DNA methylation of pck1 might contribute to the programming effects of early high-carbohydrate diets feeding to the glucose metabolism across two generations in zebrafish (Danio rerio)

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Research Article

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Abstract

To assess the effects of early high-carbohydrate stimulus on glucose metabolism across two generations in zebrafish (*Danio rerio*) and explore a mechanism to explain those nutritional programming effects via epigenetic modification. The larvae were delivered a high-carbohydrate diet (53.66%) during first-feeding to the end of the yolk-sac (FF) and after yolk-sac exhaustion for 5 d (YE) as early nutritional stimulus. Then those larvae (F0) and their offspring (F1) were both fed with control diet (22.69%) until adult (15 week), they were challenged with a high-carbohydrate diet (35.36%) at 16th week. The results indicated that early stimulus raised the mRNA levels of genes involved in glycolysis and gluconeogenesis immediately. At the end of challenge in F0, the plasma glucose levels were decreased and expression levels of glucokinase (*gck*) were increased, the transcription of genes in gluconeogenesis were inhibited in two treatment groups. When challenged in F1, the glucose levels were lower in FF (F1) and the mRNA levels of phosphoenolpyruvate carboxykinase 1 (*pck1*) were decreased in FF (F1), YE (F1). Besides, the lower expression levels corresponding with hypermethylation in CpG island of *pck1* were maintained from the adult of F0, 24 hours post-fertilization embryo to the adult of F1 in two experimental groups (F0 and F1). In conclusion, these results indicated that early high-carbohydrate stimulus could significantly reprogram glucose metabolism in adult zebrafish and those modifications could be transmitted to next generation partly, the DNA methylation of *pck1* might work as a stable epigenetic marker to contribute to those processes.

Introduction

In mammals models, it is widely accepted that nutritional perturbations at crucial developmental windows, such as fetal or postnatal stage, could permanently program metabolism and elicit lifelong effects on organism's physiology and metabolism, which was known as nutritional programming (Patel et al., 2009; Ruchat et al., 2014). The early nutritional interferences are believed to confer the adaptive advantages to the animals which could promote them to sustain themself better when the nutritional environment in later life corresponds with that experienced in early stages(K. A. Lillycrop & Burdge, 2012; Marousez et al., 2019; Zheng et al., 2014). Those changes of physiology and metabolism were often accompanied by the changes in protein or mRNA levels or even the epigenetic changes in DNA methylation or histone modifications at promoter regions of candidate genes (K. Lillycrop & Burdge, 2015; Patel et al., 2009; Randunu & Bertolo, 2020; Ruchat et al., 2014). And sometimes those modifications could be transmitted to future generations as show by the research in Dutch famine about the nutritional programming induced by starvation during World War II (Roseboom et al., 2011). Fish are traditionally considered to be glucose intolerant and have limited ability to digest, absorb and metabolize carbohydrates in diets, especially the carnivorous species (Kamalam et al., 2017; Wilson, 1994). However, there is now substantial evidences that the ability of carbohydrate utilization in fish could be modified through nutritional programming. Fang et al. (2013) found that the adult zebrafish which were fed with a high-carbohydrate diet during the first-feeding period showed a lower plasma glucose levels and altered expression and activity levels of enzymes involved in carbohydrate digestion, transport and metabolism

when challenged with a similar high-carbohydrate diet in adult. Glucose injection in the yolk (early stimulus) induced the enhancement of glycolysis and glucose catabolism, the inhibition of gluconeogenesis in zebrafish juveniles (Rocha et al., 2015). The adult Gilthead seabream (*Sparas aurata*) which were briefly exposed to a high-glucose feeding at larval stage showed higher catabolism and lower retention of glucose, and higher bio-conversion of glucose into lipids in tissues when challenged with a similar diet (Rocha et al., 2016a; Rocha et al., 2016b). In conclusion, in fish, although many studies reveled the programming effects of early high-carbohydrate/high-glucose stimulus on the glucose metabolism in later life, the mechanism behind those phenomenon and whether those effects could be inherited by the offspring were still unclear.

Discussions on the mechanisms of programming have been ongoing since the 1990s, yet they remain largely unknown. Among many mechanisms, epigenetic modification especially DNA methylation had been identified as one of the most important (K. A. Lillycrop & Burdge, 2012; McGee et al., 2018; Zheng et al., 2014). DNA methylation occurs predominantly at the CpG dinucleotide and the hypermethylation in the CpG island (CpG-rich clusters) were usually associated with transcription suppression (Jones, 2012; Wilkinson, 2015). In mammals, a series of studies have demonstrated that the nutrition environment in the crucial developmental windows could trigger the DNA methylation modification. The increased consumption of fish oil during gestation and lactation in rats (*Rattus norvegicus*) would induce the higher DNA methylation levels in the promoter region of fatty acid desaturase 2 (FADS2) gene in the liver of offspring, which decreased the expression levels of *fads2* and lead to a lower levels of arachidonic acid and docosahexaenoic acid (Hoile et al., 2013). Compared with the rats fed with milk-like high-fat formula since they were born, the DNA methylation and histone in Neuropeptide Y (NPY) gene proximal promoter and histone acetylation in pre-pro-opiomelanocortin (POMC) gene promoter were modified in rats fed with high carbohydrate milk formula whether in pups or adult, which might develop early and persistent hyperinsulinemia as well as post-weaning hyperphagia, adult onset obesity (Mahmood et al., 2013; Srinivasan et al., 2003; Srinivasan et al., 2008). Besides, the nutrition perturbations during key ontogenetic phase were considered to induce lifelong effects not only on the current generation, but also on the future descendants. Burdge et al. (2007) found that the dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations despite that dietary protein restriction was only applied in F0 generation and no dietary modification was administered in their offspring. Wei et al. (2014) revealed that prediabetes could be inherited transgenerationally through the mammalian germ line by DNA methylation.

In Summary, epigenetic mechanisms provided a compelling explanation for nutritionally programmed metabolic syndrome and the intergenerational inheritance effects in mammals, however evidences of the epigenetic mechanisms being associated with nutritional programming in fishes are still scarce (Hou & Fuiman, 2020).

Therefore, in the present study, the plasma glucose levels and key gene expression levels in glycolysis and gluconeogenesis were measured in adult zebrafish of F0 and F1 at the end of the challenge to illustrate the intergenerational effects of early high-carbohydrate stimulus. Further, we evaluated the DNA

methylation statue of phosphoenolpyruvate carboxykinase 1 (*pck1*) in two generations of the fish to explore the role of epigenetics might played in early high-carbohydrate programing. Our results could promote the understanding to the early high-carbohydrate programming effects on glucose metabolism and provide references to the studies about the mechanism of early nutritional programming in fish, especially in glucose metabolism.

Materials And Methods

Diets preparation and feeding regime

Three experimental diets were prepared in the present study and they are prepared in Huazhong Agricultural University (Wuhan, China). All the ingredients (obtained from Gao Long Dietary Company, Wuhan, China) were ground into a fine powder through a 180 μm mesh and mixed the ingredients with fish oil and water to get the compound. The compound was put into the screw extruder (Nuoda, Xingtai, China) and got the pellets, then dried for about 12 h at 25℃ and broken up, sieved into a proper size and stored at -20℃ until use. The ingredient formulation and the proximate chemical composition of the experimental diets were shown in Table 1.

During the two early developmental stages, first-feeding to the end of the yolk-sac larval (3-5 days post-hatching (dph), FF) and after yolk-sac exhaustion for 5 d (6-10 days post-hatching (dph), YE), the larvae of experimental groups were fed HS diet (53.66% carbohydrate and 25.84% protein) and the control group were fed control diet (20.53% carbohydrate and 53.11 % protein). Then all three groups (FF, YE and control group, defined as F0) were fed with the control diet until the adult (15 week). At last, a HC (35.36% carbohydrate and 43.40% protein) diet was given to the adult fish in all groups at 16th week as a challenge test (Hou & Fuiman, 2020). The next generation of the three groups were defined as F1, were fed the control diet (20.53% carbohydrate and 53.11 % protein) until the adulthood (15 week), then replaced the control diet for a HC diet (35.36% carbohydrate and 43.40% protein) for one week as the challenge test. The feeding schemes were summarized in Table 2.

Fish rearing and breeding

The embryos of the wild-type zebrafish were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The embryos were randomly divided into three groups and hatched in College of Fisheries, Huazhong Agricultural University (Wuhan, China). The larvae were reared in a filtered recirculating culture system 10 d after hatching. Six tanks (10 L) were randomly prepared for FF, YE and control group respectively. 100 fish lived in a tank, one month later, half of the fish in a tank were transferred into another tank. During the high-carbohydrate diet stimulus period, the larvae were fed six times to visual satiety from 8:00 to 19:00 daily, then the fish were fed two times at 8:30 and 18:30 daily until 16 week (include the challenge test period) (Fang et al., 2013).

The sexual maturity adult zebrafish (15 week) which were selected from each group were transferred to breeding tanks before the onset of darkness. On the next morning, after exposed to light for 20–30 min,

the eggs were collected from the spawning trays which was on the bottom of the breeding tanks and transferred to Petri dishes (90mm in diameter, 50 eggs/dish) (Santangeli et al., 2019). The embryos were incubated at 28℃ and the larvae were transferred into a filtered recirculating culture system 10 d after hatching. The fish were fed two times at 8:30 and 18:30 daily from the first-feeding to the challenge test. All zebrafish were kept at 28±1℃ and PH at 7.50–7.73 under a 14h/10h light/dark cycle.

Sampling

At the end of the early high-carbohydrate stimulus (5 dph and 10 dph), the average body weight (n = 6 samples of 15 larvae each) and body length (performed by an Olympus LG-SP2 stereoscopic microscope, SZX2-FOF 9J01021, n = 6 samples of 15 larvae each) of treatment and control group were calculated. For the molecular analyses, the whole bodies of larvae (n = 6 samples of 15 larvae each) in each group were quickly frozen in liquid N_2 and stored in -80℃.

For the adult fish (15 week) of F0 and F1, six zebrafish in each group were randomly chosen and deeply anesthetized with MS-222 (Redmond, WA, USA, 200 mg/L) (3 h after the last meal), the body weight and length were recorded and the blood were collected at the caudal vein to evaluate the plasma glucose levels with the Glucose Assay Kit (Catalog no. F006-1-1, Jiancheng Institute of Biotechnology, Nanjing, China). Given that the liver plays a central role in the production and catabolism of glucose in response to nutrition conditions to keep glucose homeostasis in fish (Enes et al., 2009; Hao et al., 2015), so it was chose for molecular analyses. The liver was quickly frozen in liquid N_2 and stored at -80℃. At the end of HC diet challenge (16 week) in F0 and F1. Six fish in each group were randomly sampled (3 h after the last meal), the way to collect the blood and liver just like previous described.

DNA demethylation in zebrafish embryos begins at nearly 24 hours post- fertilization (hpf) (Bogdanović et al., 2016) and the peak in glucose also occurred almost at this time (Jurczyk et al., 2011), so at the end of 24 hpf, the embryos (n = 6 samples 40 embryos each) were frozen in liquid N_2 and stored at -80 ℃ for DNA or RNA extraction in each group.

Real-time quantitative PCR

The isolation of total RNA was performed with Trizol Reagent (TaKaRa, Tokyo, Japan) following the manufacturer's instructions. The BioTek Synergy 2 luminometer (BioTek, Winooski, VT, USA) was used to evaluate the RNA concentration and quantity. The electrophoresis in 2% agarose gel (Biowest Agarose, Madrid, Spain) was used to assess the integrity of total RNA. The transcription of total RNA to cDNA was performed with Revert Aid™ Reverse Transcriptase (TaKaRa, Tokyo, Japan) according to the manual. The real-time quantitative polymerase chain reaction were performed with MyiQ™ 2 Two-Color Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) as the methods described by Fang et al (Fang et al., 2013). The primes for RT-qPCR were designed by Primer Premier 5.0 software and were listed in Table 3. *β-actin* gene was selected as the reference for the stable expression in zebrafish (Peng & Ge, 2000). The mRNA levels of target genes relative to *β-actin* gene were calculated by the optimized comparative Ct (2^-

$^{\Delta\Delta Ct}$) value method (Livak & Schmittgen, 2002). Data with six biological replicates and three technical replicates were presented as mean ± S.E.M.

DNA methylation analysis

The gene sequences were submitted to the online software MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) to get the distribution of CpG islands (CGIs) and the candidate CpG loci, the parameters were as follows: Island size > 100 bp, GC Percent > 50.0%, Observed/Expected > 0.6. Given that no CpG islands were found for *gck*, *pk* and *fbp1b*, and in terms of gene expression, only *pck1* expression levels were significantly changed in both two experimental groups across two generations after the challenge test. So *pck1* was chosen as the candidate gene for DNA methylation analysis. The BSP primers were designed by the online software MethPrimer and Primer Premier 5.0 (Table 4).

The genomic DNA were isolated with TIANamp Genomic DNA Kit (Tiangen, Beijing, China) following the standard procedures. The genomic DNA was treated with the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The polymerase chain reaction (PCR) was performed by Taq plus DNA Polymerase (Vazyme Biotech, Nanjing, China) in Biometra Thermo cyclers (Biometra, Göttingen, Germany). The PCR products were purified by the Gel Purification Kit (Sangon, Shanghai, China) and then the products were transformed to the pEASY-T1 Cloning kit (Transgen, Beijing, China). Five positive clones were randomly obtained from each sample and sequenced in Sangon Biotech (Shanghai) Co., Ltd (ABI3730, Applied Biosystems) and there were 30 positive clones for each group. The sequencing results were processed by online QUMA (Quantification tool for Methylation Analysis) software (http://quma.cdb.riken.jp/).

Statistical analysis

Date were expressed as means ± S.E.M. Statistical analysis was performed by SPSS 25 software. The normality of data was assessed with the Shapiro-Wilk test. Then all data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple range tests. If $P < 0.05$, differences were considered significant.

Results

Growth performance and plasma glucose levels

The growth performance and plasma glucose levels of F0 and F1 were summarized in Table 5. The early HS diet stimulus significantly increased the body weight (calculated with the averaged weight) of the larvae at the end of FF stage (Table 5). However, a reversed result was found at the end of YE stage, a significantly lower body weight was observed in experimental group (Table 5). And the nutrition stimulus did not affect the body length (individual length) in both two experimental groups (Table 5). During the adult (15 week), compared with control group, there is no significant differences in body weight, body length and the plasma glucose levels (BC glucose levels) of both FF and YE groups (Table 6). At the end

of the challenge (16 week), the postprandial blood glucose levels (AC glucose levels) of two experimental groups was both significantly decreased (Table 6).

The next generation of the three groups were fed with control diet until adult (15 week). The body weight, length and plasma glucose levels (BC glucose levels) were recorded in Table 7 and there was no differences between three groups. After the challenge test (16th week), the postprandial blood glucose levels (AC glucose levels) of FF (F1) and YE (F1) were lower than that in control group (F1), although there was no significant difference between YE (F1) and control group (F1) (Table 7).

Glycolytic and gluconeogenic gene expression

The expression levels of *gck*, *fbp1a* and *pck1* were up-regulated at the end of FF period (Fig.1(A)). At the end of YE period, the mRNA levels of *pfkla*, *pfklb* and *pck1* were significantly increased and the *pk* expression levels were significantly inhibited (Fig.1(B)). At the end of the challenge in F0, the expression of seven genes which were responsible for glycolysis and gluconeogenesis were detected in the liver of zebrafish. As the first gene to participate in glycolysis progress, *gck* expression levels were significantly increased in both FF and YE group (Fig.2). In the FF group, the mRNA levels of *pfkla* were significantly higher than the levels in control group (Fig.2). For the three key genes in gluconeogenesis, *fbp1a*, *fbp1b* and *pck1* expression levels in two experimental groups were both significantly repressed compared with the control group (Fig.2). At the end of HC diet challenge test in F1, the expression levels of *pk* in FF (F1) were higher than control group (F1) (Fig.3). And compared with control group (F1), the expression levels of *fbp1a, fbp1b* were significantly inhibited in YE (F1) group, the mRNA levels of *pck1* were both significantly lower in FF (F1) and YE (F1) group (Fig.3).

The intergenerational inheritance of *pck1* DNA methylation

The *pck1* expression levels were detected in the liver of F0 adult zebrafish, the 24 hpf embryos of F1 and the liver of F1 adult zebrafish. Compared with the control group, the significantly lower mRNA levels of *pck1* in two experimental groups were found in all three phases (Fig.4). It showed a stable tendency between the two generations. To investigate the possibility that the lower *pck1* expression levels were the result of the changes of the modification of DNA methylation, we analyzed the percent of 5-methylcytosine (5-mC) / total cytosine in the CpG island of *pck1* gene. The sequence before initiation codon for 2500bp were submitted to the online software Methprimer and a CpG island, which located at -1849 ~ -1656 bp (Initiation codon ATG is regarded as the '+1' site), was found and the length of the island was194 bp, which contained 17 CpG sites. The analysis of 17 CpG sites found a significantly increased methylation level in the liver of F0, embryos of 24 hpf and liver of F1 in both two experimental groups compared with the control (Fig.5).

Discussion

zebrafish cannot utilize exogenous food and exclusively depend on the yolk reserves when they are hatching. After the opening of the esophagus, they could ingest the artificial diet (Carvalho et al., 2006). In

the present study, the HS diet (53.66% carbohydrate and 25.84% protein) stimulus significantly increased the body weight of the larvae during the first-feeding period compared with the control group. The results of previous research in zebrafish was in line with our findings. The three days high-carbohydrate diet treatment (49.46% carbohydrate and 31.87% protein) at first feeding stage induced a significantly increased body weight of zebrafish compared with control diet (20.53% carbohydrate and 53.11% protein) feeding (Fang et al., 2013). However, the different responses were reported in Siberian sturgeon (*Acipenser baerii*) and rainbow trout (*Oncorhynchus mykiss*). Compared with the control group (fed with the diet containing 3.6% carbohydrate and 67.7% protein), the high glucose stimulus diet (59.8% carbohydrate and 21.5% protein) on the beginning of exogenous nutrition stage in Siberian sturgeon significantly repressed the body weight of the larvae (Gong et al., 2015) and the similar results were also found in rainbow trout (*Oncorhynchus mykiss*) (Geurden et al., 2007). The different response in species might be the result of the differences in the ability to digest and metabolize dietary carbohydrates. When the yolk sac was exhausted, the larvae must live on the exogenous nutrition. However, an reversed tendency in body weight was observed when the fish were fed with same HS diet.

After the high-carbohydrate stimulus, all zebrafish were fed with control diet until 15 week, then the differences in body length and weight were no longer existed between the experimental groups and control group, which indicated that briefly exposed to high-carbohydrate diet at early ontogenetic phases had no negative effects on the health of adult zebrafish. The studies in rainbow trout (Geurden et al., 2007), gilthead seabream (Rocha et al., 2016b) and zebrafish (Fang et al., 2013) also reported the similar results. However, Kumkhong et al. (2020) found that although early high-carbohydrate diet-fed tilapia (*Oreochromis niloticus*) had poorer growth (end of stimulus), the fish showed compensatory growth and it finally led to the improvement of growth performance in adult fish, which might be the results of the improvement of the ability to use glucose and to induce lipogenesis and the suppression to amino acid catabolism. In Siberian sturgeon, high-glucose diet stimulus during first feeding period disturbed the metabolism and although the fish could apparently adapt to later nutritional conditions by reversing the disturbances in the gluconeogenesis pathway in 20 weeks feeding, the growth performances were still poor (significantly lower body weight) (Gong et al., 2015). In conclusion, there were no consistent responses about the high-glucose/high-carbohydrate stimulus during early ontogenetic phase on adult growth performances in different fish. Besides, we found that there was no differences of the body weight and body length between both two experimental groups and control group in F1 generation, which suggested that the early high-carbohydrate diet stimulus would not affect the growth performances of the next generation in zebrafish.

Although fish have limited ability to use carbohydrates in diet, they have most key enzymes involved in carbohydrate metabolic pathways (Polakof et al., 2012). In fish, glucose is catabolized through the glycolytic pathway or other ways for ATP production. On the contrary, glucose requirements for metabolic purposes could be satisfied by de novo glucose synthesis through gluconeogenesis or other ways (Enes et al., 2009; Stone, 2003; Wilson, 2003). Thus, to maintain glucose homeostasis, a balance between glucose consumption and production which depend on the regulation of activity and expression of key enzymes involved in the glycolysis and gluconeogenesis pathways was critical (Enes et al., 2009). As the

key gene in glycolysis, *gck* (FF), *pfkla* (YE) and *pfklb* (YE) gene expression were up-regulated immediately after the larvae experienced the HS diet. Previous researches in fish also found that high-carbohydrate stimulus at early development stages could raise the expression of genes in glycolysis rapidly, such as *gck* in zebrafish (Fang et al., 2013), gilthead seabream (Rocha et al., 2016b), rainbow trout (Geurden et al., 2007) and European sea bass (*Dicentrachus labrax*) (Zambonino-Infante et al., 2019), *pk* in zebrafish (Fang et al., 2013), gilthead seabream (Rocha et al., 2016b) and *pfk-l* in gilthead seabream (Rocha et al., 2016b). Those results indicated that fish larvae could adapt to the utilization of exogenous glucose in early development stage by the improvement of glucose oxidation. In fact, some scholars believe that there is a closer relationship between carbohydrates and feeding habit than proteins at the early larval stages (Xiang. Fan et al., 2010; Hidalgo et al., 1999; Kapoor et al., 1976). PK catalyzes the last step in the glycolysis process, which is one of the rate-limiting steps in this process. However, the expression levels of *pk* were decreased in YE group. Previous study in zebrafish larvae also found the reduced mRNA levels when fed with high-carbohydrate diet after the yolk sac was exhausted, however the activities of PK were enhanced, which indicated the possibility of post-transcriptional regulation for *pk* gene (Fang et al., 2013). At the end of FF stage, the mRNA levels of *fbp1a* and *pck1* were up-regulated, the catabolism of the vitelline reserves might contribute to this (Fang et al., 2013). Interesting, the higher transcription levels of *pck1* were observed in YE group. Which might increase the production of endogenous glucose and diminish appetite to avoid the excessive intake of feed for the larvae. The significantly lower body weight in YE group was one of the evidences.

In the 1990s, professor Barker and Hales proposed that nutrition during early life could permanently change the phenotype along with ageing, such as impaired glucose metabolism, which was known as 'fetal programming hypothesis' (Hales et al., 1991). According to this hypothesis, underwent the special nutrition environment in the early ontogenetic phases could bring animals the adaptive advantages, and those advantages could promote them to behave better in later life, especially when they encountered the similar environment. The results of present study were in agreement with that. In the present study, after the high-carbohydrate treatment in the larvae, all fish were fed with control diet until adult and a HC diet was used to test the ability of fish to utilize carbohydrate in diet (Hou & Fuiman, 2020). At the end of the challenge, the plasma glucose levels in two experimental groups were both significantly decreased compared with the control group. For genes which were responsible for glycolysis, the transcription of *gck* were enhanced in FF and YE group, and *pfkla* mRNA levels were higher in FF group. The key genes transcription levels in gluconeogenesis including *fbp1a*, *fbp1b*, *pck1* were all significantly decreased in two treatment groups compared with control group. Those results indicated high-carbohydrate diet in zebrafish larvae might improve the utilization of carbohydrate in diet in adult thought the improvement of the glucose uptake and phosphorylation levels and the decrement of endogenous glucose production. In fish, many studies had showed that early high-carbohydrate/high-glucose diet could significantly affect the carbohydrate metabolism in later life. Gilthead seabream that experienced glucose-rich feed during the larval stage had a higher catabolism and lower glucose retention in tissues and the hepatic lipogenesis were enhanced when challenged with a high-carbohydrate diet in post-larval stage (Rocha et al., 2016a; Rocha et al., 2016b). The plasma glucose levels, mRNA levels and enzyme activity levels of

pck1 were all decreased in the adult zebrafish that had received high-carbohydrate diet at first-feeding period when challenged with a high-carbohydrate diet (Fang et al., 2013). However, the similar researches in rainbow trout, Siberian sturgeon and European sea bass showed different responses including absence of, or opposite changes in glucose homeostasis (unaltered or higher plasma glucose levels) and/or expression of some genes related to carbohydrate metabolism (Geurden et al., 2007; Gong et al., 2015; Zambonino-Infante et al., 2019). To sum up, the programming effects of high-carbohydrate diet at early development stages on carbohydrate metabolism in fish were not always consistent.

In mammal models, studies had pointed that nutrition intervention during the crucial developmental windows in parents especially in maternal line had a great influence on the carbohydrate metabolism in future generation. The rat whose mother had a protein restriction diet during pregnancy showed a decreased activities of glucokinase and an increased phosphoenolpyruvate carboxykinase activities during the weaning and adult (Desai et al., 1995). And the altered physical structure and function of the liver in the offspring might be responsible for those results (Desai et al., 1995). Prenatal protein restriction was associated with reduced pancreatic GK activity in 3-month-old rat offspring (Hales et al., 1996). And the blood glucose levels in those rat were higher when they were injected with glucose, which means a poorer glucose tolerance in those rats (Hales et al., 1996). In the present study, we also found the programming effects of early high-carbohydrate on the offspring of the experimental groups. The offspring of three groups were all fed with control diet until adult. After the HC diet challenge, compared with control group (F1), the postprandial blood glucose level in the offspring of two treatment groups were all decreased, although there was no difference between FF (F1) and control group (F1). Besides, the transcription levels of *pk* were raised in FF (F1) and the mRNA levels of *fbp1a* and *fbp1b* were all lower in YE (F1), the *pck1* expression were inhibited in both FF (F1) and YE (F1). Those results indicated that early high-carbohydrate stimulus could affect the glucose metabolism in zebrafish until the next generation. And compared with stimulus after yolk-sac exhaustion, nutritional programming in first-feeding period might be more potential to transmit the effects of early high-carbohydrate programing to next generation.

To explore the possible mechanism for the nutritional programming effects, we analyze the DNA methylation levels of *pck1* in two generations of zebrafish cause of the finds that *pck1* gene expression levels were inhibited in experimental groups in both F0 and F1 adult at the end of the challenge test. DNA methylation is a common and stable epigenetic modification in animal genomes. It could stably regulate genes expression and transmit through DNA replication as cells divide and differentiate from embryonic stem cells into specific tissues (Bird, 2002). The heritable cytosine methylation primarily occurs in the context of the symmetric CpG dinucleotide, where replication results in two daughter genomes each carrying a hemimethylated CpG that provides a substrate for the maintenance methyltransferase (Bošković & Rando, 2018). And the CpG methylation patterns are largely erased from one organismal generation to the next. However, in zebrafish, some researches had reported that the inheritance of some DNA methylation patterns could be intergenerational even transgenerational inheritance (Xiaoteng Fan et al., 2019; Kamstra et al., 2018; Olsvik et al., 2014; Santangeli et al., 2019). In zebrafish, the DNA methylation model is reset almost immediately after fertilization (Mhanni & McGowan, 2004) and subsequent de novo methylation occurs (MacKay et al., 2007; Mhanni & McGowan, 2004) after which

sperm DNA becomes hypermethylated compared with oocyte DNA in newly fertilized embryos (Mhanni & McGowan, 2004). Paternal methylation patterns are retained through early development, but maternal methylation patterns are lost by the midblastula stage and altered to resemble paternal methylation patterns (Jiang et al., 2013; Potok et al., 2013). The early developmental methylation landscapes indicated that DNA methylation might serve as a conduit for parental effects in zebrafish (Perez & Lehner, 2019). PEPCK-C, which was encoded by *pck1* in zebrafish, could convert oxaloacetate into phosphoenolpyruvate and carbon dioxide which was a rate-limiting step of gluconeogenesis in the liver. As a non-allosteric enzyme, the transcription level is the primary controller of enzyme activity as the lack of post-transcriptional regulation (Li et al., 2019). Besides, the studies in mice and fish both pointed that the over-expression of liver *pck1* expression led to hyperglycemia, hyperinsulinemia, and altered hepatic glycogen contents (Valera et al., 1994). In the present study, the lower plasma glucose levels were detected in the adult of F0 (15 week) and F1 (15 week), although there was no significant differences. Then, we found that the significantly lower transcription levels of *pck1* existed in the two experimental groups of adult F0, 24 hpf embryos and adult F1, corresponding with significantly higher global DNA methylation levels (the percent of 5-methylcytosine (5-mC)/total cytosine) in the CpG island. Those results revealed that *pck1* might be the adaptive gene, which was programmed by high-carbohydrate diet in two early stages and it persisted in the subsequent generation, which might enable future generations to maintain glucose homeostasis. Besides, we found that not all the changes of genes transcription could be transmitted to the next generation, such as the *gck*, *fbp1a* and *fbp1b*, the transcription levels of those genes were all effected in two treatment groups of F0, however the differences were no longer existed in F1 generation.

In conclusion, the present study suggested that early high-carbohydrate diet stimulus could enhance the glycolysis process and reduce endogenous glucose production by regulating the key genes expression until the adult in zebrafish and those effects could be delivered to next generation partly. The DNA methylation of *pck1* might be the possible transmission pathway to deliver the *pck1* expression model from F0 to F1. Our results could promote the research to the role of DNA methylation played in nutrition programming and provide a novel route to understand the possible mechanisms of nutrition programming, further, to improve the study and application of nutrition programming to aquaculture and fisheries, with regard to glucose metabolism.

Declarations

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Conflicts of interest

The authors declare that they have no competing interests. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript,

and in the decision to publish the results.

Ethics approval

All the fish and experiments were conducted according to the “Guidelines for Experimental Animals” published by Ministry of Science and Technology (Beijing, China). This study had been approved by the Institutional Animal Care and Use Ethics Committee of Huazhong Agricultural University.

Consent to participate Not applicable.

Consent to publication Not applicable.

Data Availability

All data are available from the corresponding author by request. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

Xu-Fang Liang and Wenjing Cai conceived and designed the experiments; Tong Liu and Wuyuan Zhuang performed the experiments; Tong Liu, Xu-Fang Liang, Wenjing Cai and Yanpeng Zhang wrote and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table1 Ingredient formulation and proximate chemical composition of the experimental diets

Diets	Control diet	High-carbohydrate (HC) diet	High-carbohydrate stimulus (HS) diet
Ingredient formulation (%)			
Casein	25	25	20
Fishmeal	32	32	12
Fishoil	6	6	6
Vitamin premix[1]	1	1	1
Mineral premix[2]	1	1	1
Pre-gelatinized Starch	18	35	60
Microcrystalline cellulose	17	0	0
Chemical composition			
Dry matter (DM) (%)	93.60	93.77	95.72
Crude protein (% DM)	53.35	43.40	25.84
Crude fat (% DM)	8.87	8.90	7.19
Ash (%DM)	15.09	12.34	13.31
Carbohydrate[3]	22.69	35.36	53.66

[1]Vitamin premix (mg/kg diet): vitamin D3, 0.05; vitamin A1, 6.9; vitamin K3, 25; vitamin E, 100; vitamin B1 (thiamin), 30; vitamin B2 (riboflavin), 30; vitamin B6, 20; vitamin B12, 0.1; nicotinic acid, 200; folic acid, 15; ascorbic acid, 1000; inositol, 500; vitamin H, 3; pantothenic acid calcium, 100 (Gao Long Dietary Company).

[2]Mineral premix (mg/kg diet): $CoSO_4.H_2O$, 0.65; $CuSO_4.5H_2O$, 9; $FeSO_4.7H_2O$, 8.34; KI, 0.5; $MnSO_4.H_2O$, 22.85; Na_2SeO_3, 0.01; $ZnSO_4.7H_2O$, 14.3; NaCl, 400; CaCO3, 1860; MgSO4, 240 (Gao Long Dietary Company).

[3]Carbohydrate = 1- (crude protein + crude fat + ash).

Table2 Feeding scheme followed in the experiment

Treatments	First trial period			Final challenge
	3-5 dph	6-10 dph	11 dph–15th week	16th week
Control group	Control diet	Control diet	Control diet	HC
FF	HS	Control diet	Control diet	HC
YE	Control diet	HS	Control diet	HC
	First trial period (3 dpf-15th week)		Final challenge (16th week)	
Control group (F1)	Control diet		HC	
FF (F1)	Control diet		HC	
YE (F1)	Control diet		HC	

Abbreviations: dph, days post-hatching; FF, fish fed with HS from the first-feeding stage to the end of the yolk-sac larval stage; YE, fish fed with HS after yolk-sac exhaustion for 5 d. Control group (F1), the next generation of Control group; FF (F1), the next generation of FF group; YE (F1), the next generation of YE group.

Table 3. List of primers used in the quantitative real-time PCR

Genes	Primer sequences	Accession no.
β-actin	F: ACAGAGAGAAGATGACACAGATCATG	NM_131031
	R: AGTCCATCACAATACCAGTAGTACG	
gck	F: TGAGGATGAAGAGCGAGGC	BC122359
	R:AGAGAAGGTGAATCCCAGTG	
pfkla	F: AGGTATGAACGCAGCCATCC	XM_693543.8
	R: TGCCAATCACTGTTCCTCCC	
pfklb	F: TTTGAGCACAGGATGCCGAA	NM_001328389.1
	R: TCGATGCTAAGGGTTCGACG	
pk	F: AGAAACAGCCAAAGGACA	BC152219
	R: ACGAGGACGATAACGAGA	
fbp1a	F: CATCTGTATGGGATTGCTGG	NM_199942
	R: TTACCCCGTCTATCTGGCTC	
fbp1b	F: GAGTCCCAAGGGCAAGCTAA	NM_213132.1
	R: TACAGGAACCCTCTGGTGGA	
pck1	F: ATCACGCATCGCTAAAGAGG	NM_214751.1
	R: CCGCTGCGAAATACTTCTTC	

Abbreviations: *gck*, glucokinase; *pfkla,* phosphofructokinase, liver a; *pfklb,* phosphofructokinase, liver b; *gk,* pyruvate kinase; *fbp1a,* fructose-1,6-bisphosphatase 1a; *fbp1b,* fructose-1,6-bisphosphatase 1b; *pck1,* phosphoenolpyruvate carboxykinase 1

Table 4. List of primers used in the BSP amplified and DNA methylation analysis

Prime name	**Sequence (5′–3′)**	**Tm (°C)**
ZF-BSP-*pck1*	F: GAATGAATGAATGATTTAGTTTGATTT	48
	R: AAACATTAAACTATAATTTTAAAAAAACAC	

Table 5. Growth performance of zebrafish during the early stages

	Control group		Experimental group	
Treatments	Mean	se	Mean	se
FF				
Wet weight[1] (mg)	0.78[a]	0.04	0.94[b]	0.04
Total length (mm)	4.46	0.05	4.35	0.06
YE				
Wet weight (mg)	1.34[a]	0.02	1.19[b]	0.03
Total length (mm)	5.04	0.06	4.87	0.05

Control group, fish fed with control diet; FF, fish fed with HS from the first-feeding stage to the end of the yolk-sac larval stage; YE, fish fed with HS after yolk-sac exhaustion for 5 d.

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

[1]Due to small size, the mean weight was used.

Table 6. Growth performance and plasma glucose level of the F0 adult zebrafish

	Treatments					
	Control group		FF		YE	
	Mean	se	Mean	se	Mean	se
Body weight (mg)	370.40	14.46	343.60	33.20	375.30	28.02
Body length (mm)	32.12	0.74	29.62	0.76	29.95	0.71
BC glucose levels (mM)[1]	3.23	0.18	2.59	0.19	2.84	0.12
AC glucose levels (mM)[2]	4.26[a]	0.16	3.45[b]	0.22	3.51[b]	0.11

Control group, fish fed with control diet; FF, fish fed with HS from the first-feeding stage to the end of the yolk-sac larval stage; YE, fish fed with HS after yolk-sac exhaustion for 5 d.

[1]BC glucose level, the glucose level before HC challenge.

[2]AC glucose level, the glucose level after HC challenge

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Table 7. Growth performance and plasma glucose levels of the F1 adult zebrafish

	Treatments					
	Control group (F1)		FF (F1)		YE (F1)	
	Mean	se	Mean	se	Mean	se
Body weight (mg)	388.80	9.96	401.43	7.35	398.03	11.65
Body length (mm)	32.51	0.75	33.22	0.47	34.25	0.63
BC Glucose levels (mM)[1]	3.33	0.11	3.14	0.16	3.19	0.02
AC Glucose levels (mM)[2]	4.67[a]	0.15	3.78[b]	0.26	4.50[a]	0.21

Control group (F1), the next generation of the Control group; FF (F1), the next generation of the FF group; YE (F1), the next generation of YE group.

[1]BC glucose levels, the glucose levels before HC challenge.

[2]AC glucose levels, the glucose levels after HC challenge

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Figures

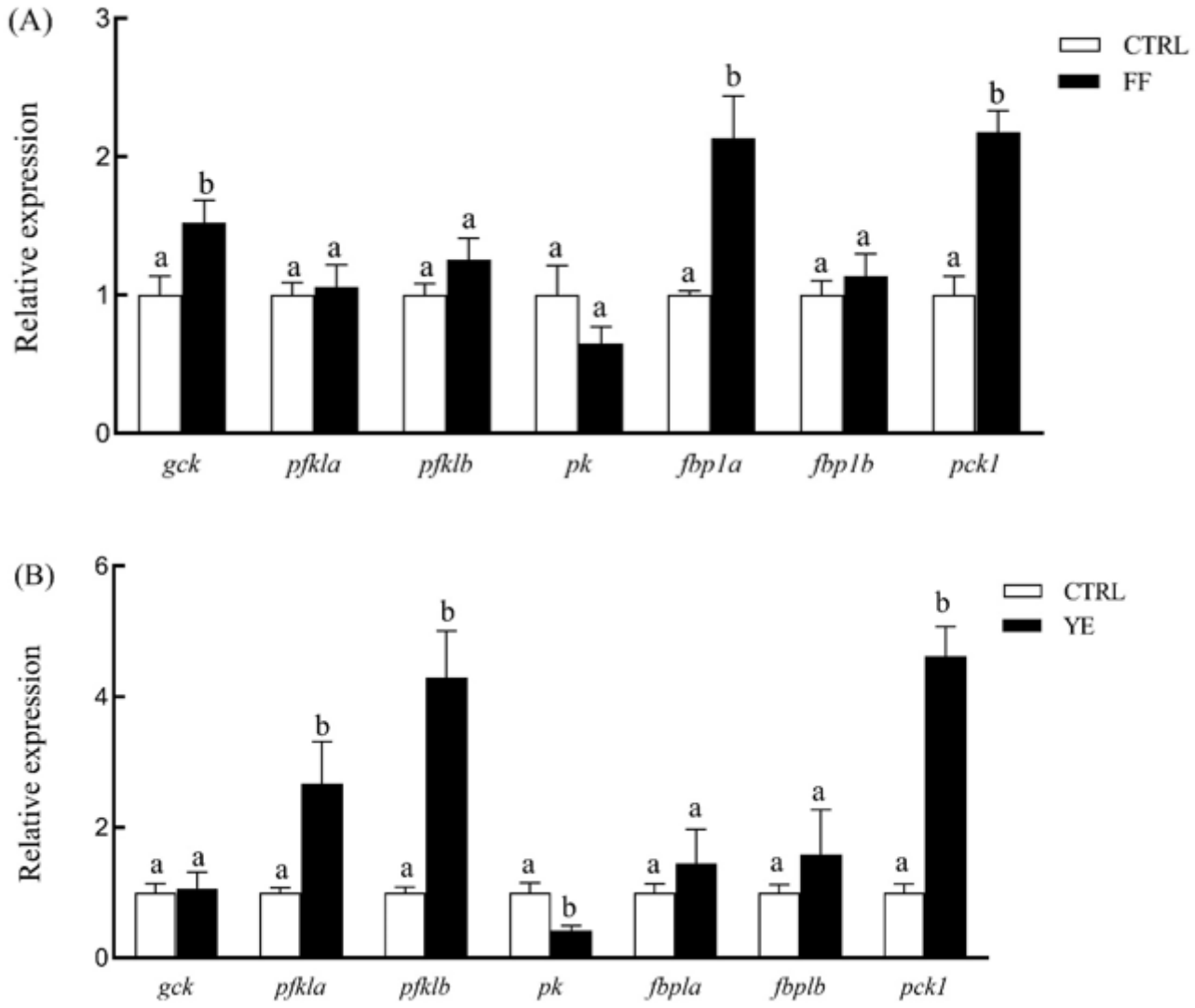

Figure 1

Expression of seven key genes in zebrafish larvae fed with HS at different stages. *gk*, glucokinase; *pfkla*, phosphofructokinase, liver a; *pfklb*, phosphofructokinase, liver b; *pk*, pyruvate kinase; *fbp1a*, fructose-1,6-bisphosphatase 1a; *fbp1b*, fructose-1,6-bisphosphatase 1b; *pck1*, phosphoenolpyruvate carboxykinase 1. (A) CTRL, fish fed with control diet ; FF, fish fed with HS from the first-feeding stage to the end of the yolk-sac larval stage; (B) CTRL, fish fed with control diet; YE, fish fed with HS after yolk-sac exhaustion for 5 d. Different letters in the bar graph indicate significant differences ($P < 0.05$, n = 6).

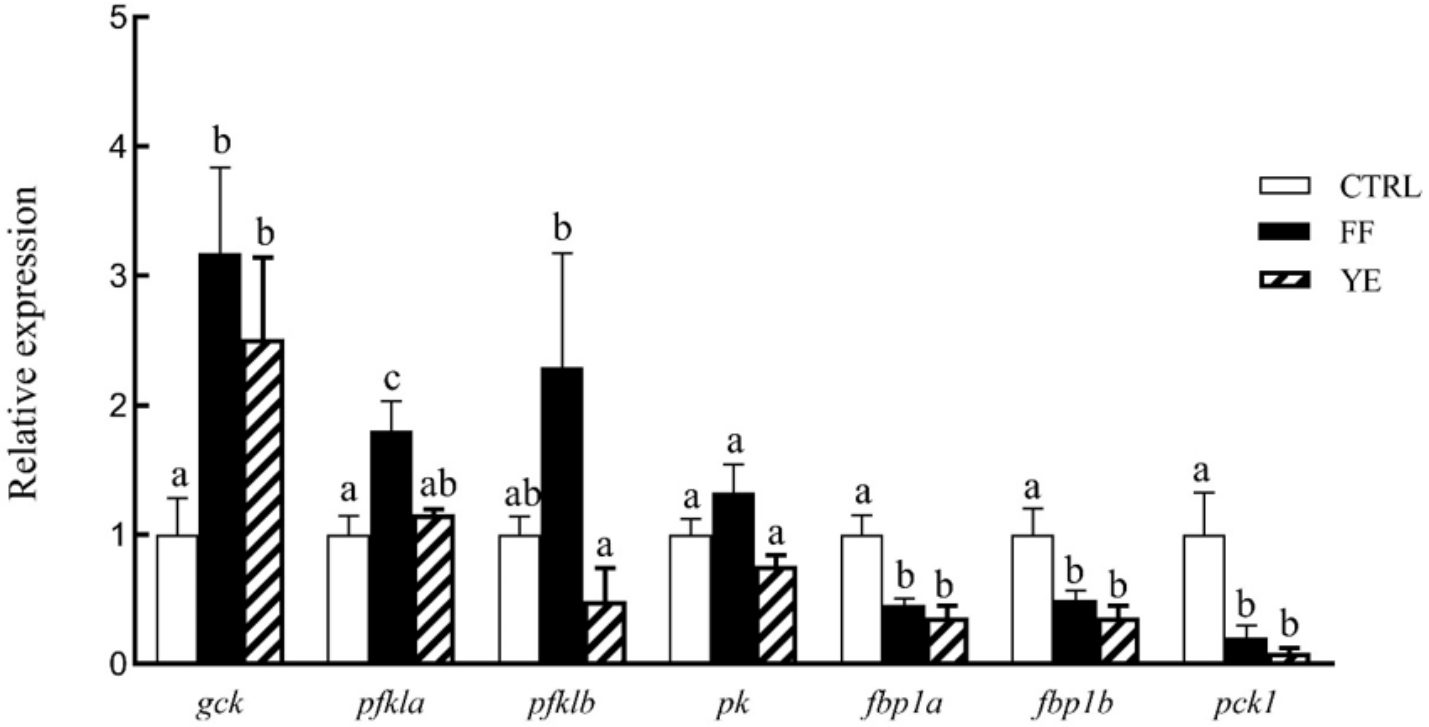

Figure 2

Long-term effect of early high-carbohydrate treatment on hepatic gene expression in adult zebrafish (one week HC challenge). *gk*, glucokinase; *pfkla*, phosphofructokinase, liver a; *pfklb*, phosphofructokinase, liver b; *pk*, pyruvate kinase; *fbp1a*, fructose-1,6-bisphosphatase 1a; *fbp1b*, fructose-1,6-bisphosphatase 1b; *pck1*, phosphoenolpyruvate carboxykinase 1. CTRL, fish fed with control diet; FF, fish fed with HS the from the first-feeding stage to the end of the yolk-sac larval stage; YE, fish fed with HS after yolk-sac exhaustion for 5 d. Different letters in the bar graph indicate significant differences ($P < 0.05$, n = 6).

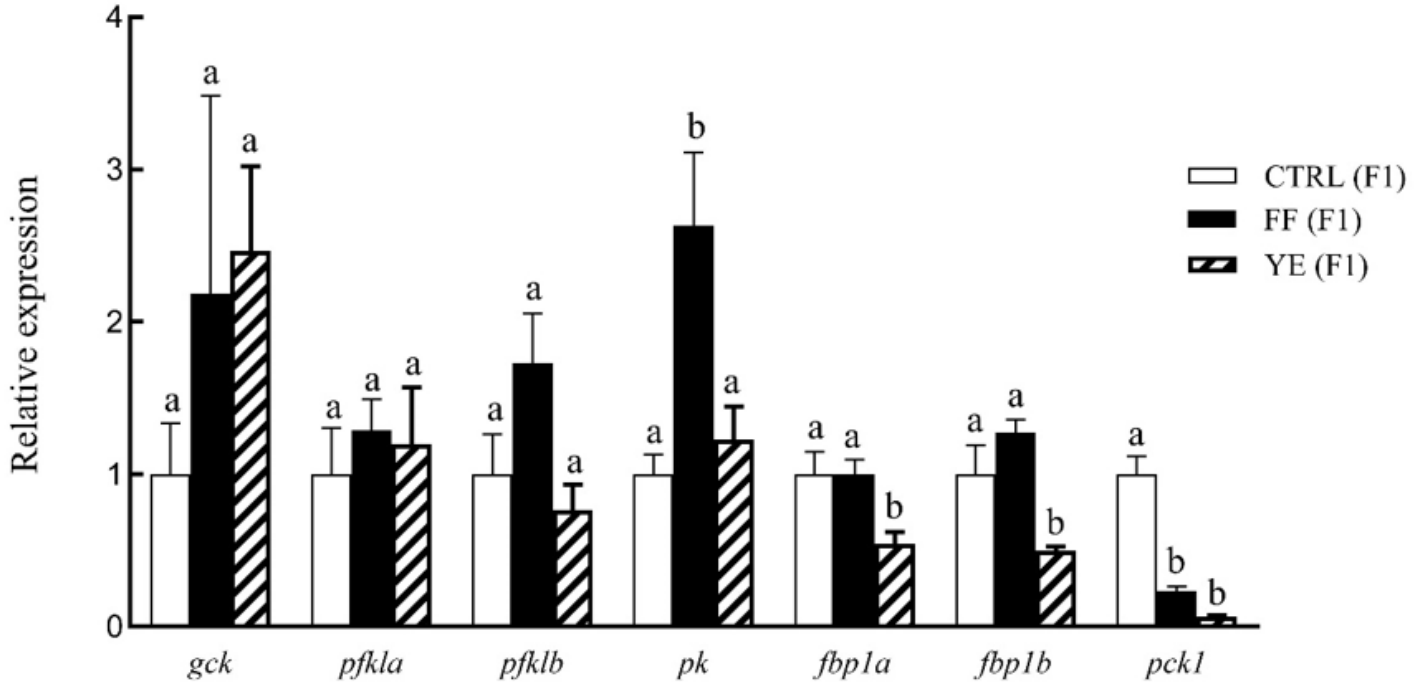

Figure 3

Effects of early high-carbohydrate treatment on hepatic gene expression in adult zebrafish of F1 generation (one week HC challenge). *gck*, glucokinase; *pfkla*, phosphofructokinase, liver a; *pfklb*, phosphofructokinase, liver b; *pk*, pyruvate kinase; *fbp1a*, fructose-1,6-bisphosphatase 1a; *fbp1b*, fructose-1,6-bisphosphatase 1b; *pck1*, phosphoenolpyruvate carboxykinase 1. CTRL (F1), the next generation of the control group ; FF (F1), the next generation of the FF group; YE (F1), the next generation of the YE group. Different letters in the bar graph indicate significant differences ($P < 0.05$, n = 6).

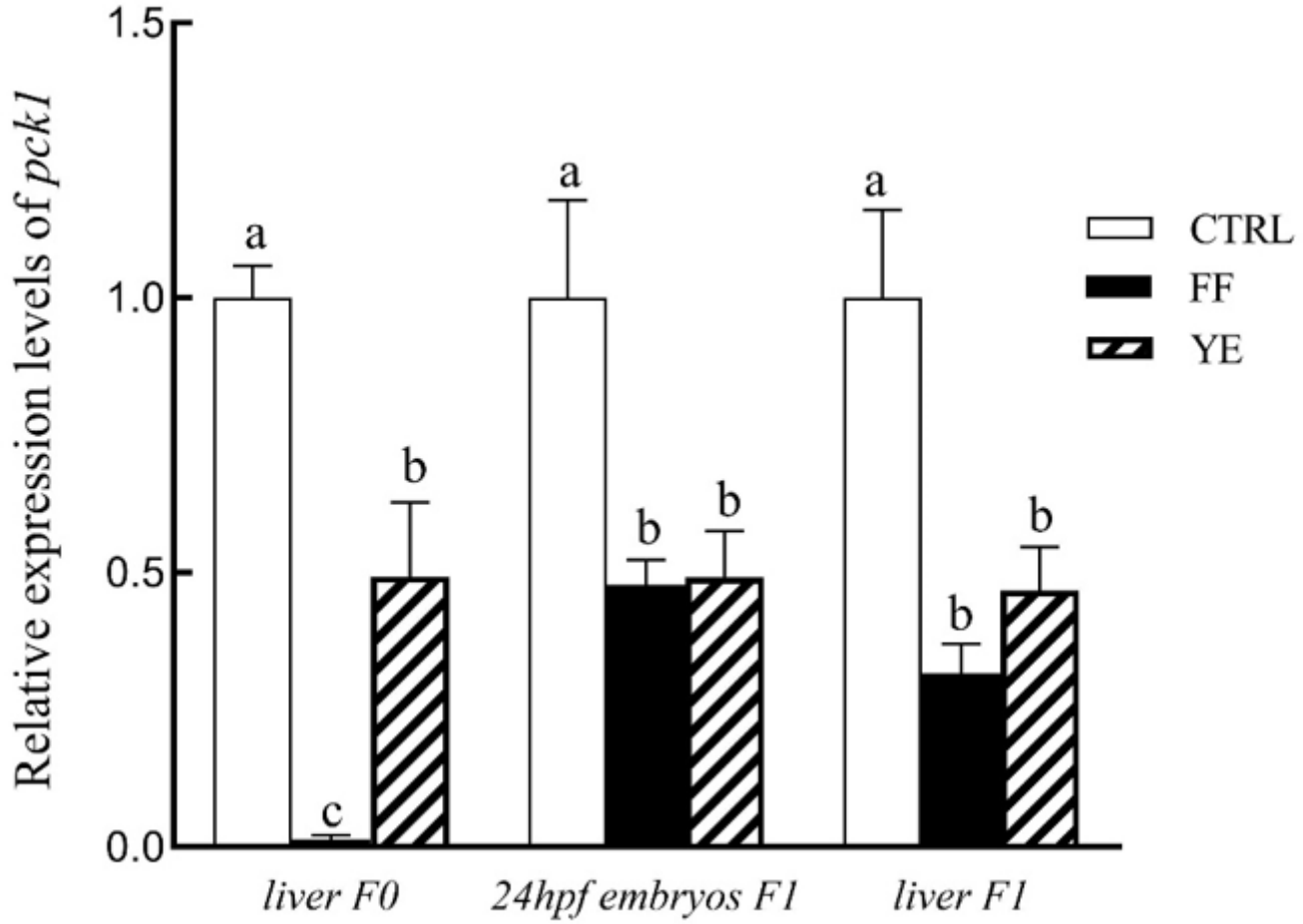

Figure 4

The *pck1* expression levels in FF, YE, control group in F0 and F1. *pck1*, phosphoenolpyruvate carboxykinase 1. Liver F0, the liver of F0 adult zebrafish; 24 hpf embryos F1, the F1 embryos of 24 hours post fertilization; Liver F1, the liver of F1 adult zebrafish. CTRL, fish fed the control diet; FF, fish fed with HS from the first-feeding stage to the end of the yolk-sac larval stage; YE, fish fed with HS after yolk-sac exhaustion for 5 d. Different letters in the bar graph indicate significant differences ($P < 0.05$, n = 6).

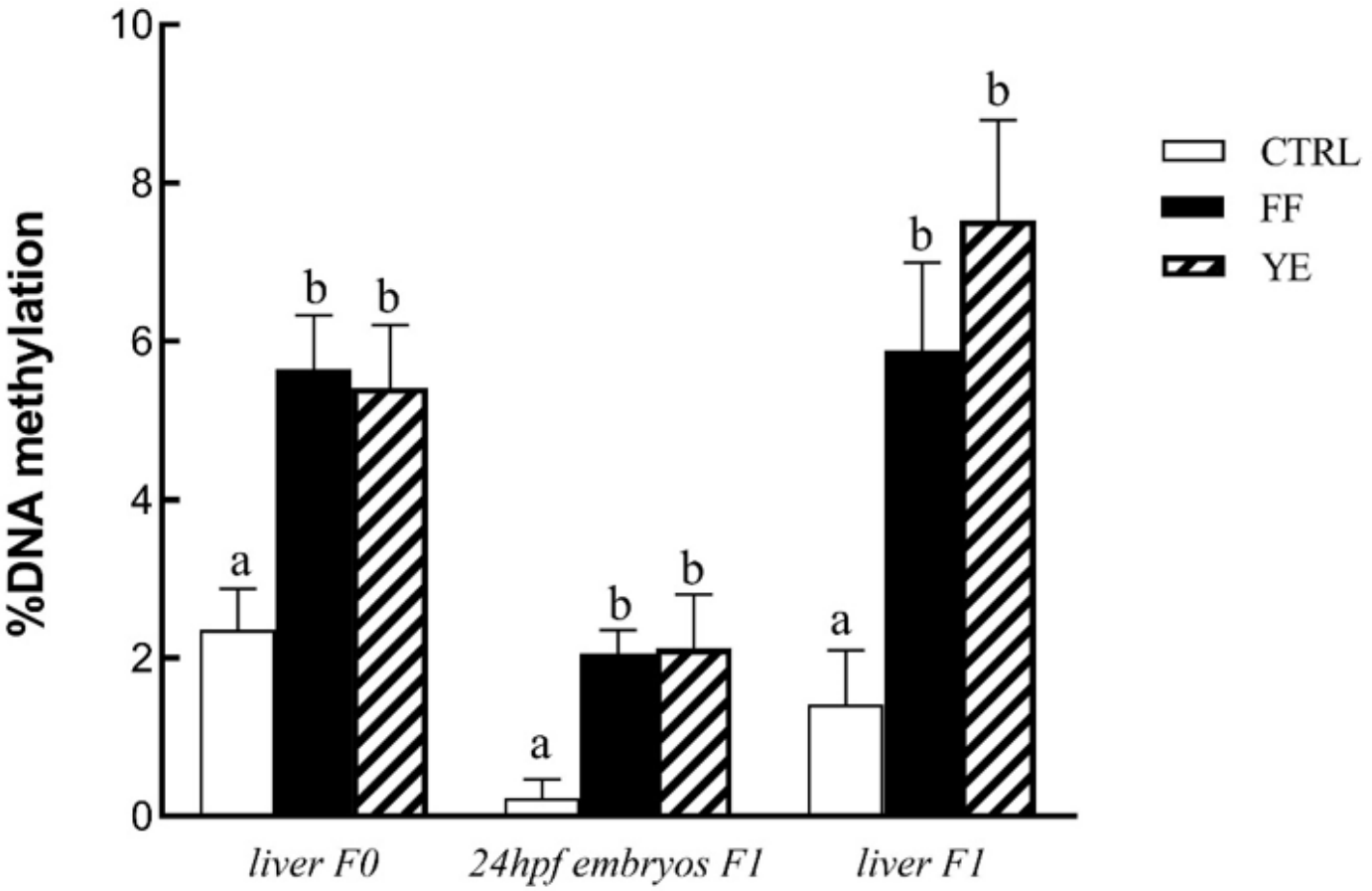

Figure 5

Gene-specific DNA methylation in the promoter region of *pck1*. 17 CpG sites in the promoter region of the *pck1* gene were analyzed in the liver of F0 adult, 24 hpf embryos F1 and the liver of F1 adult. Data are presented as the mean of the percentage in methylation for the 17 CpG sites analyzed in each groups. Different letters in the bar graph indicate significant differences ($P < 0.05$, n = 6).