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## Changes of glycogen metabolism in the gills and hepatic tissue of tilapia (*Oreochromis mossambicus*) during short-term Cd exposure

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### ABSTRACT

The aim of the study was to test the hypothesis that the mechanism of glycogen metabolism has taken place in gills rather than in liver during Cd exposure. Male tilapia were exposed to 44.45  $\mu\text{M}$  ambient Cd for 12 h, and we found blood glucose significantly increased, however, lactate levels showed no significant changes. The glycogen phosphorylase (GP) activity increased immediately after 0.75 to 3 h of Cd exposure in the gills, and after 1 to 6 h in the liver, respectively. In addition, the glycogen level depleted faster in the gills than in the liver. Plasma cortisol level increased from 0.25 to 1 h and recovered after 3 h, while the glucagon did not significantly change during Cd exposure. Glucocorticoid receptor (GR) mRNA expression decreased after 0.75 h in the gills, while it significantly increased after 6 h in the liver.  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$  significantly decreased upon Cd exposure within 6 h following Cd-induced toxic stress. We suggested that the cortisol is the spontaneous stimulation of glycogen metabolism in the gills, and it triggers a subsequent energy supply later in the liver. Taken together, the profile of glycogen metabolism between gills and liver during Cd-exposure stress provide good support to our hypothesis.

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### 1. Introduction

Animals increase blood glucose levels and regulate energy metabolism in response to stress. Glycogen metabolism is the principal energy source in both vertebrates and invertebrates, especially during responses to environmental fluctuations and stress (Bacca et al., 2005). Glucose (or glucose 6-phosphate) is released through the degradation of glycogen by glycogen phosphorylase (GP) (Roach et al., 1998), and energy is mainly supplied by the oxidation of glucose and lactate as a result of carbohydrate metabolism (Morgan et al., 1997).

Heavy metals, including cadmium (Cd), exert a wide range of pathological effects on fish (Iger et al., 1994). Teleosts are highly sensitive to Cd, as decreased growth rates were commonly observed due to the impairment of growth hormone activation by Cd (Jones et al., 2001). In addition, stress responses upon Cd exposure including increases in metallothionein expression, lysozyme content, and cortisol levels all need energy costs (Wu et al., 2007). Generally speaking, heavy metals cause changes in metabolic rate (oxygen consumption), but the direction of the effect varies with the metal: some stimulate, while others inhibit (Heath, 1995). However, the profile of energy support

system in gills and hepatic tissues during hours of Cd exposure remains unclear.

In addition to functioning in ion/osmotic regulation, acid–base balance, and gas exchange (Evans et al., 2005), gills are also the first line of defense against ambient heavy metal accumulation (McDonald and Wood, 1993). However, following heavy metal stress, there was a rise in the number of mucus-secreting cells (Wu et al., 2007), and a down-regulation in  $\text{Ca}^{2+}$ -ATPase activity (Wong and Wong, 2000). The functional morphology of chloride cells (CC) changed after Cd exposure during development of tilapia (*O. mossambicus*) (Lee et al., 1996; Wu, 2000). Various ion transporters and enzymes in CC of gills were also affected by heavy metals (Hirose et al., 2003). Tseng et al. (2007) reported that a group of cells rich in glycogen deposits, called glycogen-rich cells, were neighbored with CC. These cells expressed a specific form of GP in tilapia gills. In their study, both GP activity and protein expression in gills were up-regulated in tilapia within 1–3 h after acute transfer from fresh water to 2.5% seawater. On the contrary, glycogen levels in both gills and liver were significantly depleted after their transfer to seawater, but the depletion occurred significantly earlier in gills than in the liver. These data suggested that glycogen-rich cells are initially stimulated to rapidly provide energy for neighboring CC that triggers ion-secretion mechanisms (Chang et al., 2007; Tseng et al., 2008). Several species—including tilapia, goldfish, and rainbow trout—all experienced a modest decrease in plasma  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Cl}^-$

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under Cd exposure (Heath, 1995). It is known that heavy metal exposure causes a rather rapid decrease in plasma electrolytes and/or osmolality in fish. We have thus hypothesized that there might be a similar profile of glycogen metabolism upon Cd exposure like the salinity stress, which also has the GP activation and glycogen depletion occurring significantly earlier in gills than in the liver.

Heavy metal-induced stress is a state characterized by a specific syndrome that is induced and causes changes within a biological system. The costs of heavy metal-induced stress are associated with three phases of stress: alarm, adaptation, and exhaustion; and the alarm phase involves immediate increases in catecholamine and cortisol levels (Newman, 1995). Both endocrine pathways trigger the release of additional energy required for stress acclimation. Catecholamine is known for its role in the activation of hepatic GP and the inhibition of pyruvate kinase (PK), which increases glycogenolysis and gluconeogenesis while reducing glycolysis (Reid et al., 1992). Similarly, cortisol might have a modulatory effect on the catecholamine-stimulated adenyl cyclase system in trout hepatocytes (Reid et al., 1992). It is well known that cortisol is a classic indicator of stress in fish. Cortisol played multiple functions during stress, such as ion balance regulation (Mommsen et al., 1999); enhancement of  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  activity by stimulating CC differentiation (Veillette and Young, 2004); suppression on immune response and induce in metallothionein (Wu et al., 2006); and energy metabolism during stress (Potlunt and Tort, 1997). As regards to the effect of Cd on cortisol levels, some previous studies found that cortisol was induced upon Cd exposure (Hontela et al., 1996; Wu et al., 2006; 2007). However, there were other studies that did not find cortisol to be induced by Cd treatment (Pelgrom et al., 1995; Dang et al., 2001). The cortisol levels were not the same during Cd treatment in fish, and the cause of that may be due to Cd developing a pathological and stress responses from fish. It is possible that dietary Cd accumulates more slowly and to a lesser extent in fish gills than does waterborne Cd. Certain Cd doses fed to fish did not induce a stress response at all (Dang et al., 2001). Besides, Cd might impact the HPI (hypothalamic–pituitary–interrenal); it significantly suppressed ACTH-stimulated cortisol production (Sandhu and Vijayan, 2011). These findings result in more enigmatic roles of cortisol after Cd treatment in fish.

Glycemia is also one of the classic plasma indicators of stress in fish (Roche and Bogé, 1996). Plasma glucose was raised from fish exposed to Cd (Hontela et al., 1996; Wu et al., 2007). Cd exposure also induced increased glucose concentration in white muscle of fish (Almeida et al., 2001). The glucose concentration was proposed to be mediated by endocrine release such as cortisol (Pelgrom et al., 1995; Hontela et al., 1996). However, in cortisol and plasma glucose content, both were nonspecific stress responses as they did not exhibit a parallel change during the time course experiment. For example, the stressed juvenile common carp (*Cyprinus carpio*) did not change its cortisol and glucose levels compared to the pre-challenge values (Hosseini and Hoseini, 2010). When exposed to 3.67  $\mu\text{M}$  Cd for 2 h, the rainbow trout (*Oncorhynchus mykiss*) had a significant increase in plasma cortisol levels and glucose (Hontela et al., 1996). Thus, the level of blood glucose was probably mediated by other endocrine release during Cd stress and not only by cortisol.

Glucagon is another powerful metabolic hormone, often opposing the actions of insulin, regulating glucose metabolism. It is involved in the regulation of hepatic glycogenolysis and gluconeogenesis in fishes (Moon, 1998). *In vitro*, glucagon exposure increased GP activity 3.1-fold in fish hepatocytes (Hallgren et al., 2003). So far, it was not clear about the role of glucagon on glycogen metabolism during metal treatment in fish. We therefore measured the changes of cortisol and glucagon in order to confirm their roles on glucose releasing and GP activation within hours of Cd exposure in the present study.

In fish, cortisol plays a dual role in carbohydrate and mineral metabolism in fish. Bury and Strum (2007) reported that corticosteroids are mediated *via* corticosteroid receptors, which include the

glucocorticoid (GR) and mineralocorticoid receptors (MR). Both of them showed distinct expression patterns and transcriptional activities among tissues (Greenwood et al., 2003). But, there were many researchers who reported that the cortisol signaling in teleosts is thought to be mediated predominantly by the GR (Mommsen et al., 1999; Vijayan et al., 2003), and its expression appeared to be a tissue-specific GR transcript response in the gill and liver (Singer et al., 2007). Our previous study suggested that the multiple functions of cortisol leads to all target tissues including gills, liver, kidneys, and intestines for this hormone (Wu et al., 2005). In addition, it is known from previous studies that GR signals appeared more in chloride, pavement, respiratory and undifferentiated cells in Cd treated fish (Dang et al., 2001). It was suggested that GR expression in gills and liver was related with induction of metallothionein (one of metal binding protein) synthesis by cortisol since it occurs only after cortisol binding to GR (cited by Dang et al., 2001). Cortisol treatment *in vivo* and *in vitro* results in significant decreases in hepatic GR protein levels; but increase in hepatic GR mRNA levels suggested an autoregulation of hepatic GR mRNA by GR protein abundance (Vijayan et al., 2003). Hence, GR mRNA expression was related to the activation of cortisol.

As described above, the gills are the first target organ affected by metal exposure in fish (McDonald and Wood, 1993), and the liver is one of the major organs that stores glycogen. However, no study has clarified the partitioning of energy supplies between the energy requirements of the liver and gills during acclimation to ambient Cd. Hence, there is little understanding of the relationships among the endocrine system, the energy supply between tissues, and GP activity during the process of Cd exposure in fish.

In the present study, male adult tilapia were challenged with 44.45  $\mu\text{M}$  Cd, which is approximately half of the 96 h  $\text{LC}_{50}$  (94.84  $\mu\text{M}$ ) for adult tilapia (*O. mossambicus*) (Gaikwad, 1989; cited from Yorulmazlar and Gül, 2003). Following this treatment, we compared many parameters, including changes of blood, endocrine, and biochemistry tissues of gills and hepatic, in order to examine the profile of energy applied system. In addition, ions such as  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Cl}^-$  blood levels investigated during Cd exposure.

## 2. Materials and methods

### 2.1. Fish

Male Mozambique tilapia (*O. mossambicus*) were used as the animal models in the present study because there is a sex-specific difference between male and female fish exposed to waterborne Cd (Sellin et al., 2007). These fish were obtained from the Mariculture Research Center of the Taiwan Fisheries Research Institute, Tainan, Taiwan. The fish were reared in 182-L glass aquaria using plastic chips for gravel. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee, approval number 95021. Each tank was supplied with dechlorinated, circulated, aerated local tap water (FW) at 26–28 °C under a photoperiod of 12–14 h. Fish were fed commercial fish food pellets. The water quality parameters included a total hardness of 146.6  $\pm$  5.6 mg/L,  $\text{Na}^+$ , 35.6  $\pm$  0.3 mg/L,  $\text{K}^+$ , 3.3  $\pm$  0.1 mg/L,  $\text{Ca}^{2+}$ , 30  $\pm$  2.3 mg/L, and a pH of 8.2  $\pm$  0.3 Cd concentration was kept < 1  $\mu\text{g/L}$ .

### 2.2. Experimental design and sampling

The Cd medium was prepared using completely dried  $\text{CdCl}_2$  (Sigma-Aldrich, St, Louis, MO, USA) dissolved in 1 mL of concentrated HCl; double-deionized water was used to prepare the 0.09 mM Cd stock solution, which was then diluted with tap water before the experiments. Adult fish of 10–12 cm total length and 60–80 g of body mass were used in the present study. The time course experiment was repeated at least 4 times. In each experiment, two aquaria (at 30  $\times$  15  $\times$  24  $\text{cm}^3$ ) were used for each experimental time period after being cleaned with 10%  $\text{HNO}_3$ ,

and two adult tilapia were acclimated in each aquarium for 48 h before the experiment. Then, the aquarium of the treatment group was replaced with 44.45  $\mu\text{M}$  Cd. Two additional fish were treated with 0 mg/L Cd as controls. Four fish were sampled at 0 h, 0.25 h, 0.75 h, 1 h, 3 h, 6 h, and 12 h. Therefore, a total of 112 male adult fish were used in this study. To decrease netting stress, a fish was held in the net no longer than 1 min. In addition, all samples were collected daily at 09:30–11:30 in the morning to minimize the effects of circadian rhythms on physiological metabolism. Fish were immediately anesthetized with 80 mg/L MS222, and blood was collected from the caudal vessel within 5 min using a 1-mL plastic syringe with a 25-G needle; the syringe was rinsed with a heparin solution ( $3 \times 10^5$  units/mL, Sigma) to prevent blood coagulation. The liver, gills, brain, and intestines were excised from the body after blood collection. Hepatic and gill tissues were collected for the measurement of glycogen level, GP activity (especially for epithelial cells of the gills), GP mRNA expression and CR mRNA expression, from the methods used in our previous research (Chang et al., 2007). Glucose and lactate levels in the blood were determined with a Blood-Ions System (EML 105, Radiometer Medical A/S, Denmark). Plasma collected for cortisol measurement was allowed to sit overnight at 4 °C and then centrifuged at 2000g for 10 min. The intestines were excised from the body to extract total RNA for cloning of tilapia glucagon cDNA from this tissue.

### 2.3. Isolation of epithelial cells from gills

Isolation of epithelial cells from gill tissues was necessary to exclude the effects of non-epithelial cells. The isolation method followed a previous paper reported before (Tseng et al., 2007). To paraphrase the aforementioned method, gills were carefully rinsed with phosphate-buffered saline (PBS), and tissue was scraped from the gill filaments and immediately placed in dissociation buffer (0.5 M EDTA and 500  $\mu\text{L}$  Percoll in PBS) on ice. Gill cells were isolated from the tissue by gently filtering the solution through 100- $\mu\text{m}$  nylon mesh to remove large tissue fragments after the gill filaments were vigorously agitated with a stirring bar on ice for 30 min. The filtered cell suspension was poured into a Percoll (Sigma) solution (2:1:1 cell suspension: Percoll: PBS) and centrifuged for 10 min at 2000g at 4 °C. The epithelial cell fraction was collected, washed with PBS, and centrifuged.

### 2.4. Preparation of mRNA and cDNA synthesized from total RNA

To prepare mRNA, 200–300 mg of gill or hepatic tissue was homogenized in 3 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated according to the manufacturer's protocols. The amount and quality of total RNA were determined by measuring the absorbance at 260/280 nm with a spectrophotometer (NanoDrop ND-1000, DE, USA) and analyzing it on RNA-denatured gels. The total amount of RNA was subsequently extracted using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia, Piscataway, NJ, USA). Finally, the mRNA pellets were precipitated with 0.1 mg glycogen, a 1/10 vol. of 3 mM NaOAc, and 95% ethanol, and they were stored at –20 °C until the process of cDNA synthesis was needed to be done. Then, mRNA at 0.36  $\mu\text{g}$  was reverse-transcribed in a final volume of 20  $\mu\text{L}$  containing 0.5 mM dNTPs, 2.5  $\mu\text{M}$  oligo (dT)18, 5 mM dithiothreitol, and 200 units of PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA) for 1.5 h at 42 °C, followed by a 15-min incubation at 70 °C. The cDNA samples were finally stored at –20 °C.

### 2.5. Primers and cloning of tilapia glucagon cDNA from the intestines

Genomic sequences of tilapia (t)GCG, tGR, tGPLL, tGPGG, and  $\beta$ -actin cDNA were amplified using the following list of primers. The forward (F) and reverse (R) primers were respectively designed from the 5' and 3' ends of the cDNA for the genes listed below for glucagon

cloning (gcg); and the measurement of mRNA for GP in gills (tGPGG) and in the liver (tGPLL), GR (tGR), and glucagon (tGCG) were designed by real-time polymerase chain reaction (rt-PCR).

cDNA	Primers	
	Forward (5'-3')	Reverse(5'-3')
gcg	-GAGGGVACBTTYTCHAACGACTAC-	-VACAAABTCYTTGRYBGCCTGTC-
tGCG	-CTTTGGTTGCCTGGTCTTTG-	-ACTTTGTTCCGGTGGCTGATG-
tGR	-ACCGACCAGTTTGAGCAGATG-	-CGCTTCACAATGGCTTTTCC-
tGPLL	-GGATGCTTTTCAAACATGA-	-TCGTCCACTCCACTGGATT-
tGPGG	-CGAGCCCAGGGAAGCCATCGAA-	-TGAAGGCTTAAACCAACAGGAA-
$\beta$ -actin	-CGAATCCACGAAACCCTA-	-ATCTCCTTCTGCATCCTGTCA-

For *gcg* gene cloning, total RNA was extracted from tilapia intestines, and the cDNA was prepared as described above. The amplified cDNA fragments were inserted into a pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into competent cells (ECOS9-5) for amplification. The purified plasmids were subjected to DNA sequencing (ABI3700, Applied Biosystems, Wellesley, MA, USA).

### 2.6. Glucagon mRNA expression in the gills, liver, and intestines

Total RNA from the gills, liver, and intestines of mature tilapia was extracted using REzol™ C&T (PROtech, Taipei, Taiwan) with a ratio of 0.1 mg of tissue to 1 mL of reagent. The mRNA was separated, and 1  $\mu\text{L}$  of Random primer (PROtech) was added to 3  $\mu\text{g}$  of total RNA, followed by 15  $\mu\text{L}$  of DEPC-treated water. This mixture was denatured at 65 °C for 5 min and then placed on ice for 5 min so that the primers could anneal to the template. Components of the first-strand synthesis were supplemented with 3  $\mu\text{L}$  of an imProm-II™ Reverse Transcriptase kit (Promega, Taipei, Taiwan), 1  $\mu\text{L}$  of an RNase inhibitor, 10 mM dNTP, 25 mM  $\text{MgCl}_2$ , and 6  $\mu\text{L}$  of 5 $\times$  the RNN buffer. The 30- $\mu\text{L}$  mixture was heated to 42 °C for 60 min in order for the reaction to occur and then to 70 °C for 15 min to terminate the enzyme reaction. The single-stranded cDNA produced was kept at 4 °C. Genomic sequences of *O. mossambicus* *gcg* cDNA were amplified using the above list of *gcg* primers. The cDNA was amplified under the following PCR conditions: after denaturation at 94 °C for 1 min, the thermal cycling program consisted of 30 cycles at 94 °C for 30 s, 50 °C for 30 s for annealing, and 72 °C for 30 s before being polymerized at 72 °C for 10 min, with final cooling at 4 °C.

The amplified product was resolved on a 2% agarose gel, photographed, and analyzed using image analysis software (Kodak Digital Science, Rochester, NY, USA). The relative glucagon mRNA expression was calculated from the ratio of tGCG cDNA and  $\beta$ -actin cDNA.

### 2.7. GP activity assay and measurement of glycogen level

Measurement of gill GP activity followed a procedure described by Chang et al. (2007). Isolated gill epithelial cells were homogenized in an ice-cold homogenization solution (100 mM imidazole, 100 mM KF, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). Total GP (GP<sub>a</sub> + GP<sub>b</sub>) activity was measured by incubating samples at 25 °C in the presence of 1.6 mM 5'AMP, 45 mM potassium-phosphate buffer (pH 7.0), 0.2 mg/mL glycogen, 0.34 mM NADP, 4  $\mu\text{M}$  glucose-1,6-bisphosphate, 0.1 mM EDTA, 15 mM  $\text{MgCl}_2$ , 1.6 U/mL phosphoglucosylase, and 12 U/mL glucose-6-phosphate dehydrogenase. The differences in absorbance between the reactions with and without glycogen (the substrate) were measured at 340 nm with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Each sample was assayed in triplicate.

Glycogen determination followed a procedure described by Chang et al. (2007). Isolated gill epithelial cells and liver tissues were homogenized in 30% KOH and heated to 100 °C for 30 min. Samples were supplemented with two volumes of 100% ethanol and incubated overnight. Glycogen was precipitated by centrifugation after the

addition of two or three drops of Na<sub>2</sub>SO<sub>4</sub>. The glycogen pellets were washed with 66% ethanol and then completely dried. The glycogen level was analyzed in 0.2% anthrone reagent dissolved in H<sub>2</sub>SO<sub>4</sub> using a Hitachi spectrophotometer.

2.8. Cortisol determination by an enzyme-linked immunosorbent assay

Cortisol levels were determined by an ELISA following the general protocol of Wu et al. (2005). Briefly, diluted cortisol antiserum was coated onto a 96-well coated microtiter plate for 24 h at 4 °C. After washing three times, the plates were incubated with blocking buffer for 24 h at 4 °C. Either serum or a standard solution was then mixed with the cortisol conjugated to horseradish peroxidase (HRP); O-phenylenediamine (OPD; Sigma) was added for color development; and the results were measured at 490 nm with an automatic microtiter plate ELISA reader (Dynex MRX, Chantilly, VA, USA).

2.9. GP, GR, and GCG mRNA levels measured by real-time PCR

Total RNA was extracted and reverse-transcribed from the specific tissues of tilapia as described above. The mRNA expression of target genes was measured by qPCR using the Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). Primers for all genes were designed (see above list) using Primer Premier software (version 5.0; PREMIER Biosoft International, Palo Alto, CA, USA). In each assay, 3.2 ng of cDNA was amplified in a 10-μL reaction containing the LightCycler 480 SYBR Green I Master (Roche), 50 nM of the forward and reverse primers, and nuclease-free water. Primers designed for the consensus sequences of GP, GCR, GCG, and β-actin are listed above. All qPCRs were performed as follows: 1 cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (the standard annealing temperature of all primers). PCR products were subjected to a melting-curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with sterile water, and if either primer or template was absent it would determine the levels of background and genomic DNA contamination. The standard curve of each gene was confirmed to be in a linear range with β-actin as an internal control.

2.10. Statistical analysis

Values are presented as the mean ± S.D. (n = 4 or 6). All time-course data were statistically analyzed by one-way analysis of variance (ANOVA) with Tukey's comparisons (p < 0.05). In addition, control and treatment groups at the same time points were compared with Student's t-test, and p < 0.05 was accepted as a significant difference.

3. Results

Compared to the control group, glycogen levels in the experimental group significantly decreased in the gills by 33%, 42%, and 26% at 0.75, 1, and 3 h, respectively. However, glycogen recovered to the same level as the control group by 6 h of Cd exposure (Fig. 1). The total GP activity significantly increased by 2.4- to 3.5-fold in the gills after 0.75–3 h of Cd exposure, and it increased by 2.1- to 2.6-fold in the liver after 1 to 6 h of Cd exposure (Fig. 2). An increase in the relative GPGG and GPLL mRNA expression levels was observed earlier in the gills than in the liver. They increased by almost 2.8-fold at 3 h in the gills and by 2.3-fold at 6 h in the liver (Fig. 3).

Blood glucose significantly increased by 2-, 2.4-, 3.2-, and 2.4-fold compared to the controls at 1, 3, 6, and 12 h, respectively, following Cd treatment. Blood lactate showed no significant difference between the treatment and control groups (Fig. 4).

Cortisol levels increased by 2.7-fold over the control group after 0.25 h of Cd exposure and by 2.3- and 2-fold at 0.75 and 1 h, respectively

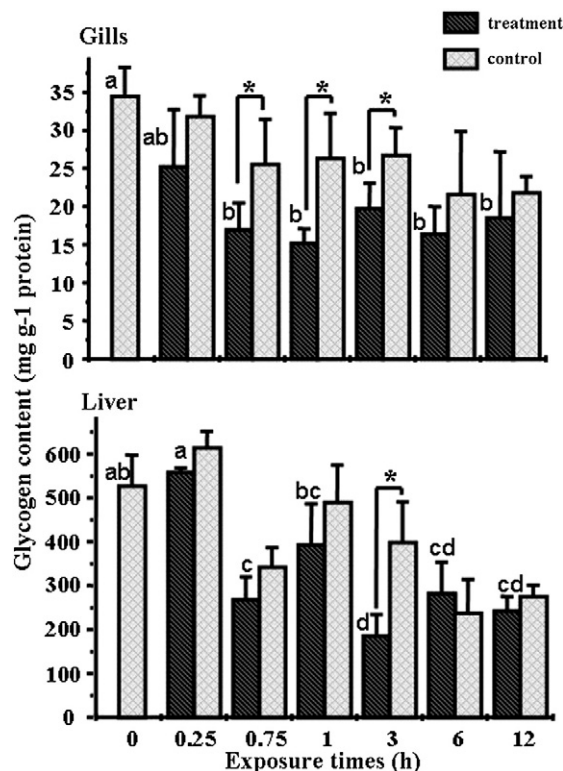


Fig. 1. Comparison of glycogen levels in the gills and liver of tilapia during 12 h of Cd exposure (44.45 μM Cd). Data are presented as mean ± S.D. (n = 6). \*Indicates a significant difference from the respective control at the same exposure time by Student's t-test. Different superscript letters indicate a significant difference (p < 0.05) among sampling times of the treatment group by one-way ANOVA.

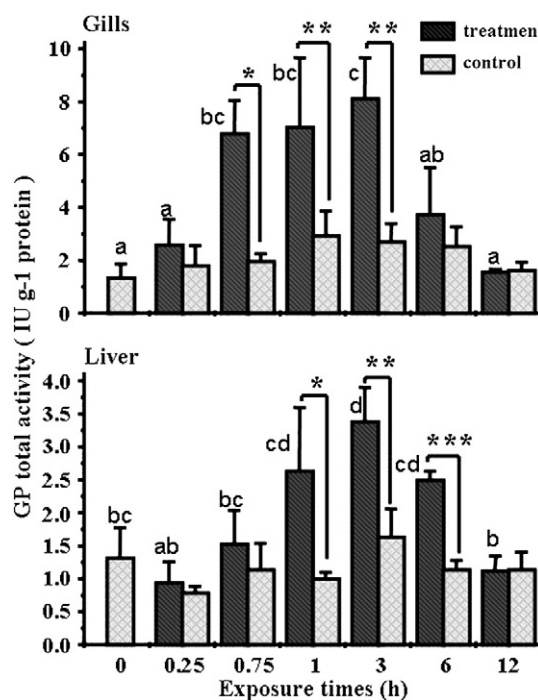


Fig. 2. Comparison of the changes in total glycogen phosphorylase (GP) activity in the gills and liver of tilapia following 44.45 μM Cd exposure. Data are presented as mean ± S.D. (n = 6). \*Indicates a significant difference from the respective control at the same exposure time by Student's t-test. Different superscript letters indicate a significant difference (p < 0.05) among sampling times of the treatment group by one-way ANOVA.

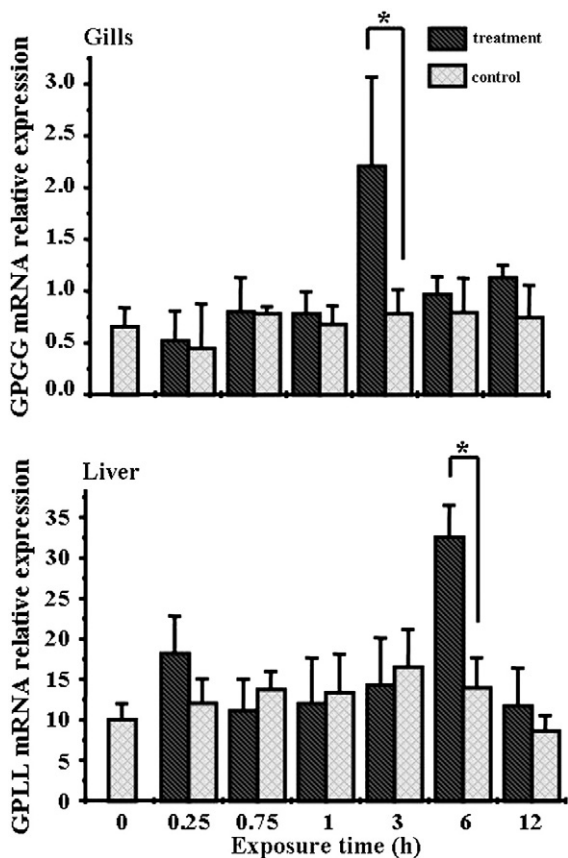


Fig. 3. Comparison of the time course of relative mRNA expression of glycogen phosphorylase in the gills of tilapia (GPGG) and liver glycogen phosphorylase of tilapia (GPLL) following 44.45  $\mu$ M Cd exposure. Data are presented as mean  $\pm$  S.D. ( $n=4$ ). \*Indicates a significant difference from the respective control at the same exposure time by Student's  $t$ -test.

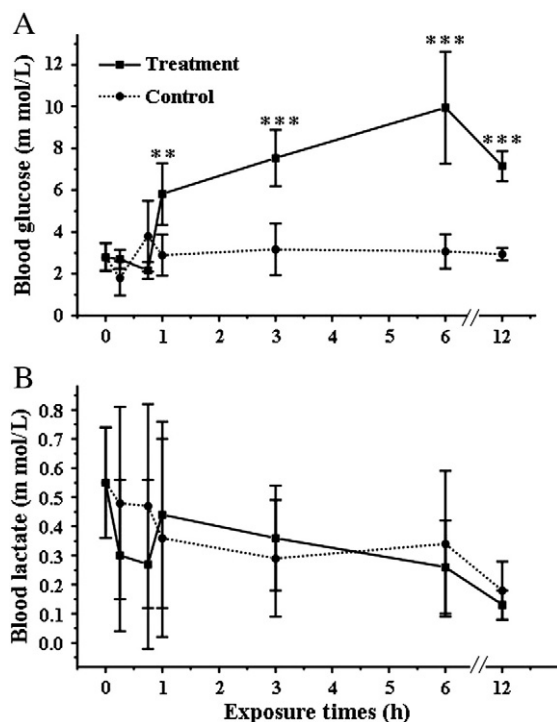


Fig. 4. Changes of blood glucose (A) and blood lactate (B) levels through the time course of Cd exposure, both were compared between treatment- (44.45  $\mu$ M Cd exposures) and control-group in tilapia. Data are presented as mean  $\pm$  S.D. ( $n=4$ ). \* Indicates a significant difference from the respective control at the same exposure time by Student's  $t$ -test.

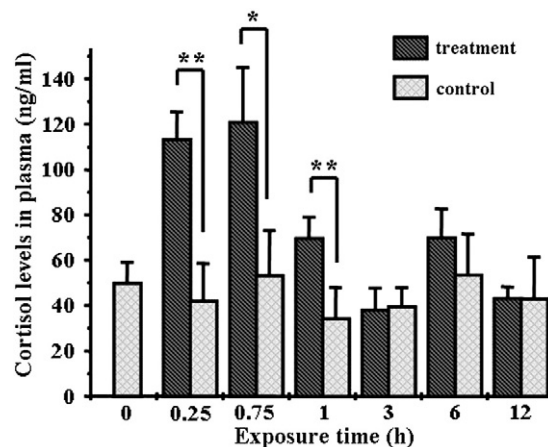


Fig. 5. Changes of plasma cortisol levels were compared with the control in tilapia after 44.45  $\mu$ M Cd exposure (treatment) at the same time; \*  $p<0.05$ , \*\*  $p<0.01$  by Student's  $t$ -test.

(Fig. 5). Tilapia GR mRNA expression was determined by real-time PCR. After 0.75 h of Cd exposure, GR mRNA expression in the gills was significantly higher in the control than in the treatment group, but in the liver, GR mRNA was higher by 1.8-fold in the treatment group compared to the control group after 6 h of Cd treatment (Fig. 6).

Glucagon cDNA (*gcg*) was cloned and sequenced from tilapia intestines (Fig. 7A) (GenBank accession no. FJ217678). The deduced amino acid sequence encodes a protein of 58 amino acids (aa). Similarity searches were performed using the National Center for Biotechnology Information (NCBI) database with the Basic Local Alignment Search Tool (BLAST). The sequence of glucagon in the *O. mossambicus* was similar to those of other fishes; 76–94% sequence

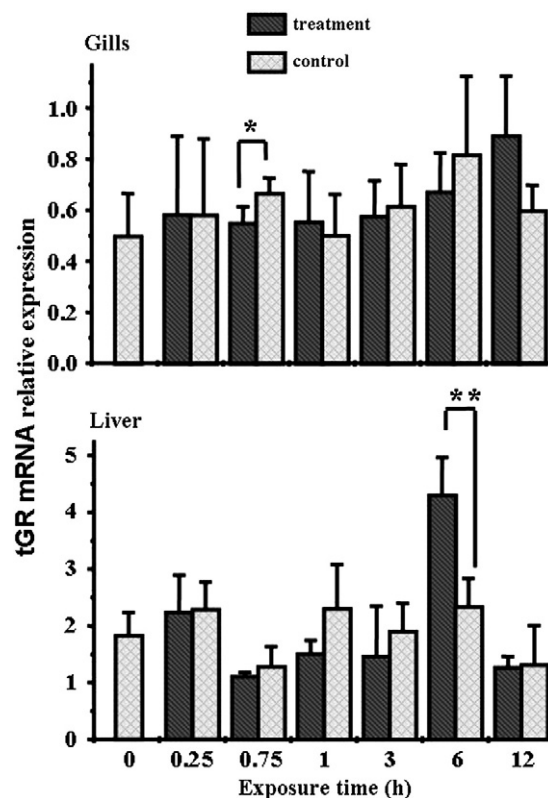
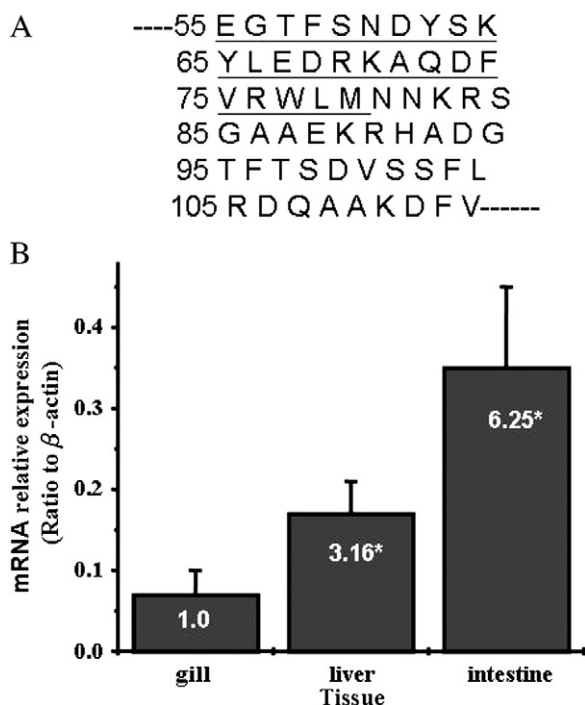


Fig. 6. The time course of changes of cortisol receptor (tGR) mRNA relative expression were compared between the gills and liver of tilapia upon 44.45  $\mu$ M Cd exposure. Data are presented as mean  $\pm$  S.D. ( $n=6$ ). \*  $p<0.05$ , \*\*  $p<0.01$  indicates a significant difference from the respective control at the same exposure time by Student's  $t$ -test.



**Fig. 7.** A fragment of tilapia proglucagon sequence from the 55th to the 113th amino acid, tGCG sequence is shown underlined (GenBank accession no. FJ217678) (A). The relative expression of tGCG mRNA were compared among the gills, intestines, and liver (B). \*The number in the column was showed increased rate as compare to the lowest value.

identity is shown in the identity matrix (Table 1). The present data showed that the tGCG gene was ubiquitously expressed in the liver, gills, and intestines, with the highest expression levels in the intestines (Fig. 7B). Therefore, tGCG mRNA was extracted from the intestines of tilapia, and measured with the real time PCR. The data showed no significant difference with the Student's *t*-test between the treatment and control groups following the same time course of Cd exposure (Fig. 8).

Ca<sup>2+</sup>, Na<sup>+</sup>, and Cl<sup>-</sup> levels significantly decreased after 1–12 h, 1–6 h and 1–6 h of Cd exposure, respectively. Both Na<sup>+</sup> and Cl<sup>-</sup> recovered to levels similar to the control group after 12 h of Cd exposure. Interestingly, K<sup>+</sup> significantly increased at 1 h but decreased within 6–12 h of Cd exposure (Table 2).

#### 4. Discussion

The changes of GP mRNA expression and glycogen contents supported the hypothesis of the present study. The GP transcripts were up-regulated and the glycogen levels were down-regulated. Furthermore, the changes were found earlier in the gills than in the liver of tilapia after Cd exposure. In addition, there were other findings in the present study: (1) blood glucose and cortisol levels significantly

increased, but glucagon did not change accordingly, (2) cortisol appeared more effective than glucagon to raise blood glucose during Cd-exposure stress; and (3) it was noted that the relative expression of GR mRNA significantly increased in the liver, but it significantly decreased in the gills, suggesting GR might play a critical role in both gills and liver under Cd-exposure stress.

Generally, any stress responses including Cd-exposure are related to energy efficiency. Furthermore, some hormones are possible to regulate the energy metabolism in fish during Cd exposure. Glycogen is primarily stored in animal tissues as a long-branched high-molecular-mass polysaccharide. Its degradation to glucose is mainly initiated by GP. Therefore, glycogen depletion is often accompanied by an increase in blood glucose, which is a common response by fish exposed to heavy metals and stressful pollutants (Cattani et al., 1996). It is possible that glycogenolysis and gluconeogenesis can be mediated by a Cd-induced increase in circulating catecholamines and cortisol (Ricard et al., 1998). However, the results may vary with different treatment conditions of Cd including treatment dose with time, the way of administration and with different animal models. For instance, plasma cortisol concentrations did not increase after waterborne 0.32 μM Cd exposure for 6 days in tilapia (*O. mossambicus*) (Pelgrom et al., 1995). An opposite result was found in hybrid tilapia (*Oreochromis* sp.) treated with 4.45 μM Cd, in which the plasma cortisol levels in the fish significantly increased from 5 h to 5 days compared to the control (Wu et al., 2007). The present study showed that plasma cortisol levels significantly increased within 0.25–1 h, but they recovered to the control level after 3 h of 44.45 μM Cd exposure in Mozambique tilapia. Depending on the data, the secretion of cortisol was significantly induced, and blood glucose rose accordingly and became hyperglycemic following toxic Cd stress. However, a hormone called glucagon did not change in tilapia when exposed to ambient Cd. It is known that glucagon is a powerful hormone regulating glucose metabolism in mammals. Generally, the hormone is instrumental in rapidly increasing blood glucose through the activation of hepatic glycogenolysis (Mommensen and Busby, 2006). Because the rainbow trout (*O. mykiss*) was fed a high carbohydrate diet, glucose affected the secretion of glucagon more than that of insulin during the postprandial period (Del Sol Nova et al., 2004). The glucagon seems to be a nutritive hormone. However, in glycogen metabolism and energy regulation, glucagon's role was still unclear during pollutants exposure or stress. The present study showed that the intestine glucagon mRNA expression did not significantly increase as the control group at each time point upon Cd exposure. We suggest that the role of glucagon might be distinct between stress and postprandial induction. Cortisol seems more important than glucagon in the glycogenolysis process under Cd exposure.

Mommensen and Busby (2006) reported that glucagon is to be presently placed into a family of peptide hormones called the secretin-glucagon family. Glucagon and the two glucagon-like peptides (GLP-1 and GLP-2) are the main functional products of the proglucagon gene. Distribution of transcripts for proglucagon includes the brain, endocrine pancreas, intestine and stomach in fish, and its

**Table 1**  
The sequence identity of the tilapia (*O. mossambicus*) tGCG was compared with other species fishes.

Identities (%)	<i>O. mossambicus</i>	<i>A. rupestris</i>	<i>D. rerio</i>	<i>I. punctatus</i>	<i>L. americanus</i>	<i>O. mykiss</i>	<i>S. caurinus</i>	<i>T. nigroviridis</i>
<i>O. mossambicus</i>	–	81	76	77	94	79	93	91
<i>A. rupestris</i>	81	–	62	64	77	66	77	75
<i>D. rerio</i>	76	62	–	91	76	84	76	77
<i>I. punctatus</i>	77	64	91	–	79	86	77	77
<i>L. americanus</i>	94	77	76	79	–	77	91	91
<i>O. mykiss</i>	79	66	84	86	77	–	81	75
<i>S. caurinus</i>	93	77	76	77	91	81	–	88
<i>T. nigroviridis</i>	91	75	77	77	91	75	88	–

*Ambloplites rupestris* (GenBank accession no: AAG16778), *Danio rerio* (NP\_001008595.1), *Ictalurus punctatus* (GAAS57648.1), *Lophius americanus* (P01278.1), *Oncorhynchus mykiss* (NP\_001118172), *Sebastes caurinus* (AAS57646), *Tetraodon nigroviridis* (CAG04974.1).

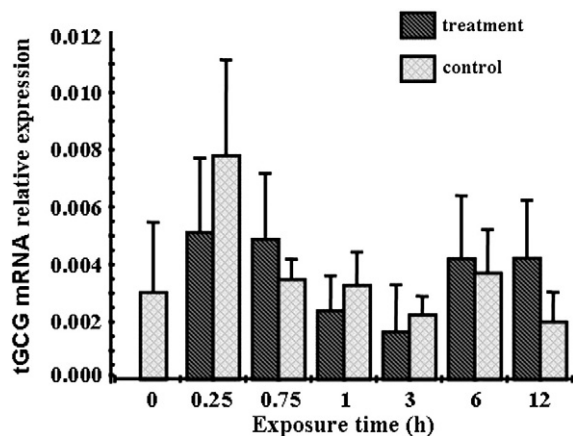


Fig. 8. Changes of relative tilapia glucagon (tGCG) mRNA expression were compared with the control after 44.45  $\mu\text{M}$  Cd exposure (treatment) at the same time, and it is no-significantly difference between the treatment and control with the Student's *t*-test. Data are presented as mean  $\pm$  S.D. ( $n=6$ ).

receptors stored at the gills, gonads, heart, intestine, kidney, muscle, and the hepatic tissue are the major target tissues of glucagon (Busby, 2002; Green et al., 2004). Glucagon is produced by  $\alpha$ -cells of the endocrine pancreas in teleostean, and they are short, single-chained peptides. In the tilapia Brockmann body, the areas that were glucagon positive accounted for only 11.5% of the endocrine cells (Mommensen and Busby, 2006). It was not easy to isolate the small islets. Thus, the present study compares the relative expression of tGCG mRNA among tissues (intestine, liver and gills). In contrast to the gills and liver, intestines had the highest GCG mRNA expression level. To date, only a few studies have investigated the physiological roles of fish glucagon *in vivo*. We compared the identity of amino acid sequences in tilapia with those of other fishes and found that the *gcg* sequence of tilapia was similar to those of other fish, showing 76–94% sequence identities. According to Mommensen and Busby (2006), the teleostean glucagon gene contains 6 exons co-encoded in proglucagon, with the first sequence as the signal peptide (20 aa), glicentin-related pancreatic polypeptide (30 aa), glucagon (29 aa), an intervening peptide-1 (6 aa), a major proglucagon fragment, GLP-1 (30 aa), and an intervening peptide-2 (12 aa), and lastly followed by GLP-2 (34 aa). The present data showed one fragment sequence that was encoded on the 55th to 113th amino acids of glucagon. Thus, we suggest that the sequence is encoded between glucagon and the GLP-1 fragment.

With respect to keeping off sampling errors in glucagon and cortisol levels, GR mRNA expression, intestine tGCG mRNA expression, and blood glucose levels, all measurements between the treatment and control groups were conducted at the same time. Plasma cortisol levels significantly increased at 0.25–3 h, and blood glucose levels increased at

1–12 h after Cd exposure. A Cd-induced acute stress response was observed, with an immediate change observed following the time course of Cd exposure at 0–12 h. The data demonstrated that cortisol was more effective at positively inducing blood-glucose than glucagon upon stress induction. The action of cortisol occurred only after cortisol bound to GR. However, the present data showed that the GR mRNA expression significantly decreased at 0.75 h in gills, followed by an increase at 6 h in the liver after Cd exposure. This phenomenon was suggested to have two possible causes. First, down-regulation of GR due to elevated cortisol levels decreases the number of GR binding sites to normalize the sensitivity of feedback (Tomy et al., 2009). Second, the GR is less sensitive to cortisol than the mineralocorticoid receptor (MR) (Prunet et al., 2006). MR-like genes have been cloned in many species such as Atlantic salmon (*Salmo salar*) (McCormick et al., 2008), rainbow trout (*Oncorhynchus mykiss*) (Küllerich et al., 2007), and eel (*Anguilla japonica*) (Hu et al., 2009). According to our knowledge, MR-like gene from the Mozambique tilapia (*O. mossambicus*) has yet to be cloned. Depending on the present data of cortisol levels and tGR mRNA expression between gills and liver, and the glycogenolysis occurs earlier in gills than in liver. We suggested that the up- and down-regulation in tGR mRNA expression were related to the cortisol level. Further, the ligand sensitivity of GR or the feedback regulation of GR differs among various organs (Greenwood et al., 2003; Stolte et al., 2008).

Cd treatment caused significant decreases in tilapia larval growth because it significantly inhibited  $\text{Ca}^{2+}$  uptake, resulting in a 31% decrease in  $\text{Ca}^{2+}$  levels within the whole body (Wu et al., 2007). Exposure of tilapia to a sub lethal Cd concentration (0.092  $\mu\text{M}$ ) for 4 days led to a rapid and substantial decrease in total  $\text{Ca}^{2+}$  and a slight drop in plasma  $\text{Na}^+$  (Fu et al., 1989). Indeed, Cd exposure disturbed the homeostasis of many ions, but  $\text{Ca}^{2+}$  levels showed the greatest response to Cd exposure. In the present study,  $\text{Ca}^{2+}$  levels decreased from 1 h until the end of the experiment. Both  $\text{Na}^+$  and  $\text{Cl}^-$  levels decreased at 3–6 h. The  $\text{K}^+$  level significantly increased at 1 h, and it was lower at 6–12 h of Cd exposure. On the other study, we found that the waterborne  $\text{Cd}^{2+}$  significantly decreased the gene expression of three  $\text{Ca}^{2+}$  transporters, *PMCA2*, *ECaC* and *ECX1b* in zebrafish (*Danio rerio*) (unpublished data). In addition, Vilella et al. (1999) reported that  $\text{Cd}^{2+}$  inhibited the activity of  $\text{Na}^+/\text{H}^+$  exchangers in a dose-dependent manner. We suggest that acute ion disequilibrium after Cd exposure may directly affect transporters and channels, or enzyme activity. Thus, the phenomenon was associated with the speculation that cortisol might have been induced to regulate its role on carbohydrate metabolism in the gills for energy support. A previous study has reported that the glycogen-rich cells are initially stimulated to prompt energy for neighboring CC that trigger ion-secretion mechanism after tilapia acutely transfer from freshwater to seawater. It was the reason to be suggested that the glycogen metabolism occurred earlier in gills than in liver (Chang et al., 2007; Tseng et al., 2008). The present study showed a similar profile;  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  ions

Table 2

Changes in calcium, sodium, chloride, and potassium levels in male tilapia blood upon 44.45  $\mu\text{M}$  Cd (T) and 0  $\mu\text{M}$  Cd (C) exposure.

Ion	Exposure condition	Exposure time (h)							
		0	0.25	0.75	1	3	6	12	
$\text{Ca}^{2+}$	T		1.5 $\pm$ 0.1	1.5 $\pm$ 0.2	1.5 $\pm$ 0.2**	1.4 $\pm$ 0.2**	1.2 $\pm$ 0.1***	1.1 $\pm$ 0.3*	
	C	1.5 $\pm$ 0.1	1.4 $\pm$ 0.1	1.4 $\pm$ 0.2	1.7 $\pm$ 0.1	1.7 $\pm$ 0.2	1.7 $\pm$ 0.1	1.6 $\pm$ 0.1	
$\text{Na}^+$	T		152 $\pm$ 3	155 $\pm$ 6	156 $\pm$ 5*	159 $\pm$ 5*	157 $\pm$ 3***	158 $\pm$ 5	
	C	150 $\pm$ 4	148 $\pm$ 3	149 $\pm$ 4	165 $\pm$ 5	164 $\pm$ 2	164 $\pm$ 4	164 $\pm$ 3	
$\text{Cl}^-$	T		135 $\pm$ 4	132 $\pm$ 11	141 $\pm$ 5**	147 $\pm$ 7*	140 $\pm$ 4**	137 $\pm$ 4	
	C	137 $\pm$ 7	131 $\pm$ 5	127 $\pm$ 11	149 $\pm$ 5	154 $\pm$ 6	146 $\pm$ 3	140 $\pm$ 4	
$\text{K}^+$	T		3.6 $\pm$ 0.4	4.1 $\pm$ 1.1	6.3 $\pm$ 2.3*	4.0 $\pm$ 0.7	4.2 $\pm$ 0.3*	3.8 $\pm$ 0.1*	
	C	4.8 $\pm$ 0.3	4.0 $\pm$ 0.7	4.1 $\pm$ 1.0	4.1 $\pm$ 0.6	4.4 $\pm$ 0.4	4.6 $\pm$ 0.2	4.1 $\pm$ 0.2	

Data are presented as the mean  $\pm$  S.D. ( $n=6$ ); \*  $p<0.05$ , \*\*  $p<0.01$ , and \*\*\*  $p<0.001$ , compared between the treatment (Cd exposure) and control groups at the same exposure time by Student's *t*-test.

were disturbed by Cd<sup>2+</sup>, and that might be to induce glycogenolysis which occurred earlier in gills than in liver. Hence, it might support energy for CC in order to compensate ions leaking under Cd exposure.

The past study reported that the cortisol has a permissive effect on glucagon-mediated glycogenolysis (Reid et al., 1992). The roles of glucagon in Cd-contaminated fish will be investigated more clearly. In addition, the relationship between carbohydrate metabolism and secreted cortisol levels may become apparent if there are further studies on the specific pathways activated by cortisol.

## 5. Conclusion

In conclusion, gill GP activity might rapidly provide energy immediately after Cd stress. Several hours later, liver GP activity began to increase to maintain subsequent energy supply. Blood glucose levels following Cd treatment were higher compared to the control group, which was possibly triggered by cortisol in the liver. However, GR mRNA expression in the gills did not rise accordingly. It is suggested that GR can down-regulate the binding site with cortisol levels, and stimulate carbohydrate metabolism. Cortisol might maintain electrolytic balance on gills during Cd exposure. Thus, under Cd-induced stress, the fish regulate GR less sensitive to ligand in gills than the control, and the fish up-regulate their GR mRNA expression in liver after the cortisol levels recover. The changes of ions including Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Cl<sup>-</sup> decreased following the time of Cd exposure.

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