicate) were dissected. The clean water and the water in controls were all dechlorinated tap water without the addition of Cu. High Cu concentration (0.60 mg/L) was chosen because this concentration may be found in some Qihe crucian carp breeding ponds in which algae have just been killed by $CuSO_4$, because the best algaecidal concentration of $CuSO_4$ is 1.5 mg/L and the Cu ion concentration is about 0.60 mg/L (Liu et al. 2007).

Fish samples were dissected on ice. The hepatopancreas and intestine were carefully removed, immediately rinsed in ice-cold physiological saline solution, and then homogenized in cold saline solution using a glass homogenizer (1 g tissue to 9 mL of buffer solution). The homogenates were centrifuged at 10,000×*g* for 10 min using a Universal 30RF centrifuge (Hettich, Tuttlingen, Germany). The supernatant was collected and stored at −80°C until enzyme activity analyses. All aforementioned operations were performed at 4°C.

Protease activity was measured according to the method of Azeez et al. (2007) using azocasein (Sigma-Aldrich, St. Louis, MO, USA) as the substrate and absorbance was read at 366 nm on a spectrophotometer. Amylase activity was determined using the method described by Rick and Stegbauer (1984). Maltose was used as the standard and absorbance was measured at 550 nm. One unit of protease

Table 1 Nominal and measured (n = 3) Cu concentrations (mg/L) during the exposure experiment

Nominal concentrations (mg/L)	Measured concentrations for each time point during the exposure experiment (mg/L) (Mean ± SD)			Means (±SE) of measured concentrations over 20 days (mg/L)	Percent differences (%)
	Day 0	Day 7	Day 14		
0.00	–	–	–	–	Not applicable
0.03	0.027 ± 0.0021	0.033 ± 0.0038	0.028 ± 0.0021	0.029 (±0.0019)	−3.33
0.06	0.060 ± 0.0044	0.061 ± 0.0031	0.065 ± 0.0026	0.062 (±0.0015)	3.33
0.15	0.153 ± 0.0047	0.160 ± 0.0042	0.158 ± 0.0042	0.157 (±0.0021)	4.67
0.30	0.281 ± 0.0032	0.302 ± 0.0040	0.279 ± 0.0040	0.287 (±0.0074)	−4.33
0.60	0.621 ± 0.0121	0.632 ± 0.0089	0.635 ± 0.0110	0.629 (±0.0041)	4.83

"–" means Cu concentrations were not detected because they were below the detection limit of Cu (0.30 μg/L)

activity was defined as the amount of enzyme that gave an increase of 0.01 in absorbance in 1 mg protein. One unit of amylase activity was defined as micromoles of maltose released per minute per milligram of protein.

SOD activity was determined according to the method of Orbea et al. (2002), which is based on the measurement of the degree of inhibition of the reduction of cytochrome c by superoxide radicals that are generated by the xanthine:xanthine oxidase system at 550 nm. CAT activity was determined according to Cakmak et al. (1993) by following the decrease in absorbance at 240 nm due to H_2O_2 consumption. One unit of SOD activity was defined as the inhibition of 50 % of cytochrome c reduction in 1 mg protein. One unit of CAT activity was defined as the quantity of enzyme that decomposes one micromole of H_2O_2 per minute per milligram of protein. The protein concentration of the supernatant solution was determined according to the method of Bradford (1976) using BSA as the standard protein.

Experimental data are presented as mean ± standard deviation. Statistical analysis was conducted using the SPSS package (version 20.0, IBM Corp, Armonk, NY, USA). One-way ANOVA was used to compare variations among different groups. Unpaired two-tailed Student's *t* test was used to analyze significant differences. The significance levels were set to $p < 0.05$ (significant difference) and $p < 0.01$ (extremely significant difference).

Results and Discussion

During the Cu exposure experiment, the average measured Cu concentrations were 0.029, 0.062, 0.157, 0.287 and 0.629 mg/L, corresponding to nominal concentrations of 0.03, 0.06, 0.15, 0.30 and 0.60 mg/L. Actual Cu concentrations of the controls were below 0.30 μg/L. The percent differences between nominal and measured concentrations were <5 % (Table 1). Nominal concentrations were used in all statistical analyses.

After the 20-day Cu exposure, body weight and length of fish declined at all Cu concentrations and significantly declined at 0.15, 0.30 and 0.60 mg/L Cu concentrations compared to the control group ($p < 0.05$) (Table 2). The results suggest that sub-lethal Cu in the aquatic environment can impair the growth of Qihe crucian carp, which is similar to many other fish species, such as *Oncorhynchu mykiss* (Hansen et al. 2002), *Xiphophorus helleri* (James et al. 2008), and *Synechogobius hasta* (Liu et al. 2010). In this study, the reduction in growth in Qihe crucian carp may be explained by reduced nutrition and energy intakes caused by decreases in digestive enzyme activity during Cu exposure. In the present study, the activities of digestive enzymes (protease and amylase) decreased in the both hepatopancreas and intestine at all Cu concentrations (Table 2; Fig. 1). Digestive enzyme activities in aquatic organisms play central roles in nutritional physiology and may directly or indirectly regulate dietary formulation and growth. Many environmental factors including salinity, pH and heavy metals may exert negative effects on digestive enzymes in aquatic organisms at unsuitable levels in water, and accordingly result in retarded growth of these organisms (Tsuzuki et al. 2007; Ye et al. 2013; Wang et al. 2015). In addition, the present study showed that fish from both the control and the 0.60 mg/L group gained weight and increased in body length during Cu exposure and post-exposure recovery, but body weight and length in the Cu-exposed fish were significantly lower than those in the controls on day 20 in exposure phase ($p < 0.05$) and on days 10 and 20 in recovery phase ($p < 0.01$), and remained less than the controls throughout the experiment (Fig. 1). These results suggest that the growth of Qihe crucian carp in this experiment was seriously deterred by the high-concentration (0.60 mg/L) and long-time (20 days) Cu exposure, and could not recover after being transferred to clean water during the 20-day recovery period.

Table 2 Changes in body weight (g) and length (cm) of Qihe crucian carp after 20 days of different Cu concentration exposures

Cu concentrations (mg/L)	Body weight (mean ± SD)	Body length (mean ± SD)
0.00	49.2 ± 3.24	12.3 ± 0.85
0.03	48.1 ± 2.71	12.0 ± 0.86
0.06	47.3 ± 2.89	11.7 ± 0.86
0.15	45.3 ± 2.50*	11.2 ± 0.84*
0.30	45.1 ± 2.60*	11.2 ± 0.64*
0.60	44.7 ± 1.88*	10.9 ± 0.49*

Data are expressed as mean ± standard deviation (n = 15)

* Significant difference ($p < 0.05$) compared with the control group

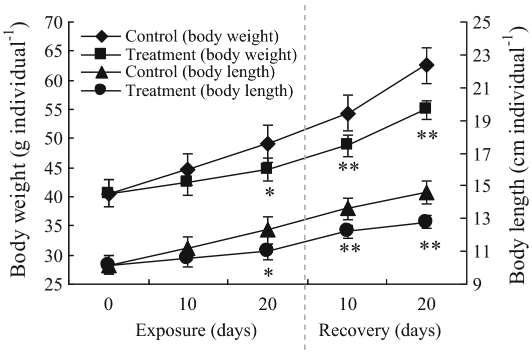

Fig. 1 Changes in body weight and length of the 0.60 mg/L group that were measured every 10 days during exposure and recovery. The values are expressed as mean ± standard deviation (n = 15). Compared with the control group, *asterisk* represents significant difference ($p < 0.05$) and *double asterisk* represents extremely significant difference ($p < 0.01$)

The hepatopancreas, a major digestive gland, can be a sensitive indicator for metabolism, nutritional status, and diseases in various aquatic organisms (Wang et al. 2008), and the intestine is a main organ closely related to digestion and absorption of nutrients. In the present study, all digestive enzyme activities decreased during Cu exposure. Protease activity in the hepatopancreas and amylase activity in the intestine significantly decreased at 0.06, 0.15, 0.30, and 0.60 mg/L Cu compared to the control group ($p < 0.01$). Protease activity in the intestine significantly decreased at 0.30 and 0.60 mg/L Cu, and amylase activity in the hepatopancreas significantly decreased at various Cu concentrations compared with the control ($p < 0.01$) (Fig. 2a, b). Some previous studies have focused on the effect of Cu on digestive enzyme activities in aquatic organisms. Li et al. (2008) observed significant inhibition of five digestive enzymes in freshwater prawn *Macrobrachium rosenbergii* by waterborne Cu exposure, in agreement with the present study. However, Li et al. (2007) reported that dietary Cu exposure increased protease activity in the hepatopancreas and lipase activity in the intestine, but markedly inhibited amylase activity in the intestine and hepatopancreas in the hybrid tilapia *Oreochromis niloticus* × *O. aureus*. This report differs from our results because of the differences in aquatic animal species and Cu exposure types used, i.e. waterborne or dietary Cu exposure.

In this study, SOD and CAT activities in hepatopancreas and intestine were also measured. Numerous reports have indicated that many contaminants, such as insecticides, oils, and phenols, can alter the SOD and CAT activities in fish depending on the organs of interest (Zhang et al. 2003, 2004; Oruc et al. 2004). In the present study, changes in the SOD and CAT activities of Qihe crucian carp under Cu exposure also displayed organ-dependent differences. SOD and CAT activities in the hepatopancreas initially increased and then decreased with increasing Cu concentration. SOD activity in the hepatopancreas significantly increased at 0.03 mg/L Cu ($p < 0.05$) and significantly decreased at 0.06, 0.15, 0.30 and 0.60 mg/L Cu compared to the control group ($p < 0.01$). CAT activity in the hepatopancreas significantly increased at 0.03 mg/L Cu ($p < 0.05$) and significantly decreased at 0.15, 0.30 and 0.60 mg/L Cu compared with the control ($p < 0.05$ or $p < 0.01$). However, SOD and CAT activities in the intestine all decreased at various Cu concentrations compared with the control group. SOD activity in the intestine significantly decreased at all Cu exposures ($p < 0.05$ or $p < 0.01$), and CAT activity in the intestine significantly decreased at 0.30 and 0.60 mg/L Cu (Fig. 2c, d). SOD and CAT can be induced by minimal oxidative stress because of compensatory effects. However, severe oxidative stress can suppress increased enzyme activity brought about by oxidative damage and loss in compensation. Higher SOD and CAT activities usually indicate that more ROS must be removed from the system (Ross et al. 2001; Chien et al. 2003). Therefore, increased SOD and CAT activities in the hepatopancreas at low Cu concentration (0.03 mg/L) indicate that Cu stress likely resulted in the accumulation of ROS, with increases in the activity levels of the enzymes for the protection of cells by scavenging surplus ROS (Fig. 2c, d). At high Cu concentrations, SOD and CAT activities in the hepatopancreas decreased (Fig. 2c, d), which may have

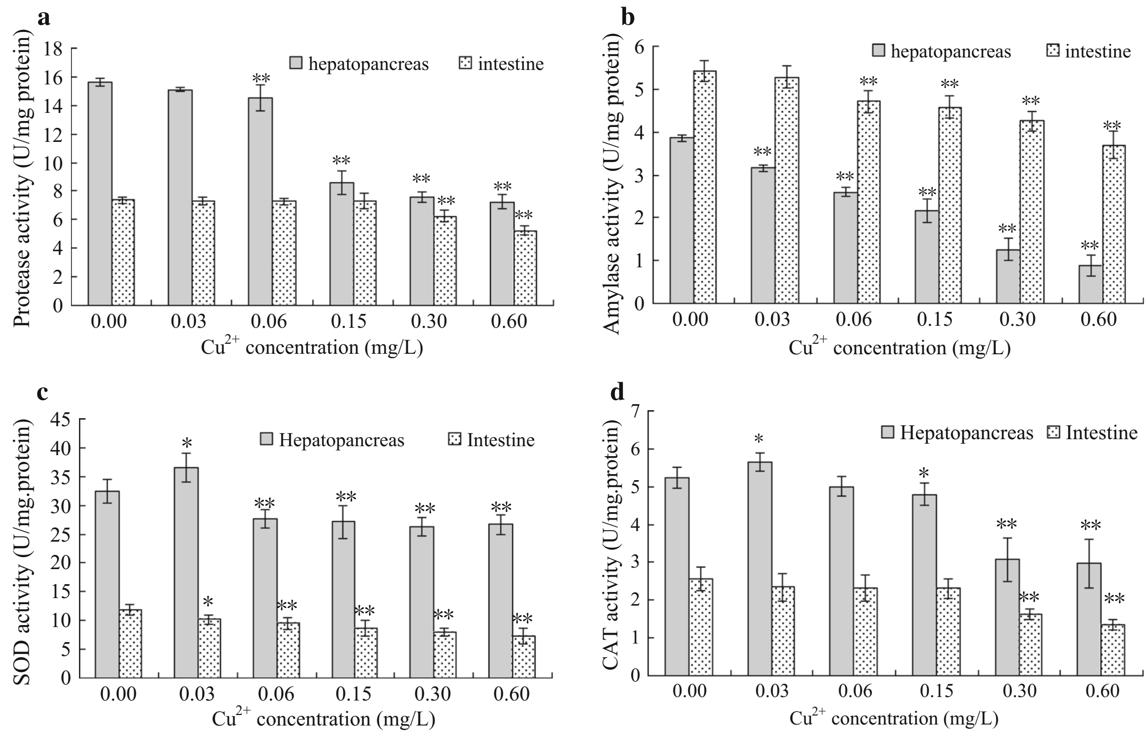

Fig. 2 Changes in digestive enzyme activities (**a**, **b**) and antioxidant enzyme activities (**c**, **d**) in hepatopancreas and intestine of Qihe crucian carp after 20 days of Cu exposure. The values are expressed as mean ± standard deviation (n = 9). Compared with the control group, *asterisk* represents significant difference ($p < 0.05$) and *double asterisk* represents extremely significant difference ($p < 0.01$)

been caused by substitution of essential metals in the active center of enzymes by excess Cu. The excess Cu may also have bound to functional groups located in enzymatic molecules, such as hydroxyl, peptidyl, and hydrosulfide groups (Von Borell 2000; Muhlia-Almazán and García-Carreño 2002). These results show that the ability of SOD and CAT to scavenge ROS is limited, in agreement with the changes in SOD activity in cadmium-treated *Oxya chinensis* reported by Li et al. (2005). SOD and CAT activities decreased in the intestine at different Cu concentrations (Fig. 2c, d), suggesting impaired antioxidant defense mechanisms resulting from the excess generation of ROS by Cu. In other words, the production of ROS overwhelmed the antioxidant system, as stated by Vutukuru et al. (2006). The discrepancy in changes of the SOD and CAT activities between hepatopancreas and intestine could be due to the difference in physiological functions of these two organs.

In the exposure phase, all digestive and antioxidant enzyme activities in the hepatopancreas and intestine of the 0.60 mg/L group significantly increased on day 1 compared to the control group ($p < 0.05$ or $p < 0.01$) (Fig. 3a–h). This increase may be an adaptive mechanism that ensures the survival of the organism at high-concentration of short-time Cu stress. All enzyme activities in the 0.60 mg/L group significantly decreased on day 20 compared the control group ($p < 0.01$) (Fig. 3a–h), suggesting the inhibitory effect of high-concentration and long-time Cu stress on enzyme activities. However, in the recovery phase, all enzyme activities were close to control levels on day 20 ($p > 0.05$) and significantly higher than those observed after 20 days of Cu exposure ($p < 0.05$ or $p < 0.01$) (Fig. 3a–h), suggesting that these enzyme activities all recovered to normal levels within 20 days after the removal of Cu stress, and attesting to the reversibility of the effects of Cu on these enzyme activities. This result was in accordance with our previous study on the reversibility of the effects of Cu on acid and alkaline phosphatase activities in freshwater fish *Carassius auratus gibelio var* (Jiang et al. 2012).

During the recovery process, the enzyme activities in the 0.60 mg/L group were restored at different rates. All antioxidant enzyme activities reached the control levels on the 15th day after the removal of Cu stress ($p > 0.05$) (Fig. 3e–h), indicating that the recovery speeds of SOD and CAT in both hepatopancreas and intestine were similar. However, protease and amylase activities in the hepatopancreas reached the control levels on the 20th day after the removal of Cu stress, while protease and amylase activities in the intestine reached the control levels on the 15th and 10th day, respectively ($p > 0.05$) (Fig. 3a–d). These data suggest that the activities of the digestive

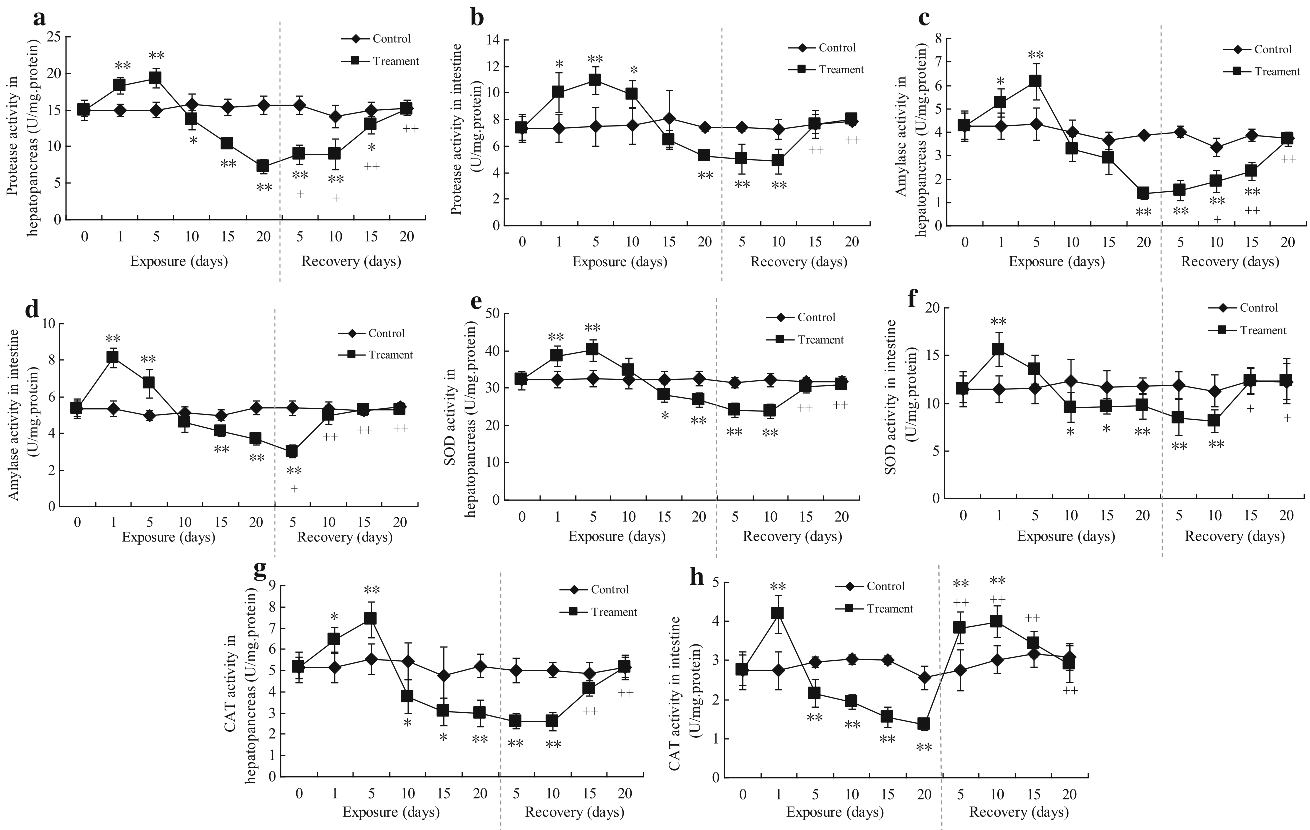

Fig. 3 Changes in the digestive enzyme activities (**a**–**d**) and antioxidant enzyme activities (**e**–**h**) in hepatopancreas and intestine of Qihe crucian carp exposed to 0.60 mg/L Cu for different days as well as subsequent recovery on different days in clean water. Data are expressed as mean ± standard deviation (n = 9). Enzyme activity unit is U/mg protein. Compared with the control group, *asterisk* represents significant difference ($p < 0.05$) and *double asterisk* represents extremely significant difference ($p < 0.01$). Compared with the 20 days exposure group, *plus* represents significant difference ($p < 0.05$) and *double plus* represents extremely significant difference ($p < 0.01$)

enzymes recovered faster in the intestine than in the hepatopancreas. This finding indicates that the intestine efficiently metabolizes or transports the toxicant to the excretory organ and ultimately facilitates its removal from fish. The intestine is used for food digestion and absorption. Decreased digestive enzyme activities in intestine in the 0.60 mg/L group after 20 days of Cu exposure may have resulted in difficulty in gaining sufficient energy to maintain normal metabolism and growth. However, the rapid restoration of digestive enzyme activities in the intestine facilitated the recovery of digestive function, enabling fish to gain energy and contribute to the restoration of enzyme activity in other organs. We believe that the recoveries of different fish organs follow a specific order after the removal of toxic stress. This restoration mechanism ensures the survival of fish.

The enzyme activities in both organs exhibited different recovery patterns in the recovery phase. Protease and amylase activities in the hepatopancreas gradually increased with increasing recovery time compared to the observed activity levels at 20 days of exposure, suggesting that Cu toxicity to digestive enzymes in the hepatopancreas was gradually weakening during the recovery period (Fig. 3a, c). Compared to activity levels after 20 days of exposure, SOD and CAT activities in the hepatopancreas, and protease, amylase and SOD activities in the intestine initially decreased and then increased with the extension of recovery time, showing the delayed toxic effects of Cu within a certain recovery time (Fig. 3b, d–g). But finally these enzyme activities were all restored to control levels on different recovery days ($p > 0.05$) and remained significantly lower than the controls before they reached control levels ($p < 0.01$) (Fig. 3a–g). CAT activities in the intestine of fish in the recovery group initially increased and then decreased compared to levels after 20 days of exposure, and were significantly higher than those in the control group on days 5 and 10 ($p < 0.01$) but close to control levels ($p > 0.05$) and significantly higher than those observed in the 20-day exposure group on days 15 and 20 ($p < 0.01$) (Fig. 3h). The change of CAT activity in the intestine was very complicated, which may be related to physiological function of fish intestine, enzymatic molecule structure of CAT, and the interaction of enzyme molecules with Cu. Further studies are needed to elucidate this pattern of change during the recovery period.

In conclusion, high-concentration Cu exposures (0.30 and 0.60 mg/L) caused significant reductions in body growth parameters of weight and length, and in digestive and antioxidant enzyme activities in juvenile Qihe crucian carp. But after fish were exposed to 0.60 mg/L of Cu for 20 days and then subsequently transferred to clean water, the digestive and antioxidant enzyme activities recovered to normal levels. However, the reduction in fish growth was not compensated for by a 20-day recovery period. The fish did not get a complete recovery in clean water after a high-concentration Cu exposure, further demonstrating the adverse effects of Cu on aquatic organisms and potential risks for aquatic ecosystems.

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