enzymes in sh 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 sh 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 suf ciently 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 sh 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 ($CuSO_4 \cdot 5H_2O$ C98 % purity). Test concentrations were triplicated and the solutions were renewed every 24 h (static renewal). The nominal Cu concentrations were con rmed 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 sh were randomly distributed into six groups, and then exposed to control and ve Cu concentrations (0.03, 0.06, 0.15, 0.30, and 0.60 mg/L) respectively. Each group had 15 sh which were divided into three replicate glass tanks (60 9 50 9 40 cm^3) with 5 sh each. After a 20-day exposure, all sh were measured for body weight and length to determine growth, and then 9 sh among them (3 sh/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 sh were randomly distributed into two groups, and then exposed to a control and the 0.60 mg/L Cu concentration. Each group had 135 sh which were assigned to three replicate plastic tanks (150 9 100 9 60 cm^3) with 45 sh each. On the rst, 5th, 10th, 15th and 20th days of exposure, 15 sh from each group (5 sh/replicate) were randomly selected and measured for body weight and length, and then 9 sh among them (3 sh/replicate) were dissected. At the end of the 20-day exposure, 60 sh 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 sh 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 sh from each group (5 sh/replicate) were randomly collected and measured for body weight and length, and then 9 sh among them (3 sh/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 9 *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 de ned as the amount of enzyme that gave an increase of 0.01 in absorbance in 1 mg protein. One unit of amylase activity was de ned 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 de ned as the inhibition of 50 % of cytochrome c reduction in 1 mg protein. One unit of CAT activity was de ned 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 signi cant differences. The signi cance levels were set to $p < 0.05$ (signi cant difference) and $p < 0.01$ (extremely signi cant 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 sh declined at all Cu concentrations and signi cantly 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 sh 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 sh 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 sh were signi cantly 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)

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

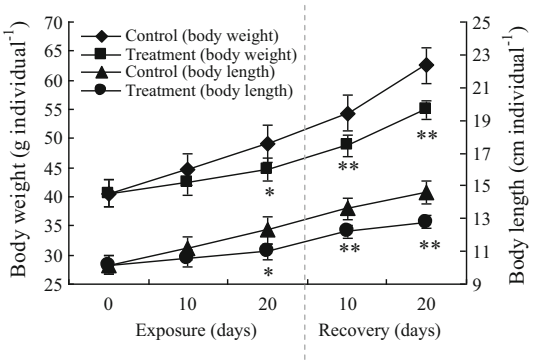

Fig. 1 Changes in body weight and length of the 0.60 mg/L group that were measured every 10 days during exposure and recovery. The values are expressed as mean ± standard deviation (n = 15). Compared with the control group, *asterisk* represents signi cant difference ($p < 0.05$) and *double asterisk* represents extremely signi cant 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 signi cantly 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 signi - cantly decreased at 0.30 and 0.60 mg/L Cu, and amylase activity in the hepatopancreas signi cantly 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 signi cant inhibition of ve 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* 9 *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 sh 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 signi cantly increased at 0.03 mg/L Cu ($p < 0.05$) and signi cantly 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 signi cantly increased at 0.03 mg/L Cu ($p < 0.05$) and signi cantly 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 signi cantly decreased at all Cu exposures ($p < 0.05$ or $p < 0.01$), and CAT activity in the intestine signi cantly 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

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 hydrosul de groups (Von Borell 2000; Muhlia-Almazán and Garc´a-Carreno 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 signi cantly 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 signi cantly decreased on day 20 compared the control group ($p < 0.01$) (Fig. 3a–h), sug-

the intestine was very complicated, which may be related to physiological function of sh 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 signi cant reductions in body growth parameters of weight and length, and in digestive and antioxidant enzyme activities in juvenile Qihe crucian carp. But after sh 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 sh growth was not compensated for by a 20-day recovery period. The sh 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.

Acknowledgments This work was sponsored by the Program for Science and Technology Innovation Talents in Universities of Henan Province (Grant No. 2011HASTIT012) and the Key Projects of Science and Technology Research of the Department of Education in Henan Province (Grant No. 13A240509). This study was also partly supported by the Key Subjects of Fisheries in Henan Province, China.

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