Dietary chromium modulates glucose homeostasis and induces oxidative stress in Pacific white shrimp (*Litopenaeus vannamei*)

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Accepted refereed manuscript of: Shi B, Tao X, Betancor MB, Lu J, Tocher DR, Meng F, Figueiredo-Silva C, Zhou Q, Jiao L & Jin M (2021) Dietary chromium modulates glucose homeostasis and induces oxidative stress in Pacific white shrimp (Litopenaeus vannamei). *Aquatic Toxicology*, 240, Art. No.: 105967. https://doi.org/10.1016/j.aquatox.2021.105967

Abbreviations:

ACC/*acc1*, acetyl-CoA carboxylase; *akt*, RAC-alpha serine/threonine-protein kinase; *bcl2*, Bcl2 protein; CAT/*cat*, catalase; CHH, crustacean hyperglycemic hormone; *cp*, ceruloplasmin; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthase; *fbp*, fructose-1,6-bisphosphatase 1; *foxo1*, forkhead box transcription factor class O1; *g6pc*, glucose-6-phosphatase; Glu, glucose; *glut1*, glucose transporter 1; GSH, oxidized glutathione; GSH-PX/*gpx*, glutathione peroxidase; *gsk-3β*, glycogen synthase kinase-3 beta; GSSG, reduced glutathione; *gys*, glycogen synthase; H_2O_2, hydrogen peroxide; HK/*hk*, hexokinase; ILP, insulin like peptide; *insr*, insulin receptor; *irs1*, insulin receptor substrate 1; MDA, malondialdehyde; MT/*mt*, metallothionein; NEFA, non-esterified fatty acids; PA, pyruvic acid; *pdpk1*, 3-phosphoinositide-dependent protein kinase 1; PEPCK/*pepck*, phosphoenolpyruvate carboxykinase; PFK/*pfk*, phosphofructokinase; *pik3ca*, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform; *pik3cd*, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform; PK/*pk*, pyruvate kinase; SCHR, scavenging capability for hydroxyl free radical; SOD, superoxide dismutase; *srebp*, sterol-regulatory element binding protein; TC, total cholesterol; TG, triacylglycerol; T-GSH, total glutathione; 8-OHDG, 8-hydroxydeoxyguanosine.

Abstract

While chromium (Cr) has been recognized as an essential nutrient for all animals, and dietary supplementation can be beneficial, it can also be toxic. The present study aimed to investigate the contrasting effects of dietary chromium in Pacific white shrimp *Litopenaeus vannamei*. Five experimental diets were formulated to contain Cr at levels of 0.82 (Cr0.82, unsupplemented diet), 1.01 (Cr1.01), 1.22 (Cu1.22), 1.43 (Cr1.43) and 1.63 (Cr1.63) mg/kg and were fed to shrimp for 8 weeks. Highest weight gain was recorded in shrimp fed the diet containing 1.22 mg/kg Cr. Shrimp fed the diet containing the highest level of Cr (1.63 mg/kg) showed the lowest weight gain and clear signs of oxidative stress and apoptosis as evidenced by higher levels of H_2O_2, malondialdehyde and 8-hydroxydeoxyguanosine, and expression of *caspase 2, 3, 5,* and lower contents of total and oxidized glutathione, and expression of *Cu/Zn sod, cat, gpx, mt, bcl2*. Chromium supplementation promoted glycolysis and inhibited gluconeogenesis as shown by increased activities of hexokinase, phosphofructokinase and pyruvate kinase, and reduced activity of phosphoenolpyruvate carboxykinase in shrimp fed the diet containing 1.43 mg/kg Cr. Shrimp fed the diet with 1.63 mg/kg Cr had lowest contents of crustacean hyperglycemic hormone and insulin like peptide in hemolymph. Expression of genes involved in insulin signaling pathway and glycose metabolism including *insr*, *irs1*, *pik3ca*, *pdpk1*, *akt*, *acc1*, *gys*, *glut1*, *pk*, *hk* were up-regulated, and *foxO1*, *gsk-3β*, *g6pc*, *pepck* were down-regulated in shrimp fed the diets supplemented with Cr. This study demonstrated that optimum dietary supplementation of Cr had beneficial effects on glucose homeostasis and growth, whereas excess caused oxidative damage and impaired growth. The results contribute to our understanding of the biological functions of chromium in shrimp.

Keywords: Chromium, Oxidative stress, Apoptosis, Glucose metabolism, *Litopenaeus vannamei*

1. Introduction

Chromium (Cr), more specifically trivalent chromium (CrIII) is an essential micronutrient for all animals, and has been used as a dietary supplement in both humans and animal feeds (Mertz, 1993; Vincent, 2004). The biologically active version of Cr is an organic, amino acid bound compound that is termed glucose tolerance factor as it activates insulin production and promotes glucose metabolism (Davis and Vincent, 1997) and, in humans, appropriate dietary Cr was found to mitigate insulin resistance and help protect against free radical damage (Tulatermed si and Rao, 2014). Consequently, Cr has insulin mimetic activity, potentiating insulin-mediated activation of Insulin Receptor Substrate-1 (IRS-1) and insulin signaling leading to glucose uptake (Miranda and Dey, 2004). Therefore, sufficient dietary Cr can promote the efficiency of insulin, thereby reducing insulin required to maintain glucose homeostasis (Anderson, 1992). However, the absorption rate of inorganic chromium is only 0.4 – 3 %, whereas absorption of organic Cr is 20 to 30 times more efficient than that of inorganic forms and, thus, chelated minerals are better sources (Starich and Blincoe, 1983; Gammelgaard et al., 1999). While several studies have been conducted to investigate the effects of dietary organic and inorganic chromium on growth and carbohydrate utilization in fish species (Shiau and Shy, 1998; Gatta et al., 2001a, 2001b; Kuykendall et al., 2006; Kubrak et al., 2010; Liu et al., 2010; Selcuk et al., 2010; Ahmed et al., 2012, 2013; Giri et al., 2014), information is very limited in shrimp.

Although an essential nutrieny, all forms of chromium (hexavalent or trivalent chromium) can be toxic and even carcinogenic at high concentration (Tulatermed si and Rao, 2014). Furthermore, Cr is one of most common and ubiquitous metal pollutants in the environment, entering aquatic systems via industrial effluents and posing a significant threat to aquatic organisms and food safety

via bioconcentration in the food chain (Velma et al., 2009). Studies have shown that excess Cr could cause damage by disrupting the redox balance in the body (Bagchi et al., 2003; Yao et al., 2008; Velma et al., 2009), with Cr specifically inducing the formation of reactive oxygen species (ROS), reducing activity of antioxidant enzymes and thus altering the oxidative status (Dazy et al., 2008; Rai et al., 2004). Other studies have shown that apoptosis is the mode of cell death caused by Cr (Blankenship et al., 1994; Singh et al., 1998; Feng et al., 2017). In fish, Cr exposure induced a variety of adverse effects including oxidative stress, DNA damage and apoptosis (Bagchi et al., 2003; Lushchak et al., 2009; Velma et al., 2009; Velma and Tchounwou, 2013; Kumari et al., 2014; Jin et al., 2015). Chromium shows a dose/exposure-response relationship and some species appear to be more sensitive to Cr suggesting that toxicity level of Cr may be species and dose dependent (Velma et al., 2009).

Insulin is a polypeptide hormone that regulates carbohydrate, lipid and protein metabolism, promotes glucose uptake, lipid and glycogen synthesis, and inhibits lipolysis, gluconecogenesis and glycogenolysis (Sonksen and Sonksen, 2000; Dimitriadis et al., 2011). While insulin plays a key role in lowering blood glucose via the insulin signaling pathway (Sonksen and Sonksen, 2000), it is actually just one member of a superfamily of polypeptides including insulin-like peptides (ILP) and insulin-like growth factors (IGF) that have a high degree of sequence homology (Wu and Brown, 2006). Increasing evidence has demonstrated that invertebrates contain peptides with similar biological functions as mammalian insulin (Gutiérrez et al., 2007) and, in crustaceans, the presence of ILP has been suggested in *L. vannamei* and other species (Sanders, 1983; Lin et al., 1993; Chuang and Wang, 1994; Gutiérrez et al., 2007; Mareddy et al., 2011; Li et al., 2019; Jiang et al., 2020).

Shrimp exhibit a wide versatility in the utilization of carbohydrates, which are regarded as a

cheap source of dietary energy (Cruz-Suarez et al., 1994) that can spare the use of protein and thus promote growth and development (Cruz-Suarez et al., 1994). However, excessive supplementation of carbohydrate-rich ingredients in feed can cause glucose metabolic disorders in animals (Cruz-Suarez et al., 1994). The overall aim of the present study was to investigate the contrasting impact of dietary Cr supplementation in Pacific white shrimp (*Litopenaeus vannamei*). The study was specifically designed to reveal the role of dietary Cr in maintaining glucose homeostasis and identify potential toxic effects.

2. Materials and methods

2.1 Experimental diets

Five experimental diets were formulated with different Cr levels using methionine chelated chromium as Cr source (Zinpro Corp., USA). A basal diet was supplemented with 0, 0.2, 0.4, 0.6 and 0.8 mg/kg Cr, with the analyzed values of Cr in the final feeds being 0.82 (Cr0.82, unsupplemented), 1.01 (Cr1.01), 1.22 (Cu1.22), 1.43 (Cr1.43) and 1.63 (Cr1.63) mg/kg (Table 1). The amino acid compositions (g/100g, dry matter) of the experimental diets list in Table S1. Amino acid profiles of diets were determined using a High-speed Amino Acid Analyzer (L-8900, Hitachi High-Technologies Co., Tokyo, Japan) based on the method described previously (Shi et al., 2021b). The feeds were produced as described in detail previously (Shi et al., 2020). Briefly, all dry ingredients were ground through 80-mesh and mineral and vitamin premixes added by the progressive enlargement method, before lipid and distilled water (35 %) were added. The ingredients were thoroughly mixed by Hobart mixer and feeds produced by cold extrusion (F-26, Machine Factory of South China University of Technology, Guangzhou, China) with pellets cut to 1.5 mm and 2.5

mm diameter (G-250, Machine Factory of South China University of Technology). Feeds were heated at 90 °C for 30 min, air-dried to 10 % moisture, vacuum-packed and stored at -20 °C until use.

2.2 Shrimp rearing and experimental conditions

The feeding experiment was conducted at the breeding base of Ningbo Ocean and Fishery Science and Technology Innovation Center (Zhejiang, China). Juvenile shrimp, obtained from a local commercial hatchery (Chia-Tai Ningbo Company, Ningbo, China) and were initially reared in cement tanks and fed a commercial diet (40 % protein, 8 % lipid; Yue-Hai Aquafeed Corp., Jiaxing, China) for two weeks to acclimate to experimental conditions. A total of 750 juveniles (3.20 ± 0.01 g) were randomly allocated to 25 tanks (30 per tank), and each diet assigned to five replicate tanks. The daily management procedure of the 8-week feeding trial (from August to October, 2019) was described in detail previously (Shi et al., 2021a). Briefly, shrimp were fed a daily ration of 6-8 % of biomass by hand 3-times per day at 8:00, 12:00 and 17:00 with shrimp in each tank weighed every two weeks and daily ration adjusted accordingly. Calculations of growth performance, feed efficiency and biometry are shown in supplementary materials. On a daily basis, over 70 % of the seawater was exchanged, waste material and exuviae siphoned prior to the 8:00 feed, and mortalities removed, weighed and recorded. Water quality parameters were measured daily including dissolved oxygen level ≥ 6.0 mg/L, temperature 26-20 °C, salinity 22-20, pH 7.5-7.7 and ammonia nitrogen ≤ 0.05 mg/L.

2.3 Sample collection

Samples were collected essentially as described previously with a few modifications (Shi et al., 2021a). At the end of the feeding experiment, shrimp were fasted for 24 h and anaesthetized with 10 mg/L eugenol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). All shrimp were counted and weighed individually to assess growth performance and feed utilization, and body length, whole body and hepatopancreas weights measured in four shrimp per tank before tissue samples (hepatopancreas, muscle and carapace) were dissected and collected for determining Cr concentrations. In the absence of anticoagulant, hemolymph was collected from a further five shrimp per tank and centrifuged at 850 × g for 10 min at 4 °C for analysis of hematological parameters. Hepatopancreas samples from ten shrimp per tank were collected and stored at -80 °C before analysis of lipid and glucose metabolism, oxidation state parameters, and gene expression.

2.4 Proximate composition and mineral analysis

Proximate compositions of diets were determined essentially according to the methods of the Association of Analytical Chemists (AOAC, 2006) as described in supplementary material. Concentrations of Cr in shrimp tissues and experimental diets were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES; PE 2100DV, Perkin Elmer, USA) at the Ningbo Institute of Materials Technology and Engineering, Chinese Academy of Sciences (Ningbo, China) as described in detail previously with a few modifications (Wu and Yang, 2011). Samples (experimental diets, hepatopancreas, muscle and carapace) were freeze-dried for 48h prior to analysis. Then, approximately 200 mg of freeze-dried samples were weighed before acid digestion, where samples were incubated in 70 % HNO_3 at 80 °C for 4 h. After cooling, the digested samples were washed into a volumetric flask and made up to 10 ml using ultrapure water before this solution

was filtered through a 0.22 μm membrane using a hydrophilic polyether sulfone syringe filter (CNW, Germany) prior to measuring emission spectrum intensity of analytical elements. A stock standard solution of Cr (1000 mg/L, GBW08614) was purchased from the National Research Center for Certified Reference Materials (NRCCRM, Beijing, China) and the validation procedure was carried out with certified reference material BCSS-1 (National Research Council of Canada). Quality assurance and quality control (QA/QC) tests were carried out in order to monitor and control the reliability of the analytical method. Recovery rate and relative standard deviation for Cr were 96.7 % and 1.2 %, respectively.

2.5 Hemolymph biochemical analysis

TG, TC, LDL-C, HDL-C and Glu in hemolymph were determined using an automatic chemistry analyzer (Hitachi 7600-110, Tokyo, Japan), and reagent kits (Biosino Bio-Technology and Science Inc., Beijing, China). NEFA, PA, PEPCK, PFK, PK and HK in hemolymph were determined by commercial assay kits (Nanjing Jiancheng Co., Nanjing, China).

2.6 Analysis of hepatopancreas parameters

Samples of hepatopancreas were homogenized in 9 volumes (w/v) ice-cold saline 8.9 g/L, centrifuged at 850×g for 10 min at 4 °C, and supernatant collected and stored at -80 °C prior to analysis. Activities of glucose metabolism related enzymes (PEPCK, HK, PFK, PK) and antioxidant parameters (CAT, SOD, GSH-PX, H_2O_2, MDA, SCHR, T-GSH, GSH, GSSG) were measured using the relevant commercial assay kits (Nanjing Jiancheng Co., Nanjing Jiancheng). Lipid metabolism related enzyme activities (FAS, CPT1, ACC), glucose metabolism related hormones (CHH, ILP)

and apoptosis-related parameter (8-OHDG) were determined with ELISA kits specific for *L. vannamei* (Jiangsu Meibiao Biological Co., Ltd., China), according to the manufacturer's protocols.

2.7 Gene expression analysis

The RNA isolation, reverse transcription, and RT-qPCR reaction system and procedures were conducted following the methods published by Shi et al. (2021b). Briefly, RNA was extracted from hepatopancreas using Trizol Reagent (Vazyme, China), with concentration and integrity of RNA confirmed by ultra-micro spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific) and agarose gel electrophoresis (Bio-Rad, USA), respectively. RNA was reverse transcribed into complementary DNA using HiScript® RT SuperMix Reagent kit (Vazyme, China) and Mastercycler nexus GSX1 PCR (Eppendorf, Germany). For amplification, the 20 μl reaction volume contained 0.4 μl primer, 0.8 μl cDNA, 10 μl 2×ChamQ SYBR qPCR Green Master Mix (Vazyme, China) and 8.4 μl DEPC-treated water. Gene-specific qPCR primers were designed using Primer Premier 5.0 software with E-values ranging from 95.8 to 108.3 % (Table S2), and β-*actin* (GenBank accession no. AF300705.2) used as housekeeping gene. The program for real-time PCR was 95 °C for 2 min, 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 20 s. Standard curves were analyzed with equation $E = 10^{(-1/slope)}-1$, and relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), with basal, unsupplemented diet used as the control/reference group.

2.8 Statistical analysis

All date were presented as means ± SEM (n as stated) and checked for normality and homogeneity of variances prior to statistical analysis. Differences among mean values were assessed by one-way

ANOVA followed by Duncan's multiple tests (IBM, SPSS Statistics 20.0). Differences were considered to be significant at $P < 0.05$.

3. Results

3.1 Growth performance, feed utilization and morphometric parameters

Survival ranged from 81.3 to 84.0 % and was independent of dietary treatment (Table 2). As dietary Cr increased, growth performance including WG and SGR initially increased and decreased with shrimp fed 1.01 and 1.22 mg/kg Cr exhibiting higher WG than the shrimp fed 0.82 and 1.63 mg/kg Cr. Lowest FI and FCR were recorded in shrimp fed the 1.01, 1.22, 1.43 mg/kg Cr diets with highest values found in shrimp fed the highest level of Cr (1.63 mg/kg) with those fed the lowest level of Cr (0.82 mg/kg) showing intermediate values. No statistically significant differences were observed in HSI and CF.

3.2 Cr concentration in tissues

The concentration of Cr in tissues was increased significantly as dietary Cr level increased, with shrimp fed 1.43 and 1.63 mg/kg Cr showing higher Cr concentrations in hepatopancreas and carapace than shrimp fed the basal diet (Fig. 1). Similarly, the highest Cr concentration in muscle was observed in shrimp fed the diet with highest Cr concentration (1.63 mg/kg), while the lowest Cr concentrations in all tissues were observed in shrimp fed the unsupplemented diet with lowest Cr concentration (0.82 mg/kg).

3.3 Oxidation and antioxidant parameters

243 *3.3.1 Hemolymph metabolite profiles*

244 Dietary Cr supplementation reduced T-GSH and GSH content and GSH-PX activity, but increased
245 MDA in hemolymph (Fig. 2). Significantly lowest levels of T-GSH and GSH and GSH-PX activity
246 were observed in 1.63 mg/kg Cr diet, while the opposite was the case for MDA, with shrimp fed the
247 diet containing 1.63 mg/kg Cr showing highest MDA in hemolymph.

248 *3.3.2 Hepatopancreas metabolite profiles*

249 Dietary Cr level affected activities of antioxidant enzymes (CAT, SOD, GSH-PX, MT, SCHR),
250 apoptosis marker (8-OHDG) and contents of oxidation and antioxidant products (H_2O_2, MDA, T-
251 GSH) (Fig. 3). Shrimp fed 1.63 mg/kg Cr had lower activities of SOD, GSH-PX and higher activities
252 of MT and 8-OHDG in hepatopancreas than the shrimp fed the other diets. Activities of CAT were
253 significantly higher in shrimp fed 1.01, 1.22 and 1.43 mg/kg Cr than those fed diets without Cr
254 supplementation (0.82 mg/kg) or supplemented with the highest level of Cr (1.63 mg/kg). In
255 addition, the highest activities of SOD and GSH-PX were recorded in shrimp fed the 1.43 and 1.01
256 mg/kg Cr diets, respectively. Shrimp fed the diets supplemented with 1.22, 1.43 and 1.63 mg/kg Cr
257 showed reduced scavenging ability towards hydroxyl free radicals in hepatopancreas. The levels of
258 MT and 8-OHDG increased with increasing dietary Cr supplementation with highest contents being
259 recorded in highest dietary Cr (1.63 mg/kg). Conversely, shrimp fed the 1.63 mg/kg Cr diet had
260 higher contents of H_2O_2 and MDA than shrimp fed lower dietary Cr, with lowest values found in
261 shrimp fed 1.01 mg/kg Cr. The content of T-GSH in hepatopancreas decreased as dietary Cr
262 increased, with the lowest content being observed in shrimp fed 1.63 mg/kg Cr.

263

264 *3.4 Key markers of the glucose metabolic pathway*

Glucose metabolism is regulated by varied enzymes and hormones and so some key markers in the pathway of glycolysis and gluconeogenesis were determined (Fig. 4). Shrimp fed the diet with 1.63 mg/kg Cr showed significantly reduced contents of CHH and ILP in hemolymph compared to shrimp fed the unsupplemented diet. Activities of HK and PK in hemolymph increased with increasing dietary Cr level, with highest values observed in shrimp fed 1.63 mg/kg Cr. Similarly, shrimp fed 1.43 mg/kg Cr had higher activities of HK, PFK, PK and lower PEPCK in hepatopancreas compared to shrimp fed the unsupplemented diet. Conversely, dietary Cr supplementation reduced glucagon in hemolymph, with the lowest level recorded in shrimp fed 1.43 mg/kg Cr. The ratio of ILP/Glu in shrimp increased with dietary Cr level up to 1.43 mg/kg, but decreased in shrimp fed 1.63 mg/kg Cr.

3.5 Lipid metabolites and key enzymes

Shrimp fed the diet containing 1.63 mg/kg Cr displayed significantly increased contents of TG and NEFA in hemolymph compared to shrimp fed the unsupplemented diet (Fig. 5A). A similar result was found for TG content of hepatopancreas, with a higher level found in 1.63 mg/kg Cr diet compared to lower dietary Cr (Fig. 5B). Shrimp fed 1.63 and 1.43 mg/kg Cr showed significantly lower CPT1 activity compared to shrimp fed the unsupplemented diet while the opposite was the case for hepatopancreas ACC, with a significantly higher activity being observed in shrimp fed the diets with 1.22, 1.43 and 1.63 mg/kg Cr compared to shrimp fed the unsupplemented diet.

3.6 Gene Expression

3.6.1 Oxidative stress and apoptosis related genes

Expression levels of *cat*, *Cu/Zn sod*, *gpx* showed a clear trend, being reduced in shrimp fed the highest level of dietary Cr (1.63 mg/kg) than shrimp fed the lower levels of dietary Cr although it was not consistently significant with all diets (Fig.6). Similarly, the expression of *bcl2* was down regulated in a graded manner with increasing dietary Cr, with shrimp fed the highest dietary Cr being significantly lower than the unsupplemented diet. In contrast, the caspase family of genes were up-regulated in a graded manner as dietary Cr increased, with shrimp fed 1.63 mg/kg Cr showing higher mRNA levels of *caspase 2*, *caspase 3* and *caspase 5* than those fed the unsupplemented diet. Similarly, the expression of *mt* was highest in shrimp fed 1.63 mg/kg Cr compared to shrimp fed lower Cr, significantly in the case of shrimp fed the diet containing 1.43 mg/kg Cr.

3.6.2 Genes involved in insulin signaling pathway

To further investigate the role of Cr on glucose and lipid metabolism, expression of genes involved in the insulin signaling pathway were determined with expression of *insr*, *irs1*, *pik3ca*, *pdpk1* and *akt* in hepatopancreas significantly affected by dietary Cr level (Fig. 7). Expression levels of *insr*, *pik3ca* and *akt* in hepatopancreas were generally increased as dietary Cr increased, with shrimp fed the highest level of Cr being significantly higher than those fed the unsupplemented diet. In contrast, the expression of *irs1*, *pik3cd* and *pdpk1* in hepatopancreas increased in shrimp fed intermediate levels of Cr compared to the unsupplemented diet, but then decreased in shrimp fed the highest level of Cr. While many of these differences were not statistically significant, the pattern was similar in all 3 genes suggesting biological significance.

3.6.3 Glycogenesis, gluconeogenesis and lipogenesis related genes

Contrasting results were found for expression of *gsk-3β*, *foxO1*, *g6pc* and *pepck* (Fig. 8). Expression

of *gsk-3β*, *foxO1* and *pepck* were generally significantly down-regulated with increasing dietary Cr level, with lowest levels observed in shrimp fed 1.63 mg/kg Cr. Expression of *gys* in hepatopancreas showed the increasing-decreasing pattern described above, being increased in shrimp fed intermediate levels of Cr (1.43 mg/kg) compared to the unsupplemented diet but then decreased in shrimp fed the highest level of Cr (1.63 mg/kg). The opposite pattern was shown in *g6pc*, with expression being significantly lower in shrimp fed intermediate levels of Cr compared to the lowest and highest levels of Cr.

As shown in Fig. 9, Cr supplementation promoted mRNA level of genes involved in glucose transport, glycolysis and lipogenesis. Compared to basal diet, expression levels of *hk* and *acc1* were significantly higher in shrimp fed the 1.63 mg/kg Cr diet compared to shrimp fed the unsupplemented diet. In contrast, expression of *pk* was lowest in shrimp fed the diet with highest Cr, being significantly lower compared to shrimp fed the diet with 1.43 mg/kg Cr. Expression levels of *glut1* and *srebp* showed the increasing-decreasing pattern, with highest expression levels observed in shrimp fed the diet containing 1.22 mg/kg Cr.

4. Discussion

Biological benefits of chromium continue to be debated, due to it having both beneficial nutritional effects as an essential trace element and detrimental side effects of a toxic metal (Vincent, 2013). In the present study, shrimp receiving dietary Cr of 1.01 or 1.22 mg/kg showed significant improvement in growth performance, with no additional benefit at higher dietary Cr supplementation levels. While similar studies in crustaceans are lacking, the results were consistent with previous studies in fish species. A study in grass carp *Ctenopharyngodon idellus* fingerlings

reported that WG increased as dietary Cr (as organic chromium picolinate) increased from 0.26 to 0.94 mg/kg, but declined when Cr in the diet increased to 3.38 mg/kg (Liu et al., 2010) Similarly, hybrid tilapia *Oreochromis niloticus*×*O. aureus* fed 205 mg/kg Cr (Cr_2O_3) showed highest WG, while lowest WG was recorded in fish fed 3421 mg/kg Cr (Shiau and Shy, 1998). Furthermore, a study with common carp *Cyprinus carpio L.* fed diets with 0 – 2 mg/kg Cr (chromium chloride) showed that 0.5 – 1.0 mg/kg promoted growth, but the highest level of Cr impaired growth and seemed toxic (Ahmed et al., 2013). In Indian major carp *Labeo rohita* fingerlings, WG and SGR were highest in fish fed 0.8 mg/kg Cr picolinate, but were reduced in fish fed 1.2 mg/kg Cr (Giri et al., 2014). The present study also found that shrimp fed the highest level of organic Cr (1.63 mg/kg) showed the lowest WG among the diets, indicating that supplementing the diet with Cr in excess of physiological requirements might lead to toxicity and growth inhibition of *L. vannamei*.

Although chromium is absorbed with low efficiency, it can still accumulate in tissues after a period of dietary management (Tacon and Beveridge, 1982). The present study demonstated that incremental dietary chromium significantly increased Cr concentrations in hapatopancreas, muscle and carapace and did not reach a plateau, implying that deposition of Cr in tissues was positively correlated with dietary Cr level in *L. vannamei*, consistent with previous studies in fish species (Küçükbay et al., 2006; Ahmed et al., 2012, 2013). For instance, in common carp, Cr concentration in liver increased as dietary Cr increased from 0.5 to 2.0 mg/kg (Ahmed et al., 2012) while Cr concentration in whole body increased with increasing dietary Cr up to 1.5 mg/kg (Ahmed et al., 2013). Thus, overfortification of chromium in feed could lead to excessive Cr deposition in tissues, which might cause both toxicity in the animal as well as food safety issues for human consumers, although further in-depth studies are required.

353 Chromium induces oxidative stress *via* multiple pathways derived from the production of
354 oxyradicals and depletion of glutathione (Hojo and Satomi, 1991; Yao et al., 2008). Depending on
355 the production of ROS, Cr-induced oxidative stress may lead to cellular redox imbalance or
356 apoptosis (Sun et al., 2015). Unstable metabolic intermediates (CrV and CrIV) and final product
357 (CrIII) produced during Cr reduction react with H_2O_2 to generate hydroxyl radicals (Yao et al.,
358 2008). Alternatively, Cr generates hydroxyl radicals *via* the Haber-Weiss reaction in the presence
359 of endogenous superoxide anions or H_2O_2 (Yao et al., 2008). In addition, chromium depletes cellular
360 antioxidants by forming chromium-glutathione (Yao et al., 2008), while CrIII exposure reduced
361 total glutathione by 34 – 69 % in liver of goldfish *Carassius auratus* (Lushchak et al., 2009).
362 Accumulation of MDA or H_2O_2 are markers for oxidative stress (Buddi et al., 2002), while cellular
363 enzymes including superoxide dismutase, catalase and glutathione peroxidase are an important
364 defense system for combating oxidative stress. Superoxide dismutase catalyzes dismutation of
365 superoxide radicals into H_2O_2, while catalase and glutathione peroxidase reduce H_2O_2 to water
366 (Chelikani et al., 2004; Hayyan et al., 2016). Moreover, the capability for scavenging hydroxyl free
367 radicals is considered another essential indicator of defense against oxidative stress (Oowada et al.,
368 2012). In this way, cysteine residues in metallothionein can capture hydroxyl radicals and thus
369 protect against metal toxicity and oxidative stress (Kumari et al., 1998), and its biosynthesis
370 appeared to increase several-fold during oxidative stress in order to protect cells against cytotoxicity
371 and DNA damage (Wang et al., 2014). In the present study, shrimp fed the highest dietary level of
372 Cr had high levels H_2O_2, MDA and MT, and low levels of expression and activities of SOD, CAT
373 and GPX-PX, and thus SCHR in hepatopancreas was reduced, indicating that this level of Cr
374 induced oxidative stress in *L. vannamei*. Similarly, increased oxidative stress has been reported in

fish species including rock fish *Sebastes schlegelii* exposed to dietary Cr (Kim and Kang 2016), and both fish European eel *Anguilla anguilla* L. (Ahmad et al., 2006) and crustacean freshwater field crab *Barytelphusa guerini* (Sridevi et al., 1998) exposed to environmental Cr. Specifically, expression of *mt* increased considerably in liver of rock fish after consuming dietary Cr over 120 mg/kg in 2-weeks or 30, 120, 240 mg/kg in 4-weeks, suggesting that Cr-induced oxidative stress was dose- and time-dependent (Kim and Kang, 2016). Water-borne inorganic $CrCl_3$ and $K_2Cr_2O_7$ induced lipid peroxidation and oxidative stress as evidenced by increased MDA and activities of SOD and xanthine oxidase in hepatopancreas and gill of freshwater field crab (Sridevi et al., 1998). Similarly, water Cr exposure caused oxidative stress in European eel as indicated by decreased glutathione and loss of DNA integrity in gill (Ahmad et al., 2006). The highest level of dietary Cr may cause oxidative damage to DNA in shrimp as evidenced by the increased level of 8-hydroxydeoxyguanosine (8-OHDG), which is a representative of oxidation of deoxyguanosine, and thus a biomarker of DNA damage and oxidative stress (Park et al., 1992; Helbock et al., 1999; Ock et al., 2012).

In addition in the present study, expression levels of apoptosis related genes (*caspase 2, 3, 5*) were significantly up-regulated and expression of anti-apoptosis gene (*bcl2*) was down-regulated in shrimp fed the highest dietary level of Cr. The synergistic effect of the caspase family is related to apoptosis and can be further subdivided into apoptotic caspases (caspase 3) and inflammatory caspase (caspase 4, 5) (Boatright and Salvesen, 2003; Fuentes-Prior and Salvesen, 2004). In contrast, the apoptosis regulator Bcl2 is the most important protein for inhibiting apoptosis (Cory and Adams, 2002). Thus, the results of the current study suggested that dietary Cr at 1.63 mg/kg may not only cause oxidative stress, but also may promote apoptosis in *L. vannamei*.

Glycolysis and gluconeogenesis are two major pathways of glucose metabolism regulated by multiple enzymes and hormone, of which insulin and glucagon are two most common regulators (Koeslag et al., 2013). The presence of insulin-like peptide (ILP) and crustacean hyperglycemic hormone (CHH) have been proposed in *L. vannamei*, and whose functions are associated with glucose homeostasis (Gutiérrez et al., 2007; Liu, 2014). A study reported that CHH elevated blood glucose and was regulated by a negative feedback mechanism through ILP, which is similar to the typical functions of glucagon and insulin in vertebrates (Jiang et al., 2020). In addtion, glucose metabolism is regulated by enzymes such as phosphofructokinase (PFK), pyruvate kinase (PK) and hexokinase (HK), which catalyze three irreversible reactions in glycolysis (Stryer, 1995). While most steps in gluconeogenesis are the reverse of glycolysis, the three steps above are replaced by irreversible reactions with PEPCK catalyzing the formation of phosphoenolpyruvate from oxaloacetate, the reverse reaction of PK (Chakravarty et al., 2005). Besides, pyruvic acid (PA) can be produced from glucose via glycolysis, and the level of glucose and PA in body partially reflects glucose metabolism (Mulukutla et al., 2014), while Evock-Clover et al. (1993) reported that the ratio of insulin/glucose can be considered an indicator of insulin sensitivity. The present study showed that shrimp fed the highest level of dietary Cr had the lowest levels of CHH and ILP in hemolymph. Furthermore, activities of HK, PFK and PK were elevated and PEPCK decreased in shrimp fed the Cr supplemented diets, which indicated that Cr promoted glycolysis and inhibited gluconeogenesis. In addition, the ILP/Glu ratio in shrimp increased as dietary Cr increased from 0.82 to 1.43 mg/kg, and then decreased at the highest level of dietary Cr, suggesting that appropriate level of Cr enhance ILP sensitivity. Similar results showing decreasing serum insulin as dietary Cr level increased suggesting that Cr might enhance insulin sensitivity were reported previously (Zha

et al., 2007; Liu et al., 2010; Mehrim, 2014; Rakhmawati et al., 2018).

The insulin signaling pathway maintains glucose homeostasis *via* increasing uptake and reducing synthesis of glucose in liver (Rhoads, 2001). Studies have shown that the functional mechanism of the insulin pathway is evolutionary conserved among multiple organisms (Wu and Brown, 2006; Boucher et al., 2010). Insulin receptor (INSR) is a type of tyrosine kinase receptor found widely in organisms (Ward and Lawrence, 2009) and the pathway is activated when insulin binds to INSR resulting in tyrosine phosphorylation of insulin receptor substrates (IRS) (Beale, 2013). Growing evidence indicated that phosphoinositide 3-kinases (P13K, including the subunits PIK3CA, PIK3CB and PIK3CD) are key components in insulin-mediated metabolism triggered by INSR and IRS (Hirsch et al., 2017). Protein kinase B (also known as AKT) is a major signaling molecule in the insulin pathway that is itself phosphorylated and activated by phosphoinositide dependent kinase 1 (PDPK1) (Jacinto et al., 2006; Beale, 2013). Activated AKT affects downstream transcription factors including forkhead box transcription factor class O1 (FOXO1), glycogen synthase kinase (GSK) and sterol-regulatory element binding protein (SREBP) to regulate gluconeogenesis, glycogenesis and lipogenesis (Beale, 2013). However, activated AKT inhibits GSK3, while phosphorylation of protein by GSK3 generally inhibits activity of its downstream targets such as glycogen synthase (GYS) (Woodgett, 1994). Therefore, deactivated GSK3 leads to activation of GYS and increases glycogen synthesis (Woodgett, 1994). In addition, AKT suppresses the gluconeogenesis pathway by phosphorylating transcription factor FOXO1, leading to its nuclear exclusion and inactivation (Tikhanovich and Weinman, 2013). Phosphorylated FOXO1 is then ubiquitinated and degraded by proteosome (Matsuzaki et al., 2003). Thus, inactivated FOXO1 cannot bind to its target genes such as fructose-1,6-bisphosphatase (FBP), glucose-6-phosphatase

(G6PC) and PEPCK, resulting in suppression of gluconeogenesis (Nakae et al., 2008). P13K/AKT enhances activity of SREBP, which is a master transcriptional regulator in lipid metabolism (Krycer et al., 2010). Overall, results of the present study clearly suggested that Cr activated the insulin signaling pathway *via* up-regulating expression of *insr*, *irs1*, *pik3ca*, *pdpk1* and *akt*. Elevated expression of *akt* triggered downstream transcription factors *srebp*, and inbibited *foxO1* and *gsk-3β* that enhanced lipogenesis and glycogenesis and inhibited gluconeogensis *via* up-regulating *acc1* and *gys*, and down-regulating *g6pc* and *pepck* (Fig 10).

5. Conclusion

In conclusion, the current study demonstrated that supplementing the diet of shrimp *L. vannamei* with 1.22 mg/kg Cr promoted growth, but the highest level of supplementation (1.63 mg/kg) caused growth suppression. Dietary Cr supplementation modulated the insulin signaling pathway to trigger glycolysis and glycogenesis and suppress gluconeogenesis to maintain glucose homeostasis. The highest level of Cr also increased oxidation products, reduced the content of cellular antioxidants, and activated expression of caspase family genes leading to oxidative stress and apoptosis. This study highlighted the contrasting effects of dietary chromium, with appropriate supplementation bringing beneficial effects on glucose homeostasis and growth, whereas in excess it can cause oxidative damage and impair growth.

Funding

This study was supported by the National Key R & D Program of China (2018YFD0900400), China Agriculture Research System (CARS-48), National Natural Science Foundation of China

(32072987), Industrial Chain Collaborative Innovation Project of Demonstration Work on Innovative Development of the Marine Economy of the State Oceanic Administration (NBHY-2017-S2), Nature Science Foundation of Zhejiang Province (LY17C190002), Key Research Program of Zhejiang Province of China (2018C02037), Zhejiang Aquaculture Nutrition & Feed Technology Service Team (ZJANFTST2017-2), and the K. C. Wong Magna Fund of Ningbo University.

Conflicts of interest

The authors declared that there were no conflicts of interest.

Animal ethics

We ensured that this experiment strictly followed the ethical guidelines of Standard Operation Procedures (SOP) of Experimental Animal of Ningbo University, and was approved by the Institutional Animal Care and Use Committee of Ningbo University.

Authors' contributions

B.S.: Conceptualization, Software, Validation, Writing - Original Draft. **M.B.B.** and **D.R.T.:** Writing - Review & Editing, Supervision. **X.Y.T.** and **J. J. L.:** Software, Writing - Review & Editing. **F.Y.M.** and **C.F.S.:** Writing - Review & Editing. **L.F.J.:** Supervision. **Q.C.Z.:** Validation, Resources, Writing - Review & Editing, Supervision, Funding acquisition. **M.J.:** Resources, Writing - Review & Editing, Supervision, Funding acquisition. All the authors read and approved the final version of the manuscript.

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Table 1

Formulations and proximate compositions of the experimental diets

Ingredients (g/kg)	Dietary chromium level (mg/kg)				
	Cr0.82	Cr1.01	Cr1.22	Cr1.43	Cr1.63
Fish meal	200.00	200.00	200.00	200.00	200.00
Soy protein concentrate	60.00	60.00	60.00	60.00	60.00
Soybean meal	230.00	230.00	230.00	230.00	230.00
Poultry meal	60.00	60.00	60.00	60.00	60.00
Krill meal	30.00	30.00	30.00	30.00	30.00
Peanut meal	50.00	50.00	50.00	50.00	50.00
Wheat flour	286.75	286.75	286.75	286.75	286.75
Fish oil	15.00	15.00	15.00	15.00	15.00
Soybean oil	15.00	15.00	15.00	15.00	15.00
Soy lecithin	20.00	20.00	20.00	20.00	20.00
Mineral premix [1]	10.00	10.00	10.00	10.00	10.00
Vitamin premix [2]	5.00	5.00	5.00	5.00	5.00
Ca $(H_2PO_4)_2$	15.00	15.00	15.00	15.00	15.00
Choline chloride	3.00	3.00	3.00	3.00	3.00
Astaxanthin	0.25	0.25	0.25	0.25	0.25
Chromium chelate of methionine (mg/kg) [3]	0.00	0.16	0.31	0.47	0.62
Proximate composition (dry matter, %)					
Crude protein	42.56	42.99	42.05	43.01	42.22
Crude lipid	8.05	8.24	7.99	8.15	8.65
Dry matter	89.42	89.64	89.41	89.15	89.33
Ash	10.57	10.59	10.99	11.04	11.15
Cr (mg/kg）	0.82	1.01	1.22	1.43	1.63

[1] Mineral premix (g/kg diet): NaCl, 0.74; K_2SO_4, 2.25; $MgSO_4 \cdot 7H_2O$, 3.62; $FeSO4 \cdot 7H_2O$, 0.25; $CaCO_3$, 0.16; $MnSO_4 \cdot H_2O$, 0.12; $CuSO_4 \cdot 5H_2O$, 0.16; $ZnSO_4 \cdot 7H_2O$, 0.27; KIO_3 (1%), 0.02; Na_2SeO_3 (1%), 0.07; $CoSO_4 \cdot 7H_2O$, 0.02; zeolite, 2.28. The mineral premix does not supply Cr.

[2] Vitamin premix were based on Shi et al. (2021a).

[3] Chromium chelate of methionine (Zinpro Corp., USA), Cr content = 1286.50 mg/kg.

Table 2

Growth performance, feed utilization and morphologic index of juvenile *L.vannamei* fed diet with different Cr levels

Items	Cr0.82	Cr1.01	Cr1.22	Cr1.43	Cr1.63	*P*-value
IBW (g)	3.20±0.01	3.20±0.01	3.19±0.01	3.21±0.01	3.20±0.01	0.643
WG (%)	227.07±6.10^{ab}	251.13±6.59^{c}	260.25±6.45^{c}	247.37±10.58^{bc}	206.40±6.42^{a}	0.000
Survival (%)	82.67±1.25	84.00±1.25	84.00±1.25	84.00±1.25	81.33±0.82	0.420
SGR (%/day)	2.42±0.04^{b}	2.56±0.04^{c}	2.61±0.04^{c}	2.54±0.06^{bc}	2.28±0.04^{a}	0.000
FI (%/body weight day)	3.53±0.03^{b}	3.30±0.04^{a}	3.24±0.04^{a}	3.33±0.06^{a}	3.73±0.06^{c}	0.000
FCR	1.88±0.03^{b}	1.64±0.04^{a}	1.58±0.04^{a}	1.67±0.05^{a}	2.15±0.08^{c}	0.000
HSI (%)	3.20±0.12	3.34±0.14	3.48±0.04	3.65±0.17	3.2±0.15	0.155
CF (g/cm^{3})	0.63±0.01	0.62±0.01	0.60±0.01	0.60±0.01	0.62±0.01	0.139

Values are means ± SEM (n = 5). Different superscript letters indicate significant different within treatment ($P < 0.05$). CF, condition factor; FCR, feed conversion ratio; FI, feed intake; HSI, hepatosomatic index; IBW, initial mean body weight; WG, weight gain; SGR, specific growth rate.

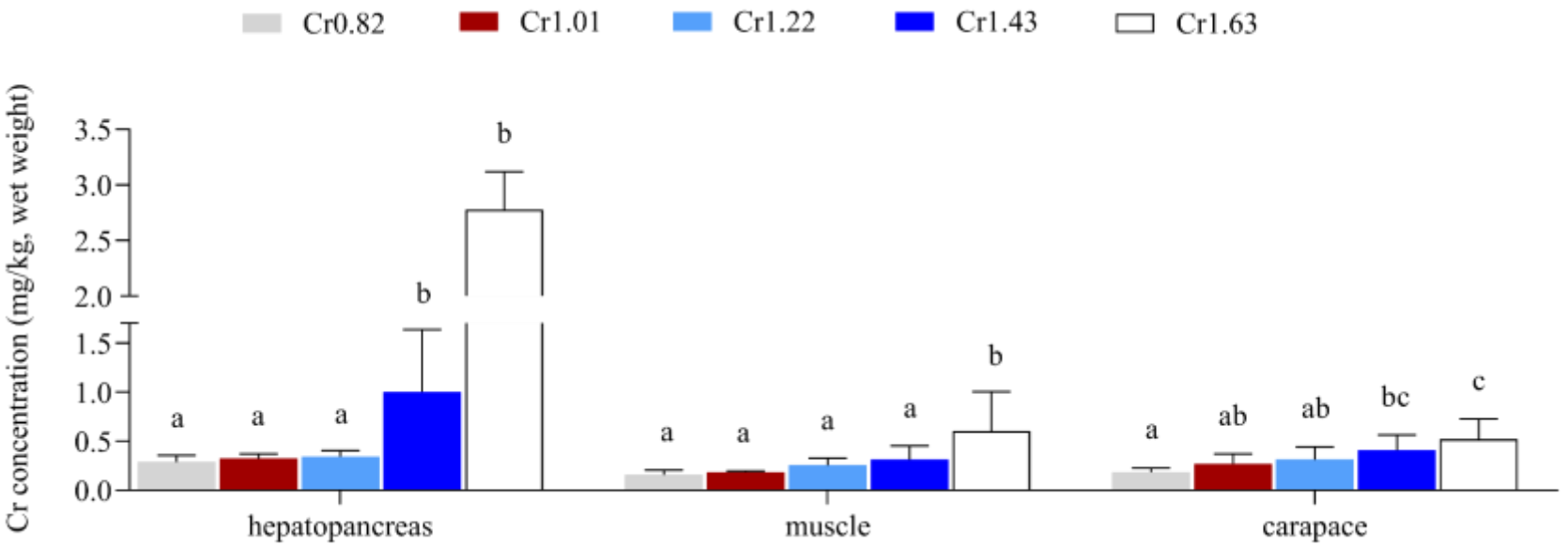

Fig. 1 Chromium concentration (mg/kg, wet weight) in tissues of *L. vannamei* fed experimental diets. Columns represent means with bars indicating standard error (n = 5).

Different letters above columns indicate significant differences between mean values.

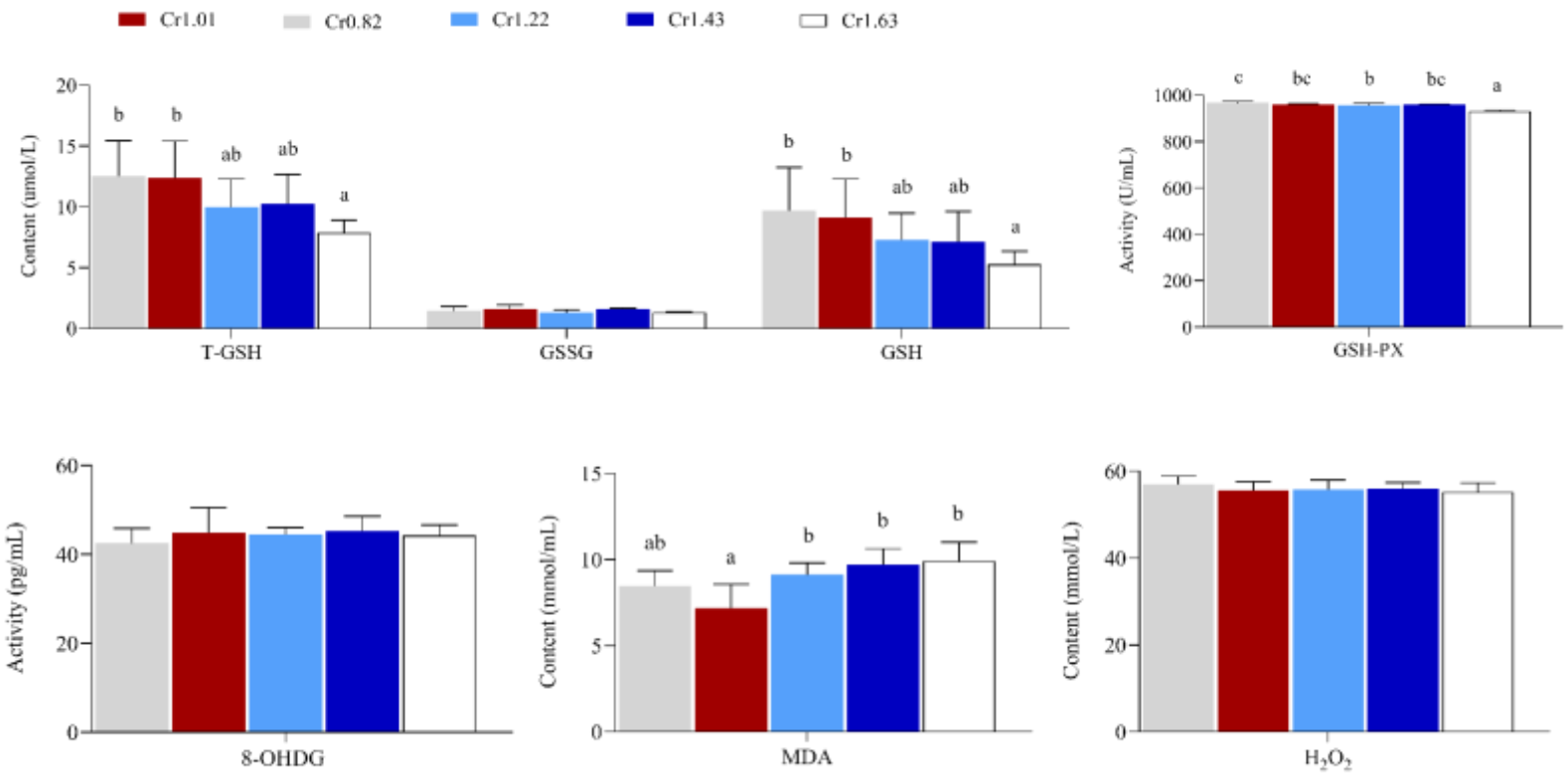

Fig. 2 Oxidation and antioxidant parameters in hemolymph of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. GSH, oxidized glutathione; GSH-PX, glutathione peroxidase; GSSG, reduced glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; T-GSH, total glutathione; 8-OHDG, 8-hydroxydeoxyguanosine.

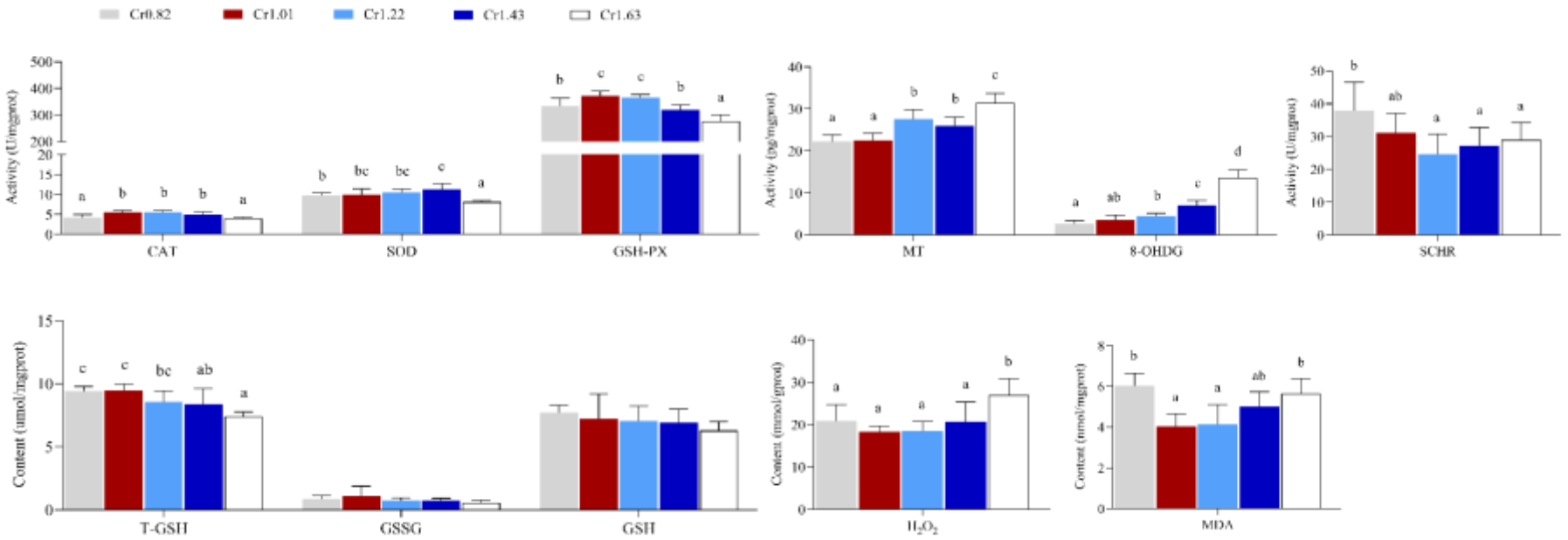

Fig. 3 Oxidation and antioxidant parameters in hepatopancreas of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. CAT, catalase; MT, metallothionein; SCHR, scavenging capability for hydroxyl free radical; SOD, superoxide dismutase.

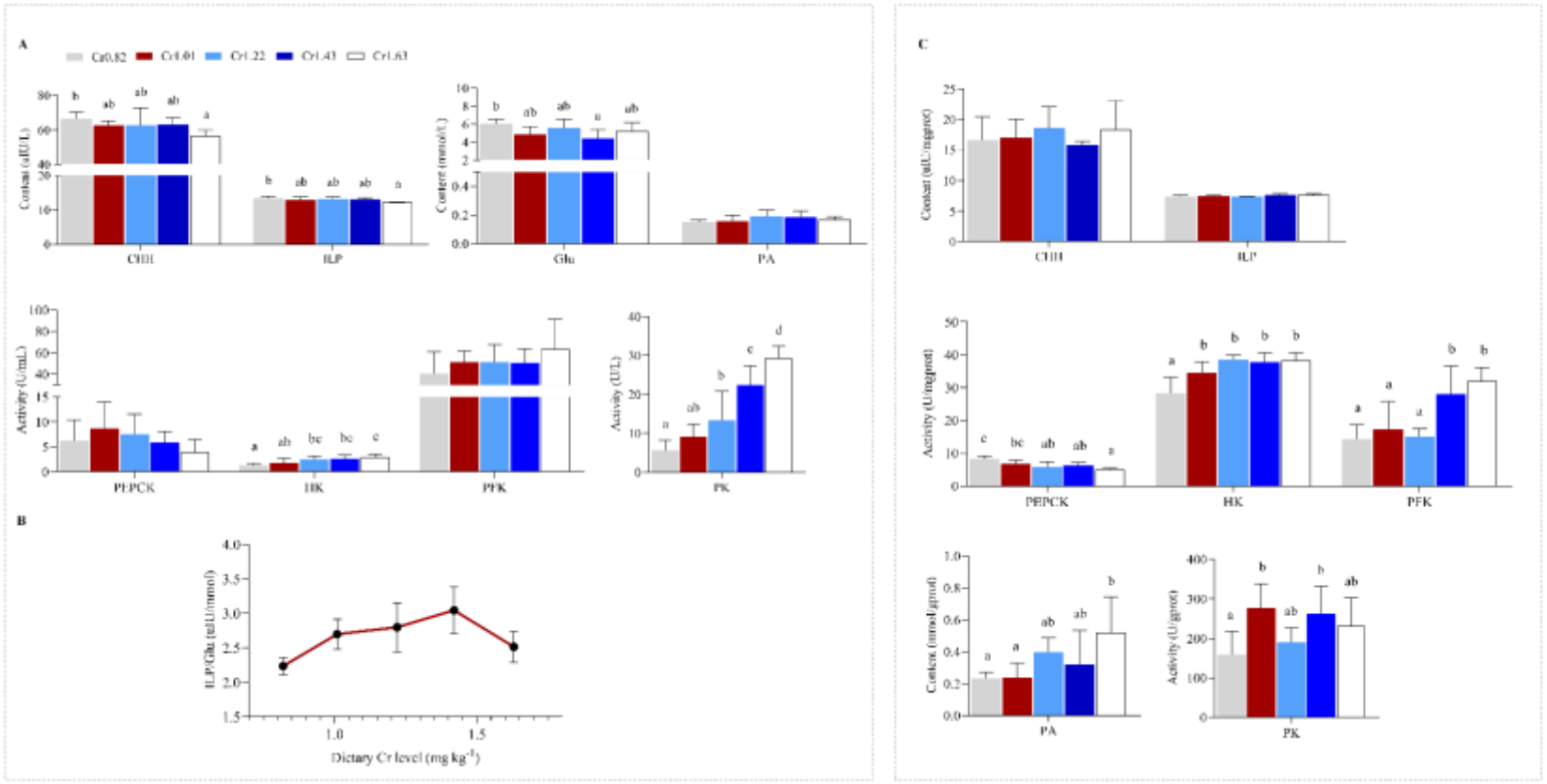

Fig. 4 Glucose metabolism related parameters in hemolymph (**A**) and hepatopancreas (**C**), and ratio of ILP/Glu (**B**) of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. CHH, crustacean hyperglycemic hormone; Glu, glucose; HK, hexokinase; ILP, insulin like peptide; PA, pyruvic acid; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PK, pyruvate kinase.

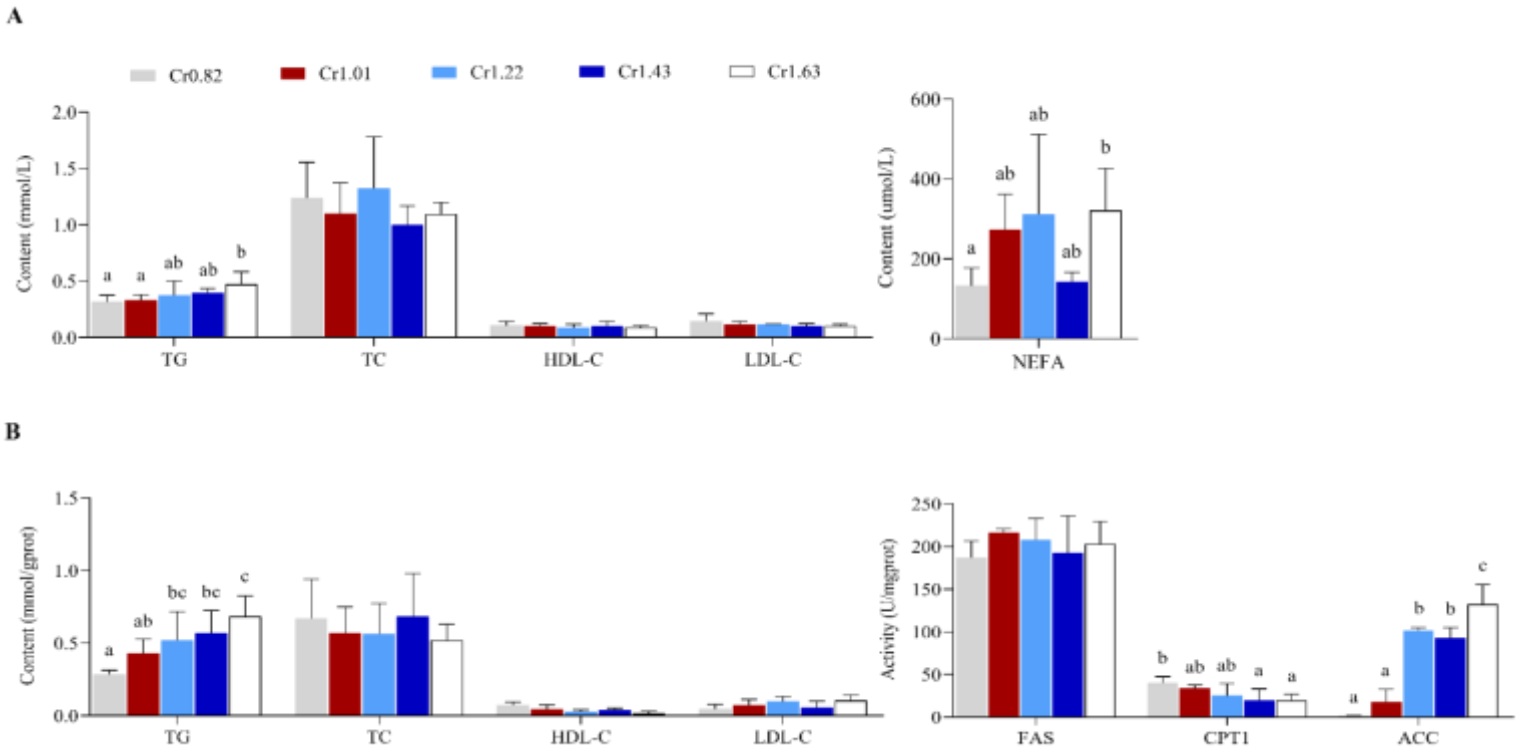

Fig. 5 Lipid metabolism related parameters in hemolymph (**A**) and hepatopancreas (**B**) of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. ACC, acetyl-CoA carboxylase; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthase; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; NEFA, non-esterified fatty acids; TC, total cholesterol; TG, triacylglycerol.

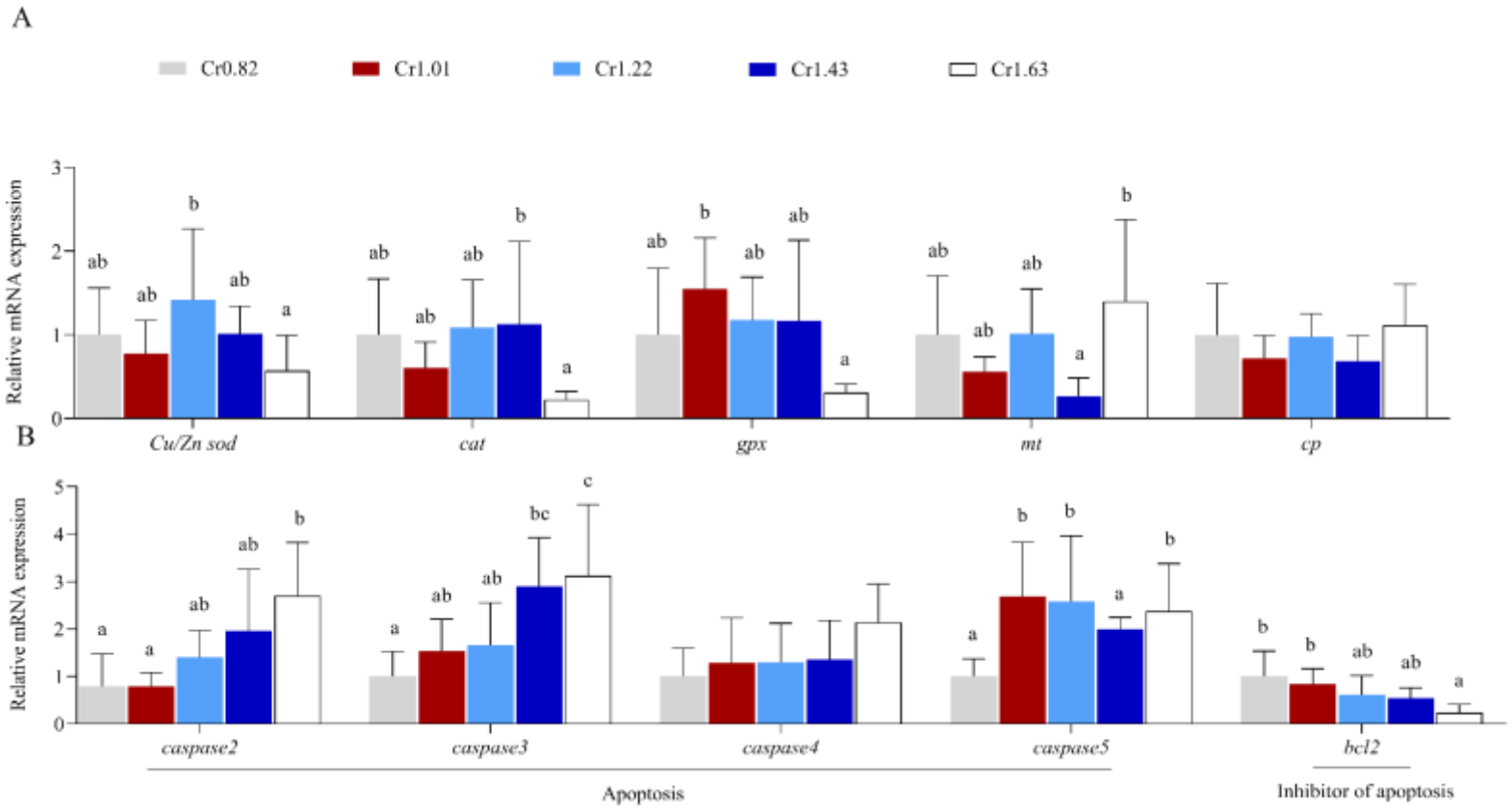

Fig. 6 Expression of genes related to oxidative stress (**A**) and apoptosis (**B**) of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. *bcl2*, Bcl2 protein; *cp*, ceruloplasmin.

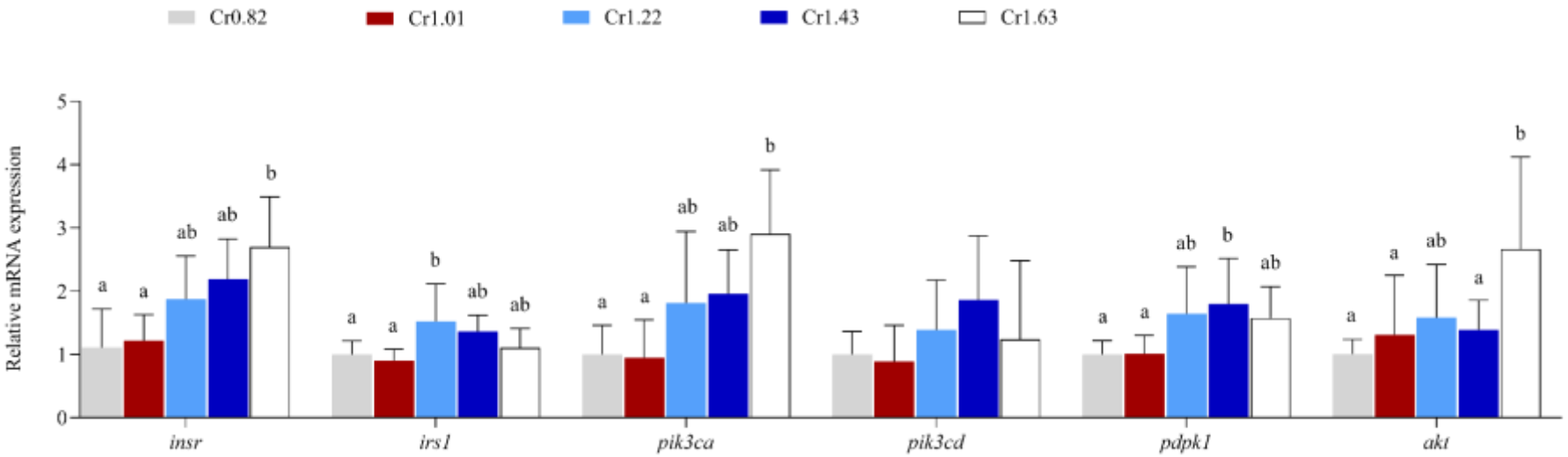

Fig. 7 Expression of genes involved in insulin signaling pathway of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. *akt*, RAC-alpha serine/threonine-protein kinase; *insr*, insulin receptor; *irs1*, insulin receptor substrate 1; *pdpk1*, 3-phosphoinositide-dependent protein kinase 1; *pik3ca*, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform; *pik3cd*, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform.

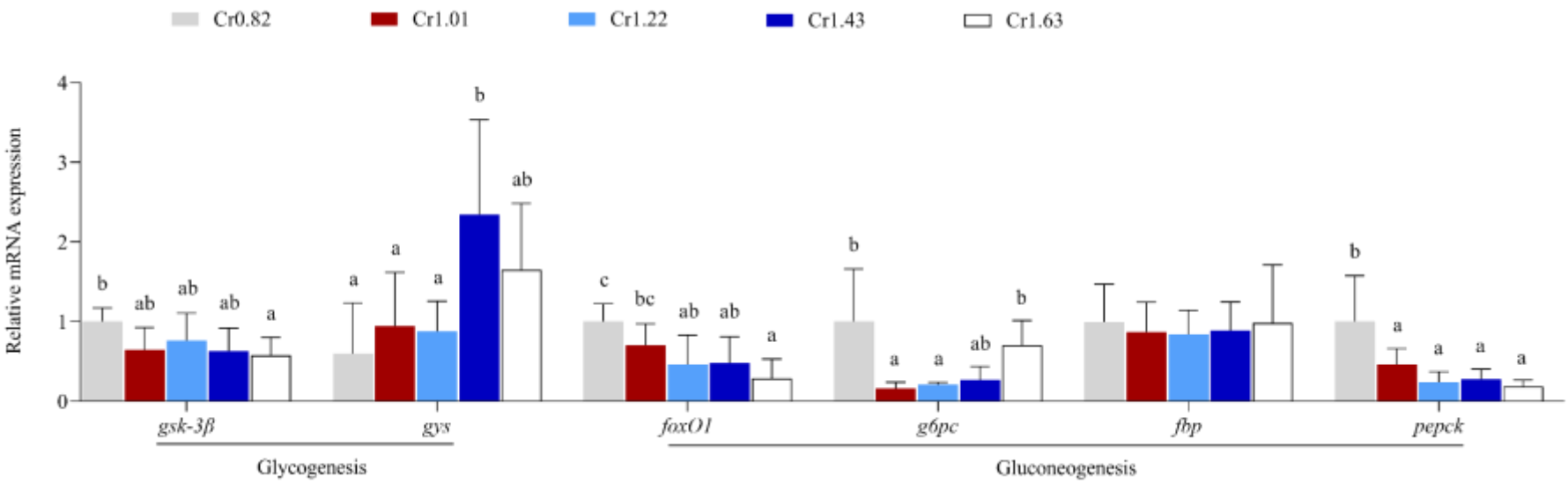

Fig. 8 Expression of glycogenesis and gluconeogenesis related genes of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. *fbp*, fructose-1,6-bisphosphatase 1; *foxO1*, forkhead box transcription factor class O1; *g6pc*, glucose-6-phosphatase; *gsk-3β*, glycogen synthase kinase-3 beta; *gys*, glycogen synthase; *pepck*, phosphoenolpyruvate carboxykinase.

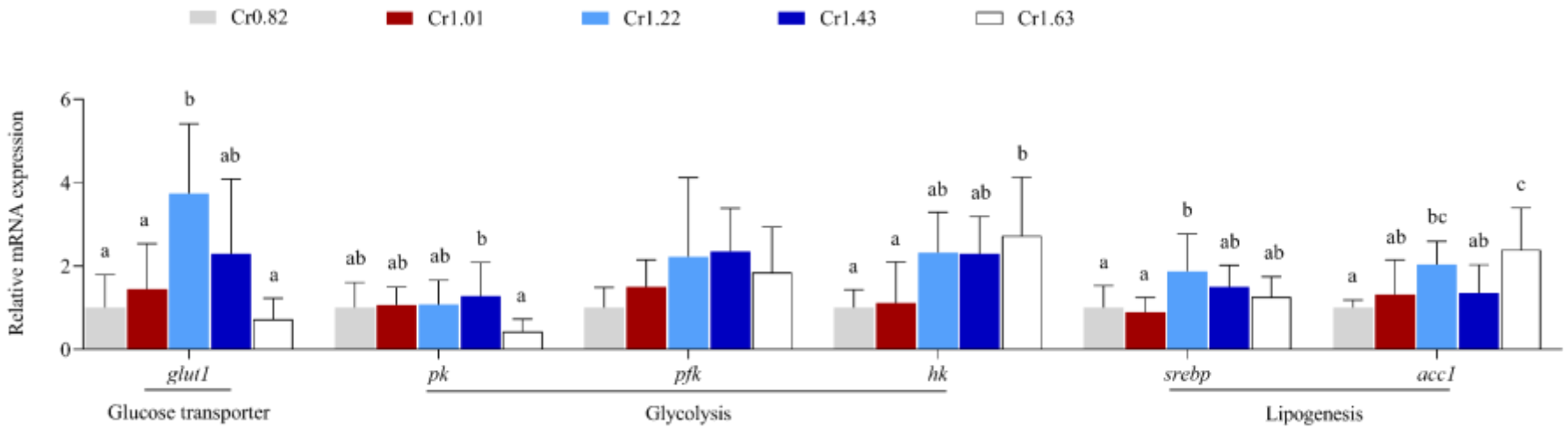

Fig. 9 Expression of genes involved in glycolysis and lipogenesis of *L. vannamei* fed the experimental diets. Columns represent means with bars indicating standard error (n = 5). Different letters above columns indicate significant differences between mean values. *acc1*, acetyl-CoA carboxylase; *glut1*, glucose transporter 1; *hk*, hexokinase; *pfk*, phosphofructokinase; *pk*, pyruvate kinase; *srebp*, sterol-regulatory element binding protein.

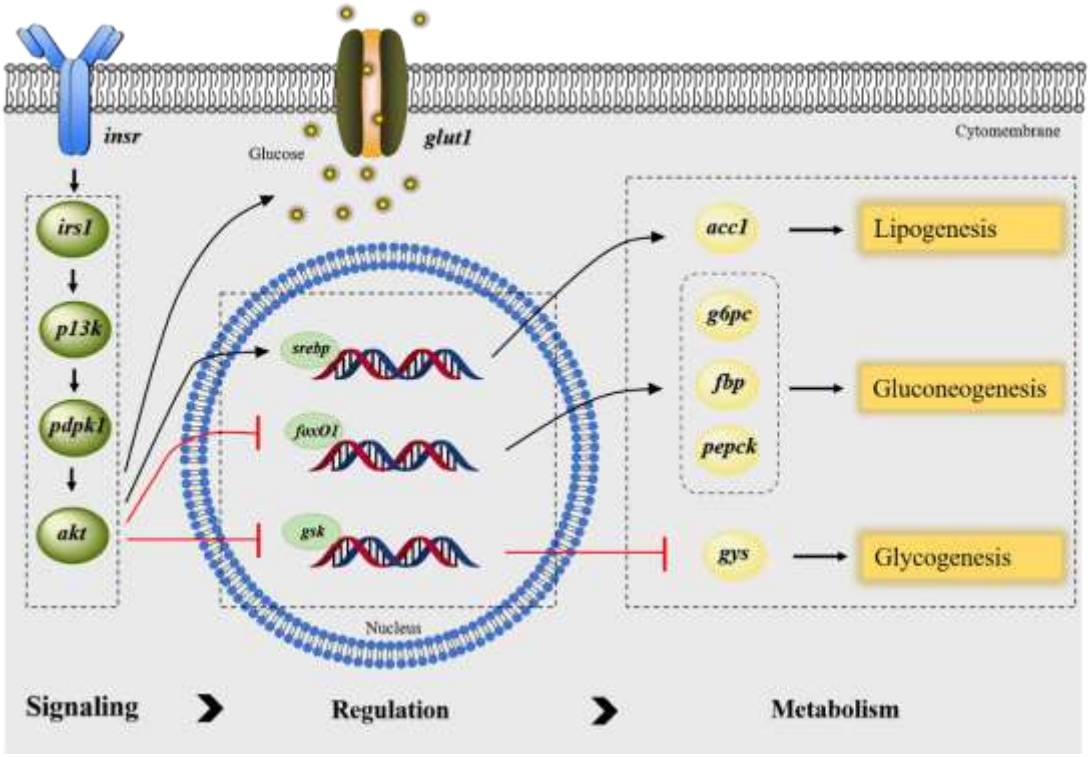

Fig. 10 A working model of chromium-mediated glucose homeostasis in hepatopancreas. The black lines indicate promotion and the red lines indicate suppression. Briefly, chromium activates *insr* and transmits signals to *akt* via *irs1*, *p13k* and *pdpk1*. Activated *akt* inbibits expression of downstream transcription factors *foxO1* and *gsk*, and promotes *srebp*. Accordingly, *srebp* induces expression of *acc1* to promote lipogenesis. Inactivated *foxO1* suppresses expression of *g6pc*, *fbp* and *pepck*, resulting in reduced gluconeogenesis. Deactivated *gsk* activates *gys* leading to increased glycogen synthesis. In addition, up-regulated *glut1* promotes transport of glucose from hemolymph to hepatopancreas.

Dietary chromium modulates glucose homeostasis and induces oxidative stress in Pacific white shrimp (*Litopenaeus vannamei*)

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Table S1

Amino acid compositions (g/100g, dry matter) of the experimental diets

Amino acids	Dietary chromium level (mg/kg)				
	Cr0.82	Cr1.01	Cr1.22	Cr1.43	Cr1.63
Arg	2.78	2.77	2.71	2.76	2.75
His	1.05	1.04	1.02	1.04	1.02
Ile	1.80	1.82	1.78	1.82	1.81
Leu	3.29	3.28	3.26	3.30	3.27
Lys	2.40	2.41	2.36	2.41	2.40
Met	0.83	0.84	0.85	0.84	0.84
Thr	1.61	1.61	1.58	1.60	1.60
Phe	1.90	1.92	1.89	1.91	1.91
Val	2.13	2.13	2.13	2.12	2.12
Total essential amino acids	17.79	17.82	17.58	17.80	17.72
Ala	1.76	1.76	1.74	1.77	1.78
Asp	3.70	3.70	3.62	3.68	3.67
Cys	0.54	0.57	0.55	0.55	0.56
Glu	6.52	6.51	6.48	6.49	6.48
Gly	1.80	1.79	1.78	1.78	1.79
Pro	2.32	2.31	2.26	2.30	2.27
Ser	1.75	1.72	1.70	1.71	1.69
Tyr	1.27	1.27	1.23	1.25	1.26
Total nonessential amino acids	19.71	19.46	19.18	19.54	19.46
Total amino acids	37.50	37.28	36.76	37.34	37.18

Table S2

Primers for real-time quantitative PCR

Gene	Primers (5′-3′)	Size (bp)	TM (°C)	Accession no./ References
β-actin	F: CGAGGTATCCTCACCCTGAA R: GTCATCTTCTCGCGGTTAGC	176	58.22 58.80	Shi et al., 2020
insr	F: CAGGTCGGTATTGATAGAAGG R: TGTAGGGGCAGTGGTGAT	127	55.30 57.42	XM_027382580.1
irs1	F: ACCGCAAGAAGGACCCGAA R: ACTATCTCCGACCCGCACGA	290	61.51 62.88	XM_027373626.1
pik3ca	F: GCTCCAAACGGAAGCAGACT R: CCCTGGTCCTTTGGTTTTCG	331	60.60 59.04	XM_027370433.1
pik3cd	F: GCCATTTATGAAGTAACCCG R: GCTGGTTGCGGTAGTCGTAT	127	54.45 60.18	XM_027364511.1
pdpk1	F: GGGAGCATAAAAATCAACCAG R: GGGAAGAGACCCTTGCGTTTA	227	55.16 60.00	XM_027361849.1
akt	F: TCACACACTGACGGAAAACC R: TTCCATTACAAAGCACAGGC	106	58.38 56.61	XM_027364781.1
foxo1	F: AATGCCCAAAGGAGATGC R: AAGAGAATGCTGAGAAGGATG	274	55.24 55.38	XM_027376335.1
g6pc	F: AAAGTTGGAACCTGCGGA R: TCTCTCCCGTCCACCAAT	255	56.68 57.11	XM_027351517.1
fbp	F: GCTGGAGGTCAGGCAACAACT R: CCATTTCAGGGGGATTATTTC	185	62.87 54.24	XM_027380587.1
pepck	F: AGACCAGTGATGGAGGAGTGT R: CTGGTTTGCCCGATTCTT	114	60.20 55.21	XM_027371589.1
gsk-3β	F: AGGGCTCAGATAGACCGCA R: CTTGGAACACAACACCGA	81	60.08 55.11	XM_027362477.1
gys	F: GCCTCCCTGAACCAGATGAA R: ATTGTGTGTGGTGATTGGCG	107	59.38 59.40	XM_027374365.1
srebp	F: ACCATTGCCACTCCCCTA R: GTTGCGTTTCTCGCCTTT	150	57.40 56.67	Shi et al., 2020
acc1	F: TGCATAGAAACGGCATTGCG R: TTTGACACCTGAGCCAGACC	134	59.90 59.89	Shi et al., 2020
hk	F: AGCCTCAACCCGACTCAGAC R: GACCACTCTGAGGAGCGACA	119	61.54 61.24	XM_027356086.1
pk	F: CCACTGGTCGCTCTGCTCAT R: TGGGAATAATGCCACGGTAG	117	60.76 58.51	EF102105.1

glut1	F: CTTCGCTGCTGTGCTTGG R: ATCCTGCTTGCTGCCTTC	139	59.44 57.67	Wang et al., 2017
pfk	F: TTGTTGCTGCTTTGACCTCT R: AACCTTCTTCACTCCTTCCG	197	55.83 55.94	EF102107.1
Cu/Zn sod	F: ACAATCCGTATATGCGCCCC R: ACCGTACGAGGTCCCACTAA	145	60.32 59.96	Shi et al., 2021
cat	F: CCATCCTTCATTCACACGCAG R: GCCTTGGTCCGTCTTGTAATG	240	61.2 59.7	AY518322.1
gpx	F: AAACGGAGAGCGGAGAAACA R: GCCCCTAACACACAAGACAT	287	59.8 54.7	AY973252.2
mt	F: ATGCAAGTGCTGCCCATAGA R: GCCTCGCTCTCACTTTCTTACT	253	59.74 60.09	Shi et al., 2021
cp	F: CAAGGACAACCTACCCCCAT R: GCCAGGCAAAGATACGAACT	266	59.00 58.26	Shi et al., 2021
bcl2	F: TGGAATCACAAGAGAGCGAA R: CTGTTCTCCACGGTGTCTCA	85	56.87 59.33	MH559339.1
caspase2	F: GCGACAATGGCAGCAATGAG R: AGTGGCGGTGGTTGAAGATG	162	60.52 60.61	KC660102.1
caspase3	F: GCCAGTGCTGTCGCCTTTA R: TCTCGCTCTTCACCCTCCA	230	60.67 59.92	KC660103.1
caspase4	F: CCGAAAGAGGTTCTCGTCAA R: TATCCTGCCACTCGCTACTG	107	57.57 58.97	KC660105.1
caspase5	F: AGAGACTGCTGGAGGGATGA R: GTATGTTGCCTTCGGGTAAA	162	59.66 55.75	KC660104.1

Calculations

Weight gain (WG, %) = 100 × [final body weight (g) - initial body weight (g)] / initial body weight (g);

Specific growth rate (SGR, %/day) = 100 × [Ln (final body weight) - Ln (initial body weight)] /days;

Survival (%) = 100 × (final number of shrimp) / (initial number of shrimp);

Feed conversion rate (FCR) = feed consumption (g) / [final body weight (g) - initial body weight (g)];

Feed intake (FI, %/bw day) =100 × feed consumption / [(initial body weight + final body weight) / 2] / days;

Hepatosomatic index (HSI, %) = 100 × [hepatopancreas wet weight (g)] / [body wet weight (g)];

Condition factor (CF, g/cm^3) = 100 × [body weight (g) / body length^3 (cm^3)].

Proximate composition analysis of experimental diets

Crude protein (N × 6.25) was determined using the Dumas combustion method with an auto-protein analyzer (FP-528, Leco, USA). Crude lipid was determined by the ether extraction method using Soxtec (Soxtec System HT6, Tecator, Hoganas, Sweden). Moisture content was determined by drying the samples to a constant weight at 105 °C, and ash content was determined in a muffle furnace at 550 °C for 8 h.

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