

# Effects of Acute Exposure to Sublethal Waterborne Cadmium on Energy Homeostasis in Silver Carp (*Hypophthalmichthys molitrix*)

Jie Pi<sup>1</sup> · Xuelin Li<sup>1</sup> · Ting Zhang<sup>2</sup> · Deliang Li<sup>1</sup>

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**Abstract** Effects of acute exposure to sublethal waterborne cadmium (Cd) on energy homeostasis in filter-feeding fishes have rarely been studied. The response patterns of energy substances were investigated in juvenile silver carp (*Hypophthalmichthys molitrix*) exposed to sublethal waterborne Cd for 96 h. The results showed the 96hLC<sub>50</sub> of Cd on juvenile silver carp was 1.723 mg/L. Sublethal acute exposure of Cd significantly affected the energy homeostasis of juvenile silver carp, including increase in plasma glucose and lactate, and decrease in plasma triglyceride, muscle glycogen and triglyceride and liver glycogen. The results indicated that glycogen and triglyceride prior to protein were mobilized to meet the increased demands for detoxication and repair mechanism to sublethal waterborne Cd exposure, and glycogen level depleted faster and restored slower in the liver than in the white muscle in juvenile silver carp.

**Keywords** Cadmium · *Hypophthalmichthys molitrix* · 96hLC<sub>50</sub> · Energy homeostasis · Sublethal exposure

✉ Ting Zhang  
zhangting@csu.edu.cn

✉ Deliang Li  
lideliang80@aliyun.com

<sup>1</sup> Collaborative Innovation Center for Efficient and Health Production of Fisheries in Hunan Province, Hunan Engineering Technology Research Center of Featured Aquatic Resources Utilization, College of Animal Science and Technology, Hunan Agricultural University, Changsha 410128, China

<sup>2</sup> Center of Environment and Water Resources, College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China

More and more attention has been attracted to the ecological and public health problems caused by the heavy metals pollution (Bendell 2010; Ings et al. 2012; Sadeghi et al. 2015; Sfakianakis et al. 2015). Cadmium (Cd) is one of the major and most widespread groups of heavy metal contaminants because of its persistent nature and slow elimination from environmental compartments (Annabi et al. 2009; Sandhu et al. 2014). This metal can enter the environment from natural sources, such as weathering of minerals, forest fires and volcanic emission, and especially from various anthropogenic sources, such as coal combustion, mine wastes, electroplating processes, iron and steel production, pigments, fertilizers and pesticides (Garcia-Santos et al. 2013). All the Cd ultimately deposits into the aquatic systems, then creates potential hazards to aquatic organisms (Cao et al. 2010; Rana 2014).

Exposures to heavy metals (including Cd) are considered as stressful conditions imposed on fishes. Increments in plasma cortisol and glucose were usually used as meaningful indicators of stressful condition of fishes (Mommensen et al. 1999; Barton 2002). Under stressful conditions, energy allocation of fishes was disturbed, because a large proportion of the ingested energy allocated primarily to maintenance is reassigned to invest in detoxification and repair mechanisms (Campbell et al. 2002). These processes resulted in rapid decrease of energy storage such as ATP, phosphocreatine (PCr) and glycogen, and rapid accumulation of blood and muscle lactic acid (Zhang et al. 2013). Moreover, these modifications in energy allocation caused by heavy metals exposure may provoke functional deficiencies in fishes, which may affect their fitness ultimately (Weis et al. 1999, 2001). Therefore, it is important to understand how exposure to heavy metals can affect their energy budgets. Although several studies have analyzed the effects of Cd exposure on the energy

metabolism of carbohydrate, lipids and protein in freshwater and marine fish (Brown et al. 1990; Soengas et al. 1996; Ferrari et al. 2011; Preto et al. 2014), information on filter-feeding fish such as silver carp (*Hypophthalmichthys molitrix*) is only poorly documented.

Silver carp is not only an economically important fish, but also a model organism for non-traditional biomanipulation to control cyanobacterial bloom in China (Liu and Xie 2003). The Yangtze River and its accessory lakes are the most important habitats of this anadromous specie, in which the various levels of heavy metals occurred in alongshore-aquatic areas with the predominant elements of Cd (Yi et al. 2008). It is necessary to study the effect of sublethal Cd on the silver carp, especially for understanding the physiological, biochemical changes and energy homeostasis, which will advance our understanding of Cd effects.

The first purpose of the present study was to define the 96hLC<sub>50</sub> value for waterborne Cd (CdCl<sub>2</sub>) in juvenile silver carp, and compare the tolerance of this specie with other teleost; the second purpose was to evaluate the effects of acute exposure to sublethal waterborne Cd on energy homeostasis in juvenile silver carp. All these results will help for predicting the possible effects of environmentally relevant Cd on the natural resources of silver carp in the Yangtze River reaches (Zhang et al. 2013).

## Materials and Methods

The juvenile silver carps from the same batch of fertilized eggs, were obtained from a fish hatchery in Xiangtan City, Hunan Province, China. All fishes were kept in a rectangular rearing pond (length × width × water depth: 22 m × 17 m × 1.2 m) with abundant phytoplankton at the Hunan Agricultural University, and were exposed to seasonal temperature. The phytoplankton community was dominated by *Microcystis aeruginosa*, *Anabaena circinalis*, *Crucigenia apiculata*, *Scenedesmus quadricauda*, *Cryptomonas ovata* and *Synedra acus*. The fishes were held for at least 2 months before the experiment. Experiments were carried out in accordance with the ethical guidelines of Hunan Agricultural University for the care and use of laboratory animals.

A static bioassay was performed to determine the 96hLC<sub>50</sub> value of CdCl<sub>2</sub> on silver carp. Three hundred and twenty four size matched silver carps (8.00 ± 0.05 cm in body length, 5.40 ± 0.01 g in wet weight) were selected from the stock pond and randomly put into 18 identical rectangular experimental tanks (60 cm × 40 cm × 50 cm). Eighteen fishes were assigned to each tank with 50 L aeration tap water. After 2 days of acclimation without feeding, the fishes were exposed to each of nine nominal

concentrations of Cd (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L) by diluting the stock solution (10 g Cd/L) prepared in ultrapure water. All treatments were carried out in duplicate. CdCl<sub>2</sub>·2.5H<sub>2</sub>O was of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (China). During the 96 h exposure period, the water did not be refreshed and no feed was provided. Mortality of fishes in each tank was recorded every 24 h, and the corpses of silver carp were removed timely. The actual Cd concentrations of 9 treatments was 0.006, 0.49, 0.99, 1.44, 1.88, 2.41, 2.93, 3.42 and 3.88 mg/L, respectively. Water quality (temperature, pH and DO) was recorded daily (T 28.38 ± 0.17°C, pH 7.25 ± 0.03, DO 7.82 ± 0.02 mg/L). The photoperiod was artificially controlled (12D:12L).

Three sublethal concentrations of Cd were as follows: 0.43 mg/L (low concentration exposure group, LC), 0.86 mg/L (medium concentration exposure group, MC) and 1.29 mg/L (high concentration exposure group, HC), corresponding to 25 %, 50 % and 75 % of 96hLC<sub>50</sub>, respectively. Two hundred size matched fishes (8.09 ± 0.05 cm in body length, 11.36 ± 0.19 g in wet weight) were selected and randomly placed into eight identical rectangular tanks (78 cm × 57 cm × 60 cm). Twenty five fishes were assigned to each tank with 100 L aeration tap water. After 2 days of acclimation without feeding, the fishes were exposed to each of four treatments. Each treatment group was duplicated. Three nominal concentrations of Cd were prepared by diluting the stock solution (5 g Cd/L) prepared in ultrapure water, respectively. The actual Cd concentrations of four treatments was 0.006, 0.46, 0.94 and 1.35 mg/L, respectively. During the 96 h exposure period, the water did not be refreshed and no feed was provided. Water quality (temperature, pH and DO) was recorded daily (T 24.74 ± 0.02°C, pH 6.52 ± 0.20, DO 8.04 ± 0.06 mg/L). The photoperiod was artificially controlled (12D:12L).

On each sampling time (24, 48, 72 and 96 h), four fishes from each tank were removed quickly with a dip net and anesthetized absolutely by clove oil. Blood samples were taken by puncturing caudal vein with heparinized needles into heparinized plastic tubes on ice. Plasma was separated by centrifugation (3 min at 10,000g) and aliquots were immediately frozen in liquid nitrogen and stored at −80°C. Slices of white muscle were removed from between the anterior insertion of the dorsal fin and the lateral line, freeze-clamped in liquid nitrogen, and stored at −80°C until later analysis. A small part of liver was excised and stored at −80°C for later analysis.

Water samples were filtered through a 0.45 μm Millipore membrane filter and acidified to pH < 2 with 2 % HNO<sub>3</sub> solution (v/v). Cd in water samples were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700x, Tokyo, Japan). For each batch of

prepared samples, a method blank was carried throughout the entire sample preparation. All analytical data were subjected to strict quality assurance and control. The precision was assessed by spiking method blank and the highest sample with 0.5 mg/L of Cd. Five independent replicate determinations were performed to estimate relative standard deviation (RSD) and recovery. The corresponding recoveries of Cd obtained were  $99.0\% \pm 2.8\%$  in method blank, and were  $102.2\% \pm 2.1\%$  in the highest water sample. Detection limits for Cd were 0.7 µg/L.

Cortisol was tested by the method of enzyme-linked immune-sorbent assay (ELISA). Other biochemical parameters were all tested by the method of ultraviolet spectrophotometry. All parameters were measured using the commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

All values were expressed as mean  $\pm$  SE. The 96hLC<sub>50</sub> values were determined by the SPSS 17.0 software (IBM, USA). Differences among exposure groups in fish weight and length were analyzed using one-way ANOVA. Physiological parameters at each sample time were statistically compared with values of the control group using one-way ANOVA with post hoc Duncan test. All comparative analyses were carried out using Statistics 6.0 software (StatSoft, Inc., Tulsa, OK, USA), with  $p < 0.05$  considered statistically significant. The figures were created using Origin 8.5 software (OriginLab Corp., Northampton, MA, USA).

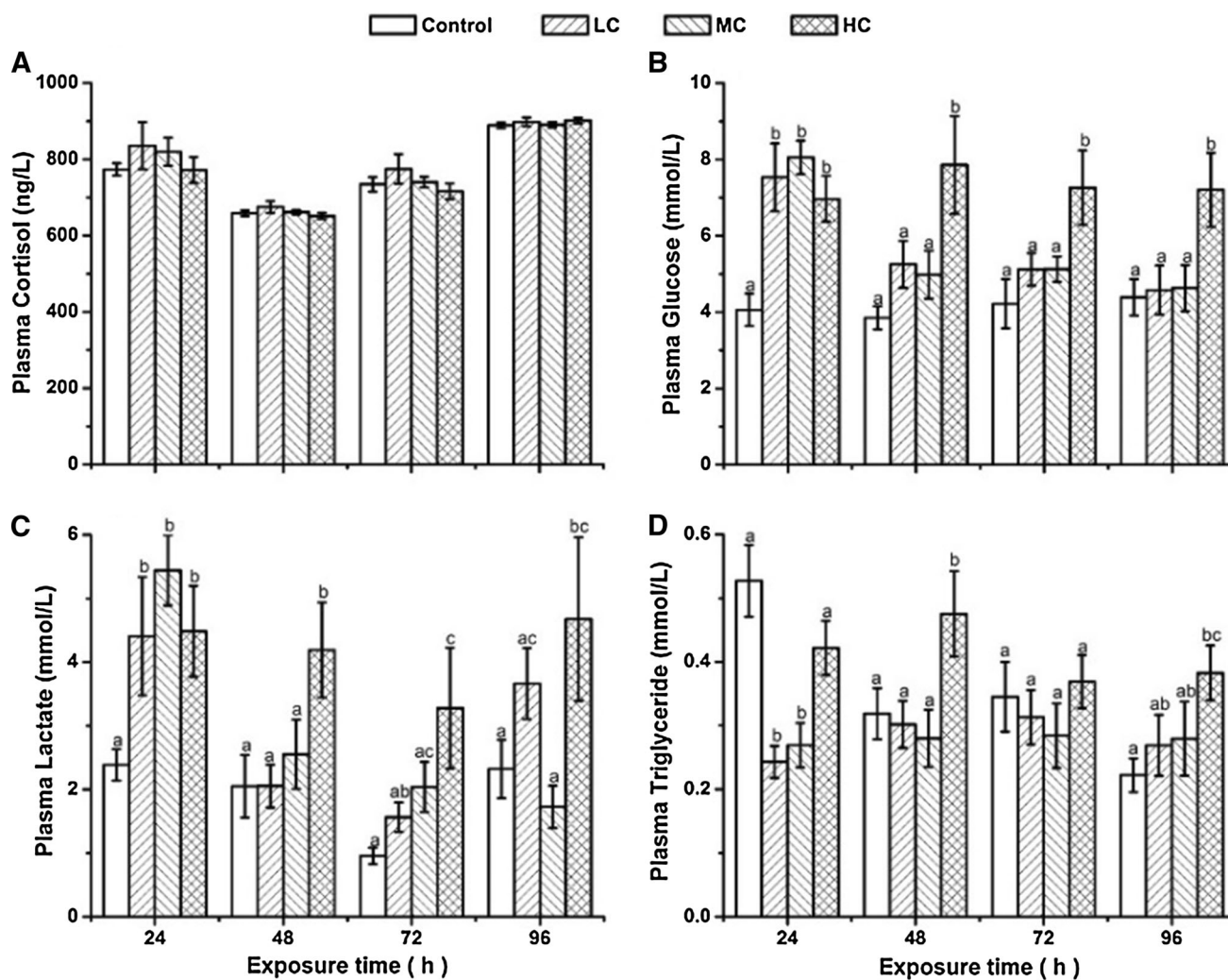
## Results and Discussion

At the end of 96 h exposure, the mortality of different exposure groups (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg Cd/L) was 0, 0, 2.78 %, 11.11 %, 86.11 % and 97.22 %, respectively and all fishes were dead in 3.0, 3.5 and 4.0 mg Cd/L exposure groups. The 96hLC<sub>50</sub> of Cd on juvenile silver carp was determined to be 1.723 mg Cd/L. It was lower than that in smaller silver carp (about 6.5 cm, 2.80 mg/L) (Yin 1979), bighead carp (*Aristichthys nobilis*) ( $6.48 \pm 0.44$  cm, 2.25 mg/L) (Chen 1991), smaller (4.10 g, 24.05 mg/L) and larger grass carp (*Ctenopharyngodon idellus*) ( $10.0 \pm 2.0$  cm, 3.49 mg/L) (Hou and Ma 2002; Wang et al. 2007). Although direct comparison cannot be made due to lack of similar information on black carp (*Mylopharyngodon piceus*), it may indicate that silver carp is more sensitive to Cd than the other three major Chinese carps. However, the acute toxicity of Cd on black carp should be carried out to broaden our understanding on tolerance of Chinese major economically important species.

During the sublethal exposure period, two fishes were dead in control and high Cd exposure group, respectively.

The increments in plasma cortisol and glucose were usually used as meaningful indicators of stressful condition of fishes (Mommensen et al. 1999; Barton 2002). Plasma cortisol and glucose increasing significantly after Cd exposure have been reported in many fishes such as rainbow trout (*Oncorhynchus mykiss*) (Hontela et al. 1996), Persian sturgeon (*Acipenser persicus*) (Zahedi et al. 2013). The present results showed plasma glucose of all exposed groups increased significantly compared to control group after 24 h exposure, although the significant differences were only observed in high concentration exposure group from then on (Fig. 1b), which indicated that acute exposure to sublethal Cd made juvenile silver carp in stressed condition. However, the cortisol in plasma was not altered during 96 h exposure (Fig. 1a). It is generally recognized that increased plasma glucose level found in stressed fishes was sustained by increased cortisol level after the initial catecholamine-induced increase in response to stressors (Mommensen et al. 1999; Barton 2002; Chowdhury et al. 2005). So the unchanged plasma cortisol in exposed groups may be explained by the likelihood that the cortisol reached the peak value and restored to normal before 24 h which was not detected with the sampling protocol time-course used. The increased plasma glucose was the lagged gluconeogenesis and/or glycogenolysis effects induced by cortisol's permissive and stimulatory actions on epinephrine or glucagon (Sapolsky et al. 2000; Garcia-Santos et al. 2015). However, some reports also showed that cortisol is increasingly induced several hours after Cd stress, and restored to normal with several days (Wu et al. 2006, 2007; Lin et al. 2011). It may be related to the cortisol's suppressive actions. That is because it was suggested that the physiological function of stress inducing increases in cortisol was considered to be against the normal defense reactions activated by Cd through turning off those defense reactions, then preventing them from overshooting and threatening homeostasis (Sapolsky et al. 2000; Wu et al. 2006). Plasma lactate of silver carp, as the main end product of anaerobic metabolism, increased significantly in high concentration exposure group for all exposure period, but only for the first 24 h in two other exposed groups, which showed a typical dose-dependent effect (Fig. 1c), and may indicate metabolic disorders and a severe respiratory stress in the tissue (Pretto et al. 2014).

There were no significant differences in white muscle cortisol between any treatment groups and control group (control group:  $617.25 \pm 71.09$  ng/L, treatment groups:  $581.65 \pm 41.17$  ng/L,  $p > 0.05$ ), which may be reflected the accumulation of cortisol in white muscle did not be detected due to the rapid response pattern of plasma cortisol. Previous studies showed that 96 h Cd exposure steadily depleted the muscle ATP with a marked decrease to about half of the control values (Zhang et al. 2013).



**Fig. 1** Time course of changes in plasma constituents of juvenile silver carp exposed to sublethal waterborne Cd. **a** Cortisol; **b** glucose; **c** lactate; **d** triglyceride. The values are presented as the mean  $\pm$  SE

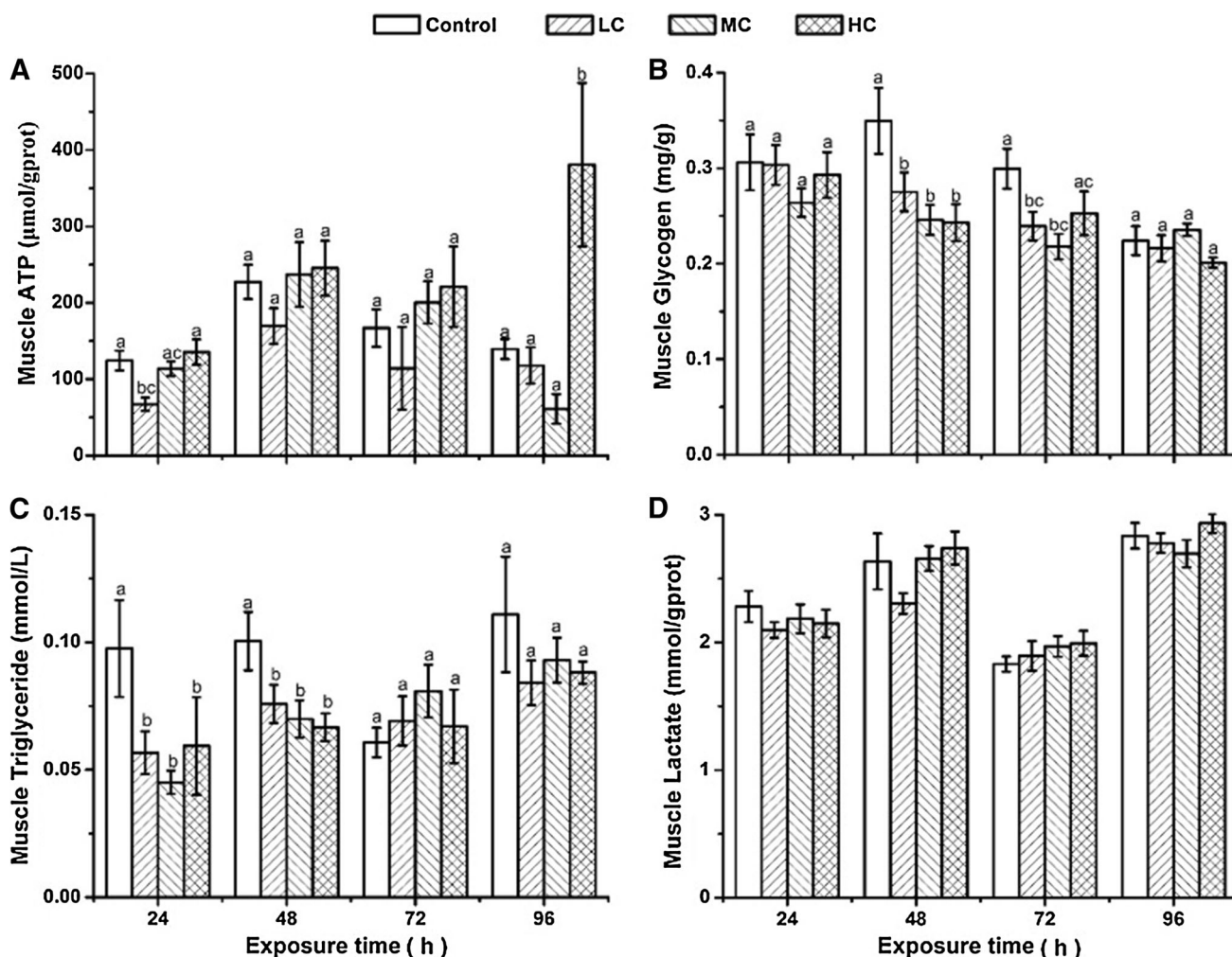
( $n = 8$ ). The different letters at each sampling time represent significant difference ( $p < 0.05$ ) among groups

Exposure to Cd did not produced significant declines in white muscle ATP of treatment groups except for lower concentration group at 24 h in the present study. Conversely, significantly higher concentration of white muscle ATP was observed at the end of 96 h exposure (Fig. 2a). The discrepancy may be partly attributed to differences on the exposure dose used, since it was approximately 430–1290 fold higher than that (0.01 mg/L) in former study (Zhang et al. 2013). However, the significant increase of white muscle ATP in high concentration exposure group at the end of exposure period was unexplainable.

A key metabolic role for muscle and liver is the production of glucose by glycogenolysis and/or gluconeogenesis, to meet the tissue energetic requirements in response to increased energy demands during the stressed condition (Vijayan et al. 1994, 2003). In the present study, the glycogen in white muscle of exposure groups was

decreased with time during the first 72 h of exposure (Fig. 2b). During the first 48 h, exposure to Cd also elicited significant decreases in liver glycogen in all exposed groups. From then on, the liver glycogen restored to the control level in low concentration exposure group, but significantly lower in medium and high concentration exposure groups than that in control group, which showed typical dose-dependent response (Fig. 3). The clear fall of glycogen in white muscle and liver was in agreement with previous reports in sea bass (*Dicentrarchus labrax*) (Cattani et al. 1996), Atlantic salmon (Soengas et al. 1996), tilapia (Lin et al. 2011). It also supports that the mobilization of glycogen in white muscle and liver is a common response in fish exposed to heavy metals and stressful pollutants to cope with the enhanced energy demand for detoxification and repair (Cattani et al. 1996). The sharply decrease of liver glycogen immediately occurred at 24 h



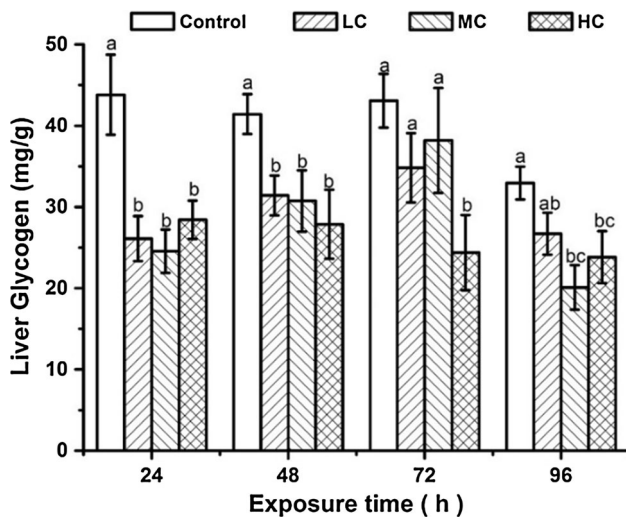


**Fig. 2** Time course of changes in muscle constituents of juvenile silver carp exposed to sublethal waterborne Cd. **a** ATP; **b** glycogen; **c** triglyceride; **d** lactate. The values are presented as the mean ± SE

(n = 8). The different letters at each sampling time represent significant difference (*p* < 0.05) among groups

and continued until 96 h, depending on the exposure dose. Otherwise, the similar downward trend of white muscle glycogen persisted from 48 to 72 h, and recovered to control level at 96 h. Then the different responding patterns between these two tissues may draw a conclusion that the glycogen level depleted faster and restore slower in the liver than in the white muscle when exposed to waterborne Cd. This conclusion may be supported in part by the different capacity between liver and white muscle for glycogen metabolism in fishes. Liver plays a major role in maintaining the plasma glucose via glycolysis and/or gluconeogenesis, while the white muscle glycogen is just used via glycolysis and restored via lactate-based in situ glycogenesis due to the minor direct contribution of blood glucose (Milligan 1996). Moreover, further studies should be performed to confirm this hypothesis and clarify the underlying mechanism.

Act as a major energy store, lipids support various physiological activities, developmental and reproductive processes in fishes (Polakof et al. 2010). The significant decreases were observed in white muscle triglyceride of exposure groups after 24 and 48 h exposure (Fig. 2c). It suggested that the Cd exposure induced the lipid catabolism and inhibited lipids synthesis in white muscle of juvenile silver carp. During the first 24 h of exposure, the plasma triglyceride of low and medium concentration groups showed similar declining trend which may be mobilized from white muscle. It is interesting that the plasma triglyceride content in the high concentration group began to increase significantly from 48 to 96 h, when the muscle triglyceride had restored the control level. Previous studies indicated the hepatic lipid content of yellow catfish (*Pelteobagrus fulvidraco*) (Chen et al. 2013) and javelin goby (*Synechogobius hasta*) (Huang et al. 2014) was



**Fig. 3** Time course of changes in muscle triglyceride of juvenile silver carp exposed to sublethal waterborne Cd. The values are presented as the mean  $\pm$  SE ( $n = 8$ ). The different letters at each sampling time represent significant difference ( $p < 0.05$ ) among groups

increased with increasing waterborne Cd, Cu and Zn levels. Thus, whether the increased plasma triglyceride content in high concentration exposure group was synthesized and transported from liver, it should be clarified further. There were no significant alteration in white muscle lactate during 96 h exposure (Fig. 2d). It may be that silver carp used muscle lactate as the primary substrate in situ for the glycogen resynthesis to meet the extra need of the energy for detoxification and repair (Milligan 1996).

Proteins are the major constituents in the metabolism of animals (De Smet and Blust 2001) and play a central role in the energy production during the stress caused by toxicants (Ferrari et al. 2011). Several studies showed that fishes present a significant decrease in protein levels when exposed to Cd or other heavy metals (Almeida et al. 2001; Pretto et al. 2014), while others showed that waterborne Cd can increase the synthesis of metallothionein (Cattani et al. 1996; De Smet and Blust 2001) and total protein contents (Zhang et al. 2013). Exposure to sublethal Cd did not induce any significant alternations in plasma protein (control group:  $18.62 \pm 0.71$  g/L, treatment group,  $17.81 \pm 0.43$  g/L,  $p > 0.05$ ) and white muscle protein (control group:  $0.38 \pm 0.01$  g/L, treatment groups:  $0.37 \pm 0.01$  g/L,  $p > 0.05$ ) in the present study. Similar results have also been reported in carp (*Cyprinus carpio*) (De Smet and Blust 2001). It may be suggested that a balance was reached between the degradation and synthesis of proteins in juvenile silver carp exposed to acute sublethal waterborne Cd (Pretto et al. 2014). In addition, some results showed that protein usually was spared during chronic period of pollutant stress (Garg et al. 2009). So the

decreased glycogen and triglyceride in muscle and liver coupled with maintenance of plasma and muscle protein may suggest that extensive protein depletion did not occur due to the ample energy supplied by glycogen and lipids (Almeida et al. 2001).

In conclusion, during acute exposure to lethal waterborne Cd, silver carp met the high basic metabolic energy demand (presumable for detoxification and damage repair) by breaking down tissues energy reserves (glycogen and triglyceride stores), and accompanied with hyperglycemia and hyperlactatemia. In order to maintain the energy hemostasis, silver carp should increase their rate of food consumption and/or change the energy allocation at the expense of growth and reproduction which may affect their fitness ultimately (Sokolova et al. 2012). However, further study should be carried out to confirm and clarify.

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