EFFECT OF NUTRITIONAL PROGRAMMING OF DIETARY ENERGY SOURCES ON LONG-TERM METABOLIC PATHWAY IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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ผลของ nutritional programming ของสารอาหารพลังงาน ต่อกระบวนการเมทาบอลิซึมของปลานิลในระยะยาว

นายสุขสันต์ ขำคง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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สุขสันต์ ขำคง : ผลของ nutritional programming ของสารอาหารพลังงาน ต่อกระบวนการเมทาบอลิซึมของปลานิลในระยะยาว (EFFECT OF NUTRITIONAL PROGRAMMING OF DIETARY ENERGY SOURCES ON LONG-TERM METABOLIC PATHWAY IN NILE TILAPIA) (*Oreochromis niloticus*) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. สุรินทร บุญอนันธนสาร, 207 หน้า.

การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของ nutritional programming ของคาร์โบไฮเดรต ซึ่งเป็นสารอาหารที่ให้พลังงานต่อกระบวนการเมทาบอลิซึมของปลานิลในระยะยาว โดยในการทดลองที่ 1 เป็นการศึกษาการปรับกระบวนการเมทาบอลิซึมของกลูโคสในปลานิลที่ได้รับอาหารที่มีคาร์โบ-ไฮเดรตแตกต่างกันได้แก่ อาหารที่มีเด็กซ์ตรินที่ระดับ 0% 30% และ 50% เป็นระยะเวลา 90 วัน ผลการศึกษาพบว่าอาหารที่มีระดับเด็กซ์ตริน 30% ส่งผลต่อการเพิ่มสมรรถนะการเจริญเติบโตที่ดีที่สุด ($P<0.05$) คาร์โบไฮเดรตในอาหารที่สูงขึ้นมีผลต่อการเพิ่มระดับไกลโคเจนในตับ และกล้ามเนื้อ เพิ่มขนาดของตับ สารเมทาบอไลท์ในพลาสมาที่สูงขึ้น และเพิ่มระดับ mRNA ของยีนที่ควบคุมการขนส่งกลูโคสในกล้ามเนื้อ (glucose transporter) รวมถึงการลดระดับการแสดงออกของยีนในกลุ่มการสลายกรดอะมิโน (Catabolism of amino acids) ($P<0.05$)

งานวิจัยต่อมาเป็นการศึกษาหลักการ nutritional programming ด้วยสิ่งกระตุ้นทางสภาวะโภชนาการสองแบบ โดยในการทดลองที่ 2 และ 3 เป็นการกระตุ้นทางสภาวะโภชนาการในลูกปลาวัยอ่อนด้วยการฉีดน้ำตาลกลูโคสที่ระดับความเข้มข้น 2 โมลาร์เข้าสู่ถุงไข่แดง ผลการศึกษาพบว่าที่ระยะเวลาหลังการฉีด 1 สัปดาห์ ปลาที่ได้รับการฉีดน้ำตาลกลูโคสที่ระยะวัยอ่อน มีระดับการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการไกลโคไลซีส (Glycolysis) การขนส่งน้ำตาลกลูโคส (glucose transport) เพิ่มขึ้น ($P<0.05$) และระดับการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการสร้างน้ำตาลกลูโคส (gluconeogenesis) และการสลายกรดอะมิโน (Catabolism of amino acids) ลดลงอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) โดยการเปลี่ยนแปลงการแสดงออกของยีนเหล่านี้พบต่อมาที่ปลาช่วงวัยรุ่น (juvenile fish) แต่ไม่พบอีกในปลาตัวเต็มวัย (adult fish) ต่อมานำมาปลาที่มีประวัติการฉีดน้ำตาลกลูโคสมาทดสอบด้วยอาหารที่มีระดับของคาร์โบไฮเดรตที่แตกต่างกัน 2 ระดับ ผลการศึกษาพบว่าประวัติการฉีดน้ำตาลกลูโคสส่งผลต่อการเพิ่มขึ้นของเอนไซม์ในกระบวนการไกลโคไลซีส และการขนส่งกลูโคส รวมถึงการลดลงของเอนไซม์ในกระบวนการกลูโคนีโอจีนีซีสในปลาวัยรุ่นอย่างมีนัยสำคัญทางสถิต ($P<0.05$) ในปลาระยะตัวเต็มวัยพบว่า ปลากลุ่มที่มีประวัติการฉีดน้ำตาลกลูโคส มีการเพิ่มการแสดงออกของยีนที่สร้างเอนไซม์ในกระบวนการไลโปจีนีซีส (lipogenesis) ไกลโคไลซีส และกลูโคนีโอจีนีซีส ประวัติการฉีดน้ำตาลกลูโคสที่ส่งผลต่อสมรรถนะการเจริญเติบโตที่เพิ่มขึ้นนั้น พบเฉพาะในปลาช่วงวัยรุ่นแต่ไม่พบในปลาระยะโตเต็มวัย โดยอิทธิพลของ nutritional programming

นี้เกี่ยวข้องกับภาวะดีเอ็นเอเมทิลเลชั่น (DNA methylation)ในตับและกล้ามเนื้อที่ศึกษาในปลาระยะวัยรุ่น การทดลองที่ 4 เป็นการกระตุ้นทางสภาวะโภชนาการด้วยการให้ลูกปลาวัยอ่อนกินอาหารที่มีคาร์โบไฮเดรตสูง ตั้งแต่ระยะเริ่มกินอาหารเป็นระยะเวลา 4 สัปดาห์ ผลการศึกษาพบว่า ปลาที่มีประวัติได้รับอาหารคาร์โบไฮเดรตสูง มีระดับการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการกลูโคนีโอจีนีซีส และการสลายกรดอะมิโนลดลงอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) และมีระดับการแสดงออกของยีนเฮกโซไคเนส-2 (hk2) ในกระบวนการไกลโคไลซีสเพิ่มขึ้น ต่อมานำปลาที่มีประวัติการกินอาหารที่มีคาร์โบไฮเดรตสูง มาทำการทดสอบด้วยอาหารที่มีระดับของคาร์โบไฮเดรตที่แตกต่างกัน 2 ระดับ ผลการศึกษาพบว่า ประวัติการกระตุ้นด้วยทางสภาวะโภชนาการด้วยอาหารที่มีคาร์โบไฮเดรตสูงส่งผลต่อ 1) การเพิ่มการใช้ประโยชน์ของน้ำตาลกลูโคสซึ่งมีอิทธิพลต่อการใช้ประโยชน์โปรตีนเพื่อการเจริญเติบโต (protein-sparing effects) 2) การเพิ่มการสร้างไขมัน และ 3) ลดการสลายกรดอะมิโนเพื่อเป็นแหล่งพลังงาน โดยสรุปปลาที่ได้รับการการฉีดกลูโคสที่ระยะวัยอ่อนและการกระตุ้นสภาวะทางโภชนาการด้วยอาหารคาร์โบไฮเดรตสูง ทำให้เกิดสภาวะ nutritional programming เมทาบอลิซึมของกลูโคสในทางบวก โดยสิ่งกระตุ้นทางสภาวะโภชนาการนี้มีความสัมพันธ์กับการเพิ่มความสามารถการใช้กลูโคส การเหนี่ยวนำการสร้างไขมัน และการลดการสลายกรดอะมิโน จึงส่งผลต่อการเพิ่มสมรรถนะการเจริญเติบโตในปลานิล

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา _____

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SUKSAN KUMKHONG : EFFECT OF NUTRITIONAL PROGRAMMING OF DIETARY ENERGY SOURCES ON LONG-TERM METABOLIC PATHWAY IN NILE TILAPIA (*Oreochromis niloticus*). THESIS ADVISOR : ASSOC. PROF. SURINTORN BOONANANTANASARN, Ph.D., 207 PP.

NILE TILAPIA/NUTRITIONAL PROGRAMMING/GLUCOSE INJECTION/ EARLY FEEDING/GENE EXPRESSION/GLUCOSE METABOLISM

This study aims to investigate the effect of nutritional programming of dietary carbohydrate, an energy source, on long-term glucose metabolic pathway in Nile tilapia (*Oreochromis niloticus*). First, in experiment I, the molecular adaptation of the glucose metabolism was investigated by feeding Nile tilapia with 3 different levels of carbohydrates including 0% 30% and 50% dextrin for 90 days. The best growth performance was found for fish fed 30% dextrin containing diet ($P<0.05$). Increased hepatic and muscle glycogen, hepatic somatic index, plasma metabolites, mRNA levels for glucose transporter in muscle and down-regulation of amino acid catabolism ($P<0.05$) mRNA levels was linked to the increased dietary carbohydrates.

The concept of nutritional programming was explored in two different nutritional intervention stimuli. In experiments II-III, a nutritional stimulus was accomplished by microinjecting 2M glucose into yolk reserves. At 1 week post-injection, glucose stimuli were associated with the up-regulation of genes involved in glycolysis, glucose transport and down-regulation of genes related to gluconeogenesis and amino acid catabolism ($P<0.05$). These effects were able to later observed in juvenile fish but not in adult fish. The effects of the glucose injection stimulus history were also examined in fish fed with two different dietary carbohydrate levels. The

early glucose injection led to up-regulation of glycolytic enzymes and glucose transporter as well as lower gluconeogenic gene expression ($P<0.05$) in juvenile fish. In adult fish, the early glucose stimulus enhanced the expression of lipogenesis, glycolysis and gluconeogenesis. The early glucose stimulus was associated with better growth performance of juvenile fish but not in adult fish. These nutritional programming effects were associated with DNA hypomethylation in the liver and muscles in juvenile fish. For experiment IV, early nutritional intervention stimulus was achieved by feeding fry with high carbohydrate diet since first feeding for 4 weeks. Our findings indicated that high carbohydrate stimulus diet-fed fry had lower levels of mRNA for genes coding enzymes in gluconeogenesis and amino acid catabolism and higher levels of *hk2* ($P<0.05$). Finally, experimental fish were challenged with different dietary carbohydrate levels. The early high-carbohydrate stimulus had significant effects on adult tilapia by 1) promoting efficient use of glucose, which had protein-sparing effects for better growth, 2) inducting lipogenesis, and 3) decreasing amino acid catabolism. In conclusion, early glucose injection and high carbohydrate diet feeding were effective for positive nutritional programing of metabolism. Early hyperglucidic stimuli are linked to a better ability to use glucose, to induce lipogenesis, and to suppress amino acid catabolism, leading to the improve of growth performance in Nile tilapia.

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Advisor's Signature
Co-advisor's Signature

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LIST OF ABBREVIATIONS

wpi	=	Week post injection
°C	=	Degree Celsius
µL	=	Microliter
AA	=	Amino acid
ADG	=	Average daily weight gain
ADG	=	Average daily gain
Alat	=	Alanine aminotransferase
ANOVA	=	Analysis of variance
Asat	=	Aspartate transaminase
BUN	=	Blood urea nitrogen
Cm	=	Centimeter
Cm^3	=	Cubic centimeter
DNA	=	deoxyribonucleic acid
Dpf	=	day post-fertilization
DTT	=	Dithiothreitol
ef1α	=	Elongation factor 1-alpha 1
fasn	=	Fatty acid synthase
FCR	=	Feed conversion ratio
FCR	=	Feed conversion ratio
FI	=	Feed intake
G	=	Gram

LIST OF ABBREVIATIONS (Continued)

g kg^{-1}	=	Gram per kilogram
g kg^{-1}	=	Gram per kilogram
g6pca1	=	Glucose-6-phosphatase-1
g6pca2	=	Glucose-6-phosphatase-2
g6pd	=	Glucose-6-phosphate dehydrogenase
gck	=	Glucokinase
gdh	=	Glutamate dehydrogenase
glut4	=	Glucose transporter type 4
glut2	=	Glucose transporter type 2
HCD	=	High carbohydrate diet.
HSI	=	Hepatosomatic index
hk1	=	Hexokinases
hk2	=	Hexokinase 2
HP	=	High protein diet;
HPR	=	High protein restinction.
kJ g^{-1}	=	Kilojune per gram
kJ g^{-1}	=	Kilojune per gram
KOH	=	Potassium hydroxide
LC-PUFA	=	Long-chain polyunsaturated fatty acids
LP	=	Low protein diet
M	=	Meter
M	=	Molar

LIST OF ABBREVIATIONS (Continued)

m^2	=	Square meters
mg	=	Miligram
mg mL^{-1}	=	Miligram per microlite
mg g^{-1}	=	Milligrams per gram
mg l^{-1}	=	Milligram per liter
miRNAs	=	MicroRNAs
mL	=	Milliliter
mM	=	Millimolar
NaCl	=	Sodium chloride
NaCl	=	Sodium chloride
NADP	=	Nicotinamide adenine dinucleotide phosphate
NFE	=	Nitrogen-Free Extract
nL	=	Nanoliter
NPY	=	Neuropeptide y
pck1	=	Phosphoenolpyruvate carboxykinase 1
pck2	=	Phosphoenolpyruvate carboxykinase 2
pfklr	=	Phosphofructo kinase
pfkma	=	Phosphofructokinase
pfkmb	=	Phosphofructokinase
pklr	=	Pyruvate kinase liver
pkma	=	Pyruvate kinase muscle
PPT	=	Part per thousand

LIST OF ABBREVIATIONS (Continued)

SD	=	Standard deviation
SGR	=	Specific growth rate
SGR	=	Specific growth rate
WG	=	Weight gain
WPI	=	Week post injection

CHAPTER I

INTRODUCTION

1.1 Introduction

Global tilapia production increases every year for sustainable fish food (FAO, 2018). A continuous increase in tilapia production has been predicted to be a sustainable fish food worldwide. There are various researches and development to improve tilapia production in terms of production system, environmental impact and feed management. Generally, for intensive farming system, feed accounts for over 50 percent of production costs; therefore, tilapia nutrition has been extensively studied in order to develop least cost feed with high quality. For example, alternative ingredients for protein sources have been searched and tested for the potential use in practical diet, since among nutrients, protein has been the most expensive ingredient for practical diet (Khan et al., 2013, Montoya-Camacho et al., 2019, Soltan et al., 2008). In addition, optimal protein levels were revealed at various growth phase (Choudhary et al., 2017, Daudpota et al., 2014). For energy source, optimum carbohydrate and lipid levels in diet were also demonstrated (Xie et al., 2017, Ali et al., 2001, Kabir et al., 2020).

Among tilapia, Nile tilapia has dominated fish farming. Nile tilapia (*Oreochromis niloticus*) is an omnivorous fish species that can efficiently use high levels of dietary carbohydrates as an energy source (Shiau and Peng, 1993; Wang et al., 2005; Kamalam et al., 2017). Practical diets for Nile tilapia contain carbohydrate

in range of 20-50%, demonstrating that tilapia is a good carbohydrate user. Therefore, tilapia would be a good model for omnivorous species to study carbohydrate utilization and metabolism. Understanding carbohydrate metabolism could lead to improve carbohydrate utilization, and the efficiency use of dietary carbohydrate would contribute to a sparing effect for protein for growth. Therefore, investigation of the metabolic response of different levels of dietary carbohydrate at molecular level would provide valuable information of carbohydrate metabolism in Nile tilapia.

Nutritional programming has been an issue that is interested to examine how it modulates metabolism in fish (Geurden et al., 2007,2013,2014; Fang et al., 2014; Rocha et al., 2014, 2015;2016 a,b; Panserat et al., 2017; Song et al., 2019). Metabolic programming is defined as the long-term consequences of environmental events or nutrient stimuli during early development that exerts permanent effects on metabolism and physiology later in life (Lucas, 1998; Symonds et al., 2009). The concept of nutritional programming might offer a potential application in fish nutrition for the goal of modifying fish metabolism for efficient use of alternative feed such as plant-based ingredients, high-carbohydrate diet and low-fish-oil/fishmeal-containing diet (Geurden et al., 2013; Rocha et al., 2014, 2015; Lazzarotto et al., 2016; Clarkson et al., 2017). Several factors were demonstrated to affect programming of nutrients including types of the early environmental/nutritional stimuli, different developmental windows for applying the stimulus and the types of challenges at later in life (Geurden et al., 2007, 2014; Mennigen et al., 2013; Fang et al., 2014; Rocha et al., 2014, 2015, 2016a,b; Gong et al., 2015; Marandel et al., 2016a,b). Persistence of long-term effects of nutritional stimuli was proposed to be linked with epigenetics, which might be transmissible from one cell generation to another (Gavery and Roberts, 2017; et al.,

2017; Veron et al., 2018). Recently, nutritional programming effects of carbohydrate were demonstrated in carnivorous and experimental fish (Geurden et al., 2007; Fang et al., 2014; Rocha et al., 2014, 2015). More investigations of the nutrition programming of carbohydrate in Nile tilapia, a model of omnivorous fish would provide comparable metabolic and physiologic of carbohydrate utilization in fish.

1.2 Research objectives

1.2.1 To study the effects of different levels of dietary carbohydrates on growth performance, plasma metabolites, nutrient composition in muscle and whole body, and glucose metabolism in Nile tilapia.

1.2.2 To study the effect of nutritional programming of early glucose stimulus in the alevin stage on growth performance, plasma metabolites, nutrient composition in muscle and liver, glucose metabolism and DNA methylation in Nile tilapia during juvenile stage (20-24 week post injection; body weight 107-116 gram)

1.2.3. To study the effect of nutritional programming of early glucose stimulus in the alevin stage on growth performance, plasma metabolites, nutrient composition in muscle and liver and glucose metabolism in Nile tilapia during adult stage (32-37 week post injection; body weight 222.7-305.8 gram).

1.2.4. To study the effect of nutritional programming of early high-carbohydrate intake at first feeding on growth performance, plasma metabolites, nutrient composition in muscle and liver and glucose metabolism in Nile tilapia during adult stage (37-41 weeks after stimulus for 4 weeks; body weight 300.9-331.2 gram).

1.3 Hypotheses

1.3.1 The metabolic responses including growth performance, plasma metabolites, nutrient composition in muscle and whole body, and glucose metabolism in Nile tilapia depended on different levels of dietary carbohydrates.

1.3.2 There were nutritional programming of carbohydrate in Nile tilapia, and early glucose stimulus in the alevin stage was effective to generate the glucose history effects on growth performance, plasma metabolites, nutrient composition in muscle and liver, glucose metabolism and DNA methylation in Nile tilapia during juvenile stage.

1.3.3. The early glucose stimulus in the alevin stage was effective to generate the glucose history effects on growth performance, plasma metabolites, nutrient composition in muscle and liver, glucose metabolism and DNA methylation in Nile tilapia during adult stage.

1.3.4. Early high-carbohydrate intake at first feeding was effective to generate the hyperglucidic history effects on growth performance, plasma metabolites, nutrient composition in muscle and liver and glucose metabolism in Nile tilapia during juvenile stage

1.4 Scope of the study

1.4.1 To study the effects of different levels of dietary carbohydrates on growth performance, plasma metabolites, nutrient composition in muscle and whole body, and glucose metabolism in Nile tilapia.

1.4.2 To study the effect of nutritional programming of early glucose stimulus in the alevin stage on growth performance, plasma metabolites, nutrient

composition in muscle and liver, glucose metabolism and DNA methylation in Nile tilapia during juvenile stage.

1.4.3. To study the effect of nutritional programming of early glucose stimulus in the alevin stage on growth performance, plasma metabolites, nutrient composition in muscle and liver and glucose metabolism in Nile tilapia during juvenile stage.

1.4.4. To study the effect of nutritional programming of early high-carbohydrate intake at first feeding on growth performance, plasma metabolites, nutrient composition in muscle and liver and glucose metabolism in Nile tilapia during juvenile stage.

In order to test the metabolic response and also the nutritional programming in Nile tilapia, a model fish of omnivorous species. First, the effects of different levels of dietary carbohydrates were determined. In addition, this study investigated whether there was nutritional programming of carbohydrate in Nile tilapia by different nutritional intervention such as early glucose injection and early high-carbohydrate intake at first feeding. The metabolic responses to dietary carbohydrate and nutritional programming were evaluated on several parameters including growth performance, plasma metabolites, nutrient composition in muscle and whole body, and glucose metabolism were determined. A schematic view of the plan work, detailing the four experimental trails developed under this thesis is provided in Figure 1.1.

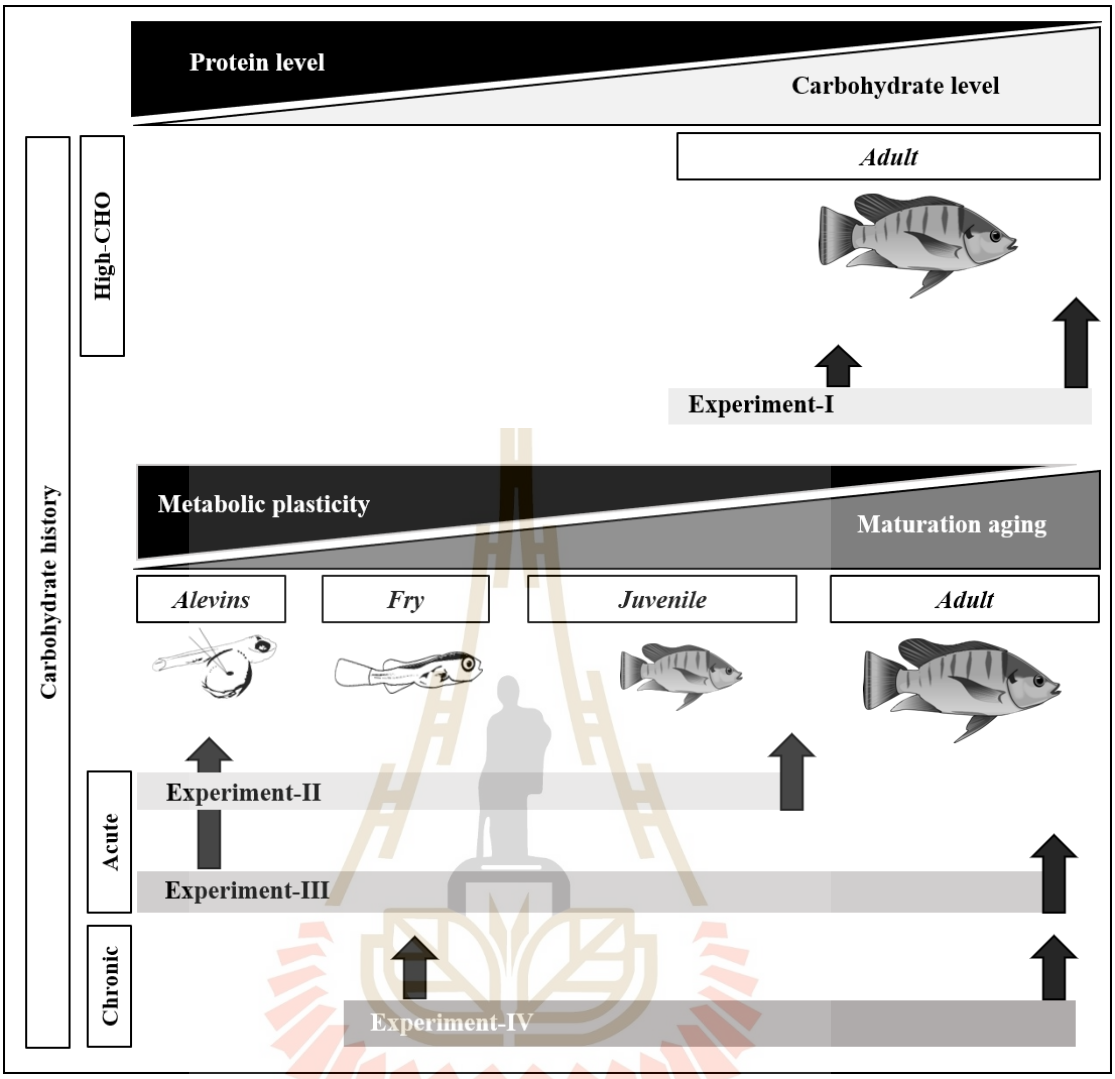

Figure 1.1 Schematic plan of the four experiments developed in Nile tilapia focusing the carbohydrate responded and early nutritional programming with glucose history.

1.5 Expected results

These findings provide a comparative study on metabolic responses to carbohydrate intake and nutritional programming of carbohydrate between carnivorous fish (i.e. Rainbow trout) and omnivorous fish (Nile tilapia). The resulting

knowledge from this thesis can potentially pave the way towards a better use of carbohydrates as energy substrates for fish, and therefore allow a higher incorporation of carbohydrate ingredients in fish diets, necessary for a sustainable.

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CHAPTER II

REVIEW OF THE LITERATURE

2. 1 Review of the literature

Aquaculture is expected an important role in global food supply for human demand (FAO, 2018). There have been a number of farm species which have been produced for aquaculture including aquatic plant, molluscs, crustaceans and fish (Cai et al., 2018) (Table 2.1). Fish from aquaculture has contributed for human consumption which has increased from 6% (1970) to 49% (2012) (FAO, 2014). Fish farming products have been provided for food industry and staple protein source for human consumptions. About 17% global animal proteins were obtained from fish products. In addition, fish products are important animal protein sources for populations in Low Income Food Deficit Countries in Africa and Asia (FAO, 2014). Comparing to marine fish, freshwater fish are mainly obtained from aquaculture (Table 2.1).

Among freshwater fish, tilapia has become the second economic fresh water fish species in aquaculture later carp (FAO, 2018). Worldwide tilapia production more than 1,500,000 metric tons in 2002 (Fessehaye, 2006) and has increased annually year by year (Figure 2.1). Tilapia prefers tropical environments with water temperatures in the 26-32ºC range (Agbo, 2008). It is an omnivorous grazer that naturally feeds on phytoplankton, periphyton, aquatic plants (FAO, 2019). Tilapia is able to withstand a wide range of environmental conditions.

Table 2.1 Top ten ASFIS species items by quantity in the world aquaculture production, 2017.

Top 10 ASFIS species items		World aquaculture (2017 quantity)		
ASFIS species	**Scientific name**	**Number of countries farming the species item**	**World production quantity of the species item (live weight; tonnes)**	**Share of world production quantity of all species (%)**
1. Japanese kelp	*Laminaria japonica*	4	11,174,505	9.98
2. Eucheuma seaweeds nei	*Eucheuma spp.*	13	8,637,534	7.72
3. Grass carp (= white Amur)	*Ctenopharyngodon idellus*	38	5,519,487	4.93
4. Cupped oysters nei	*Crassostrea spp.*	9	4,905,215	4.38
5. Silver carp	*Hypophthalmichthys molitrix*	37	4,704,673	4.20
6. White leg shrimp	*Penaeus vannamei*	36	4,456,603	3.98
7. Gracilaria seaweeds	*Gracilaria spp.*	7	4,311,040	3.85
8. Japanese carpet shell	*Ruditapes philippinarum*	7	4,228,206	3.78
9. Nile tilapia	*Oreochromis niloticus*	78	4,130,281	3.69
10. Common carp	*Cyprinus carpio*	78	4,129,100	3.69
Other species		n.a.	55,749,978	49.80
All species		196	111,946,623	100

Data source: FAO Global Fishery and Aquaculture Production Statistics 1950–2017 (v2019.1.0), published through FishStatJ (March 2019). Available at www.fao.org/fishery/statistics/software/fishstatj/en.

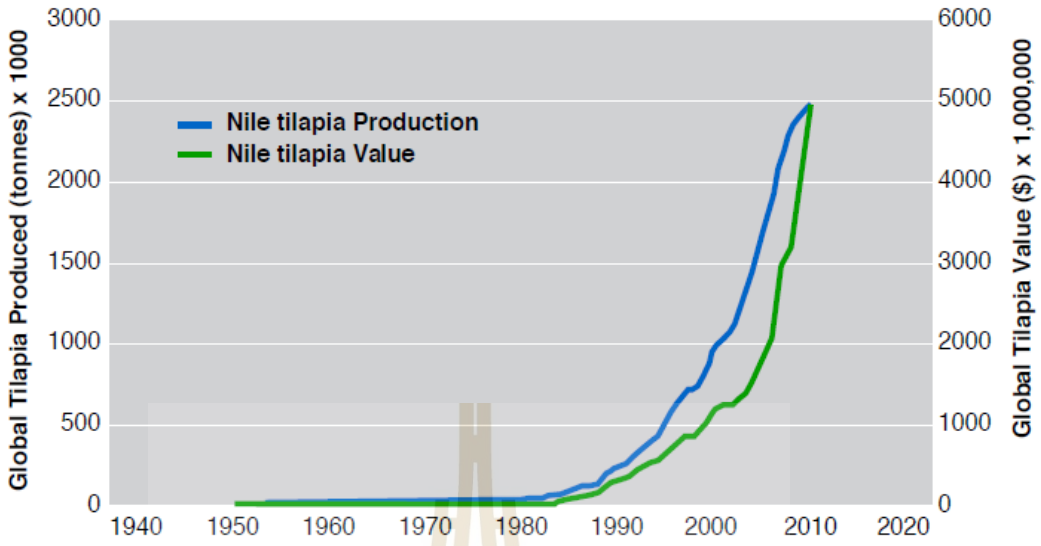

Figure 2.1 Global tilapia production and trend value, Source; Economic Research Service (ERS), USDA. 2010.

Figure 2.2 Nile tilapia (*Oreochromis niloticus*).

This makes tilapia very suitable for aqua farming. The water qualities that are suitable for tilapia are present in the Table 2.2. Among tilapia, Nile tilapia (*Oreochromis niloticus*) (Figure 2.2) dominates world tilapia culture.

Table 2.2 Water quality parameters for tilapia (*Oreochromis niloticus*).

Water parameters	Desirable level	Tolerance range
Salinity PPT	0-20	3-25
Temperature °C	26-32	12-35
Dissolved oxygen (DO) mg l^{-1}	3.0 - 5.0	2.0-8.0
pH	6.5-8.5	5-10
Ammonia (NH_3) mg l^{-1}	-	0.0125
Nitrite (NO^-_2) mg l^{-1}	-	0.1-0.2
Nitrate (NO^-_3) mg l^{-1}	-	0.0-3.0

Source: Agbo. (2008)

In commercial, all male tilapia culture are preferred. Sex reversal of Nile tilapia can be achieved by feeding fry with dietary containing 17 α-methyltestosterone (17 α-MT) at 60 mg/kg feed at first-feeding for 21-28 days (Rima et al., 2017). Generally, commercially practical diets for Nile tilapia could be divided according to the requirement of each growth stage. The chemical composition of commercial diet for Nile tilapia is showed in Table 2.3. In addition, practical diets for Nile tilapia were reported to contain crude protein (25-45%), crude fat (5-12%), carbohydrate (interm of NFE) (20-50%), fiber (≤ 6%), total n-6 and n-3 fatty acids (0.5-1.0%), minaral (1%, calcium 0.3% and phosphorus 0.7%) (Pichet, 2016). In commercial tilapia production, feed generally is the major part (50-60%) of the production cost. In order to expand the production of tilapia for world consumption, cost-effective diet with high quality, understanding on metabolic responses are required to develop to reduce the production cost as well as stainable of tilapia production.

Table 2.3 The commercial diet for Nile tilapia (*Oreochromis niloticus*).

Growth stage	Nutrient composition (%)					
	Moisture	**Protein**	**Fat**	**Fiber**	**Ash**	**NFE***
Fry	Powdered fish meal > 40% protein					
Fingerlings	12	40	5	4	8	31
35 - 100 g	12	32	4	6	8	38
100 - 300 g	12	30	4	8	8	38
300 ~	12	25	4	8	10	41

NFE = Nitrogen-Free Extract [NFE% = 100% -[Moisture% + Fat% + Protein% + Ash%]

Source; Aqua feed company, THAILAND (2019).

Generally, nutrients are categorized according to their function such as body building, energy giving, protective and regulatory nutritive values. Adequate level of protein and amino acids contents are mainly important for growth and body building. Energy yielding nutrients contain protein, lipid and carbohydrate. Vitamin and minerals provide the protective role for normal health and growth. Ingredients use for practical diet and their composition are showed in Table 2.4.

Table 2.4 Ingredients use for practical diet in Nile tilapia (*Oreochromis niloticus*).

Ingredient (%)	**Moisture**	**Protein**	**Fat**	**Fiber**	**NFE**	**Ash**
Fish meal	9.7	55	6	2.4	3.3	24.6
Soy bean meal	11.8	46.9	1.3	6.5	25.1	8.4
Broken rice	11.6	7.1	1.4	0.5	75.2	4.2
Rice bran	10.0	12.2	11.8	12.3	40.6	13.1
Corn	11.2	9.4	0.2	0.8	77.2	1.2
Cassava	13.5	2.2	0.5	3.0	77.2	1.2
Groundnut	7.0	48.0	5.8	7.0	27.1	5.1
Wheat	12.1	12.0	1.7	2.5	70.0	1.7

Tanaporn, (2014) (Department of Fisheries).

In order to develop low cost feed with high quality, a number of cheap ingredients with high quality are investigated the potential use in fish diet. To date, protein has been the most expensive ingredient in fish diets. Alternative ingredients for protein to replace fish meal content were investigated (Azaza et al., 2015; Honorato et al., 2010). The protein content in cost-effect feed contains the protein at the optimum level for high growth performance. Dietary energy yielding sources in fish feeds are mostly obtained from non-protein energy sources such as carbohydrate (Table 2.5) and lipid (Silva et al.1993). Dietary energy level is one of the significant factors affecting the utilization of protein for growth efficiently. Therefore, since ingredients yielding carbohydrate are cheap, comparing to protein ingredient, investigate of utilization and metabolism of dietary carbohydrate is important to develop cost- effective feed with high quality.

Table 2.5 Sources of carbohydrates in aqua feed.

Source (%)	Moister	Protein	Fat	Fiber	NFE	Ash
Broken-rice	11.6	7.1	1.4	0.5	75.2	4.2
Bran	10.0	12.2	11.8	12.3	40.6	13.1
Corn	11.2	9.4	0.2	0.8	77.2	1.2
Rice bran	12.1	14.7	4.0	9.9	53.5	5.8
Cassava	13.5	2.2	0.5	3.0	71.8	5.0

Tanaporn, 2014 (Department of Fisheries).

Carbohydrate has been the cheapest source in fish feed. The ability of fish to utilized carbohydrates base on natural feeding habits (Table 2.6). Carnivorous species they are considered to be poor carbohydrates user compare to herbivorous fish (Wilson, 1994; Legate, 2001; Hemre et al., 2002). Most omnivorous and herbivorous fish have a good ability to use starch as energy source. Enzyme alpha amylase hydrolyzes the starch components to short chain oligosaccharides. After various brush border enzymes further hydrolyze these oligosaccharides into their constituent monosaccharides mainly glucose.

Table 2.6 The optimum inclusion carbohydrate level for Carnivorous, Omnivorous and Herbivorous species under specified conditions.

Fish species	CHO source	Optimum level (%)	Reference
Carnivorous			
Rainbow trout	Gelatinized potato starch	18-27	Yamamoto et al. (2001)
(*Oncorhynchus mykiss*)	Gelatinized maize starch	20	Suárez et al. (2002)
Gilthead sea bream	Gelatinized maize starch	18	Fernández et al. (2007)
(*Sparus aurata*)			
European eel	Gelatinized maize starch	30	Suárez et al. (2002)
(*Anguilla anguilla*)			
Japanese flounder	Dextrin	18-24	Lee et al. (2002)
(*Paralichthys olivaceus*)			
Chinese long snout catfish	Maize starch and α-starch	17	Tan et al. (2007)
(*Leiocassis longirostris*)			
Herbivorous			
Grass carp	Maize starch	38	Li et al. (2014)
(*Ctenopharyngodon idellus*)	Wheat starch	33	Tian et al. (2012)
Blunt snout bream	Cassava starch	30	Zhou et al. (2013)
(*Megalobrama amblycephala*)	Dextrin and plant meals	29	Li et al. (2013)
Omnivorous			
Gibel carp	Maize starch	30	Li et al. (2014a)
(*Carassius auratus gibelio*)	Maize starch and α-starch	28	Tan et al. (2006)
Nile tilapia	Maize grain and wheat	48	Ali and Al-asgah
(*Oreochromis niloticus*)	bran		(2001)
Hybrid tilapia	Maize starch	22-46	Wang et al. (2005)
(*Oreochromis niloticus x O. aureus*)			

Table 2.6 (Continous).

Fish species	CHO source	Optimum level (%)	Reference
Silver barb (*Puntius gonionotus*)	Dextrin	26	Mohanta et al. (2009)
White sea bream (*Diplodus sargus*)	Waxy maize starch	36	Sá et al. (2007)
Yellow fin sea bream (*Sparus latus*)	Raw maize starch	20	Wu et al. (2007)

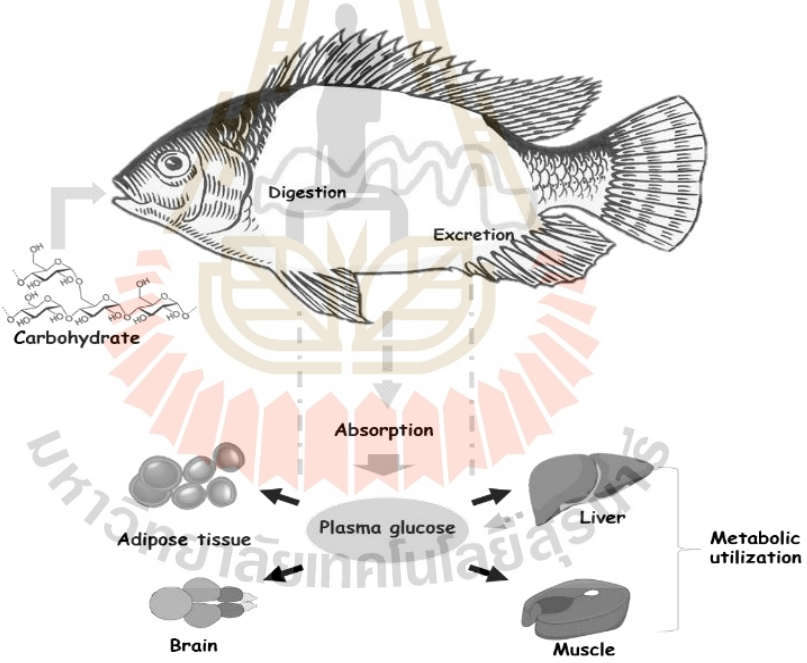

Figure 2.3 Digestion and absorption carbohydrate adapted from Kamalam et al., (2017).

Monosaccharides (mainly glucose an energy source for muscle cells, brain and red blood cells) are then transported past to the villi (Figure. 2.3) (Krogdahl et al., 2005; NRC, 2011). Sodium dependent glucose symporter (SGLT1) and glucose transporter (GLUT2) transport glucose molecule from intestinal lumen into blood vessel (Epithelium cell). Generally in fish species, it was demonstrated that the metabolic pathway for exportation of glucose to blood stream by phosphorylation of glucose via enzyme in the liver (hexokinase; HK) and dephosphorylation to glucose-6-phosphatase (G6Pase, the main glucose converse) (Mithieux, 2005; Polakof et al., 2010).

Glycolysis is the major pathway for glucose metabolism, the phosphorylation of converse glucose molecule to pyruvate ($C_3H_4O_3$) and its contrary metabolic pathway, the glucose production (gluconeogenesis), where synthesized glucose molecule from non-carbohydrate (glucose amino acid and fatty acid) (Figure.2.4) (Pilkis and Granner, 1992). The glycolytic pathway are regulated at three enzyme limiting steps including catalysed by the hexokinase (HK, the first step of glycolysis), 6- phosphor fructo-kinase (6PFK) and pyruvate kinase (PK, the last step of glycolysis) (Figure 2.4) (Oosterveer and Schoonjans, 2014).

When fish intake carbohydrate (glucose) in excess, the hepatic glucokinase (GK), acts the first to introduce the storage glycogen from exceeds glucose, concrary to supporting the glycolytic pathway. Generally, the activity of this enzyme is related to nutrient intake and hormonal control (Iynedjian, 1993).

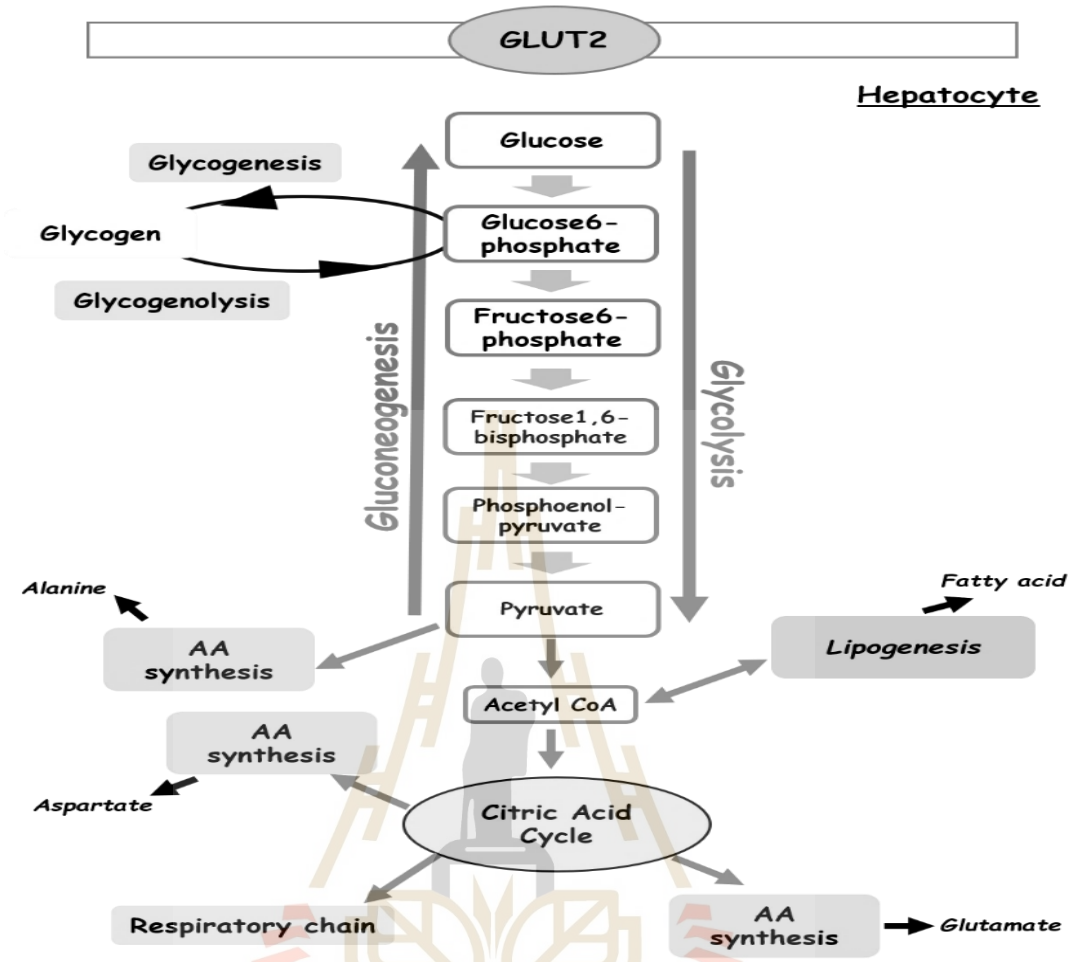

Figure 2.4 The hepatic major enzyme intermediary in glucose metabolism: glucose metabolism are including glycolysis (converse glucose to pyruvate), gluconeogenesis (glucose production from non-carbohydrate), glycogen syntase, lipogenesis and amino acid catabolism. The main metabolic pathway are, as well, presented.

Glucokinase is the major enzyme were already characterized by Panserat et al., 2014 at a biochemical and molecular levels in various fresh water and marine fish species. The up-regulated of GK enzyme activity in trout (*Oncorhynchus mykiss*) when they fed with high carbohydrates diet (Panserat et al., 2001; Seiliez et al., 2011)

feeding was often connected with a up-regulated induction of hepatic glucokinase gene, which strongly suggests the quantity of carnivorous species (i.e. Rainbow trout) to adapt to carbohydrate intake and, therefore, to future commercial farm scale use of plant-based diets. The regulation of the hepatic gluconeogenic is dependent on three irreversible key-enzymes, 1) the phosphoenolpyruvate carboxy kinase (PEPCK), 2) the fructose-1,6-bisphosphatase (FBPase) and 3) the glucose-6-phosphatase (G6Pase) (Figure 2.4) (Oosterveer and Schoonjans, 2014; Pilkis and Granner, 1992). Gluconeogenesis takes place mainly in the liver, less extent in the muscle, kidney and intestine (Kirchner et al., 2008). Also, this pathway was found to be sensitive to a nutritional and hormonal regulation in omnivorous fish, such as common carp (*Cyprinus carpio*) (Shimeno et al., 1995; Sugita et al., 2001), in a similar way to that observed in mamals. Thus, the induction of the gluconeogenic enzymes its repression occurs with intake of dietary carbohydrates (Pilkis and Granner, 1992). The opposite way, in non-herbivorous they are limited in their ability to use carbohydrates. For example; metabolic responses of rainbow trout (carnivorous fish) when they intake higher carbohydrate have been intensively researched (Polakof et al., 2012). Molecular regulation of metabolic pathways in the liver and muscles explain to inhibit hepatic gluconeogenesis, poor induction of hepatic lipogenesis and less effective cycling between glucose and glucose-6-phosphate in the liver (Figure 2.4). Moreover, molecular regulation of glucose transport (GLUT-4) and glycolysis were absent in muscle tissue (Panserat et al., 2000,2001,2002,2009; Polakof et al., 2012; Seiliez et al., 2011; Marandel et al., 2015; Kamalam et al., 2017). The activity and gene of the major enzymes involved in glucose metabolism are including, glycolysis, gluconeogenesis, glycogen synthesis and breakdown (glycogenolysis) and the

presence of glucose transporters have been confirmed and characterized in several fish species (i.e. Rainbow trout, European sea bass) (Figure 2.4) (Enes et al., 2009; 2011; Krogdahl et al., 2005; Panserat et al., 2000).

Existing literature have generally proved that gut plays an important role in the glucose homeostasis of carnivorous rainbow trout, while information in non-carnivorous fish is very limited. Generally, a better use of dietary carbohydrates is observed with omnivorous fish (common carp, gibel carp, Nile tilapia) and herbivorous fish (grass carp) as shown previously. They appear to be able to effectively adapt to the intake of high level of carbohydrates, as responed by: (1) the weak postprandial hyperglycemia (5.0-5.5 mmol) an increasing plasma triglyceride, plasma cholesterol, (2) the higher level of hepatic and muscle glycogen, hepatic lipid and body lipid contents, (3) the higher pyruvate kinase enzyme in muscle (mRNA level and activity) and (4) the suppression of the gluconeogenic enzyme for glucose-6-phosphatase and phosphoenolpyruvate carboxykinase enzymes (at mRNA and enzymatic activity) could account for a better use of carbohydrates in tilapia as examples (Li et al., 2013; Song et al., 2018; Li et al., 2016,2019; Cai et al., 2018; Tan et al., 2006; Tian et al., 2012; Boonanuntanasarn et al., 2018; Song et al., 2018.). Therefore, tilapia would be a good model in omnivorous fish for investigation of the molecular responses of the metabolism to different levels of dietary carbohydrates. In addition, mechanism which could modulate carbohydrate metabolism is interesting to study in order to improve the utilization of dietary carbohydrate as an energy.

The term of programming describes the process through which organism experiences any environmental stimulus during critical phases of development. Consequently, this stimulus from the first experience affects permanent changes to the

physiology or metabolism of the particular nutrient in organism (Langley- Evans 2009). For instance, dietary stimulus exerts during early life period at critical developmental time window may subsequently have effect on physiological functions and metabolism pathways at later life phase (Rocha et al., 2015; Rocha et al., 2016a,b and Turkmen et al., 2017). This phenomenon is called nutrition programming. Evidence for nutritional programming has been investigated in mammal (human and rodents) (Langley-Evans 2015). For example, it was investigated that whether n3-LC PUFA intake of lactation might be important for young children. Lactating Danish mothers obtained fish oil which is rich in n3-LC PUFA, comparing to olive oil, during 0-4 months. The result demonstrated that although the fish oil intake during lactation had no effect on growth in weight, length and head circumference of infants up to 9 months, at 2.5 year, children in the fish oil group had larger head circumference and body mass index (Laurizen et al., 2005). In Sprague-Dawley rat, there were interaction effects between maternal and post-weaning diet on hypothalamic gene expression in adult offspring. Adult male offspring from chow-fed dam and high saturated fat diet-fed dam were tested two different diets including standard chow diet and diet with high saturated fat. The results showed that both offspring from control and high-fat dams if fed a high-fat diet from weaning to adulthood had significantly higher body weight, energy intake, fat deposition, serum leptin and insulin. Only offspring from high-fat dam if fed a high-fat diet were hyperglycemic. In offspring exposed to high-fat diet during gestation and into adulthood, the significant increases in hypothalamic gene expression including leptin receptor, proopiomelanocortin, neuropeptide y (NPY) were observed whereas decrease in the expression of NPY receptor was found, supporting the hypothesis that early life exposure to high fat is associated with

hypothalamic regulation of body weight and energy homeostasis (Kathleen et al., 2009). Additionally, the nutritional programing was demonstrated in avian model. The effects of albumen removal (albumin-deprived group and replacement with saline) during early embryonic development on growth and metabolism were demonstrated. Compared to non-manipulated or sham-manipulated hens, albumin-deprived hens had significantly lower body weight. In addition, albumin-deprived hens had lower number of eggs and egg weight, suggesting that albumen removal diminished the reproductive capacity. Plasma triiodothyronine of albumin-deprived hens was enhanced. Reduction in glucose tolerance was observed in albumin-deprived hens. The effects of albumen removal were transmitted into their offspring. The offspring of albumin-deprived hens had low body weight and relative residual yolk weight at hatching. Therefore, prenatal protein under nutrition had long-term effects on growth characteristic and reproductive system (Willems et al., 2015).

Nutritional programing of carbohydrate metabolism was demonstrated in zebrafish (*Danio rerio*) (Fang et al., 2014;Rocha et al., 2014; Rocha et al., 2015) gilthead seabream (*Sparus aurata*) (Rocha et al., 2016a,b) and rainbow trout (*Oncorhynchus mykiss*) (Marandel et al., 2016) (Table 2.4). Long-term effects of glucose microinjection in the yolk (early stimulus) and subsequently challenge with a high-level of carbohydrate (CHO) and low protein diet on CHO metabolism and expression of gene related to CHO metabolism were investigated in Zebrafish. It was revealed that glucose stimulus at embryo stage altered several steps of glucose metabolism. Up-regulation of hexokinase and phosphofructokinase in muscle of juvenile fish exposed to glucose stimulus, indicating that glucose oxidation was possibly improved. Glucose stimulus also affected gluconeogenesis in liver which was

indicated by inhibition of the expression of phosphoenol pyruvate carboxykinase. In addition, glucose stimulus at early stage would affect the regulation of the fructose-1,6 bisphosphatase (FBP)- phosphofructokinase-6 (6 PFK) metabolic cycle (Rocha et al. 2015). Whether high glucose levels injection during the embryonic stage could be a lifelong modulator of genes related to carbohydrate metabolism in juvenile was studied in zebrafish. Zebrafish egg yolk was enriched with glucose, and acute effects of glucose injection were evaluated by determination of gene related to carbohydrate metabolism in larvae up to 10 day post-fertilization (dpf). At 4 dpf, downregulation of several genes related to glycolysis, glycogenolysis, lipogenesis and carbohydrate digestion were observed in glucose-enriched eggs. However, at 10 dpf larvae, there were little downregulation of these gene expression. The programming concept was evaluated in juveniles (41 dpf) challenged with a hyperglucidic diet. The results showed that early glucose stimulus had non-significant effect on the expression of gene involved in carbohydrate metabolism, suggesting that glucose supplementation in egg yolk had little effect on the long-term modulation of carbohydrate metabolism in zebrafish (Rocha et al., 2014). Nevertheless, early nutritional programing of carbohydrate modulated carbohydrate digestion, transport and metabolism of adult, demonstrating permanently nutrition programing effect in zebrafish. It was also revealed that different particular ontogenic phase of high carbohydrate stimulus had different effects of long-term metabolism in adult fish (Fang et al., 2014). The study on nutritional programming in rainbow trout demonstrated that the time point of nutritional stimulus was important. It was reported that nutritional stimulus at first feeding was too late for the programming of glucose metabolism in the long term (Marandel et al., 2016). Furthermore, the nutritional programming of carbohydrate

was demonstrated in seabream. Although early glucose stimuli at larval stage did not modulate the expression of several genes related to glycolysis, gluconeogenesis, glucose transporter, lipogenesis, energy metabolism and amino acid catabolism, it increased carbohydrate digestion in juvenile fish. Therefore, nutritional programing of carbohydrate metabolism was presented in experimental fish and carnivorous fish, and more investigations of the nutrition programming effects of carbohydrate in other fish remained to be studied. Further studies to investigate the effects of other types of carbohydrate nutrient stimulus and window of plasticity during early development are also required.

Epigenetic refer to changes of level gene expression without any change of the nucleotide (DNA sequences). There are several mechanisms that control gene expression in animals. The major regulation mechanisms are including 1) DNA methylation 2) histone modification and 3) microRNA-mediated genetic silencing change the genomic structure and thereby consequence gene expression without any modifying the DNA sequence (Abukiwan and Berger. 2018) (Figure 2.7).

Table 2.7 Study nutritional programming in fish.

Stimulus	Dietary stimulus	Stimulus duration	Challenge feed/Time	Reference
First feeding (Trout)	60 % Dextrin	3 days 5 days	25 % Dextrin/7days	Geurden et al., 2007
First feeding (Zebrafish)	60% Dextrin	FF3,FF5,YE3,YE5 (3 days)	35 % Dextrin/7days	Fang et al., 2014
First feeding (Trout)	65% Starch + Glucose	5 days	28% Starch /65 days	Geurden et al., 2014
Injection (Zebrafish)	2M Glucose injection	0.4 day post fertilized (30% epiboly)	20% glucose + Gelatinised starch 17 days	Rocha et al., 2014
Injection (Zebrafish)	2M Glucose injection	1 day post fertilized	50% Dextrin/11 days	Rocha et al., 2015
First feeding (gilthead seabream)	Glucose mix Artemia	60 days	33% Starch /36 days	Rocha et al., 2016 (PartA)
First feeding (gilthead seabream)	Rotifer + D-glucose (57%) Artemia + D-glucose (57%) Diet D-glucose (50%)	At day 8 At day 25 At day 35 5days	HCD (50% D-glucose)/10 days	Rocha et al., 2016
First feeding (Trout)	HP-diet LP-diet HPR-diet	4 weeks	LP-diet/11 Weeks	Song et al., 2019

FF-3: the first feeding stage to the end of yolk sac; FF-5: the first feeding stage to 2 day after yolk-sac exhaustion; YE-3: after yolk-sac exhaustion for 3 day (No yolk); YE-5: after yolk-sac exhaustion for 5 day (No yolk); HCD: High carbohydrate diet. HP: High protein diet; LP: Low protein diet; HPR; High protein restinction.

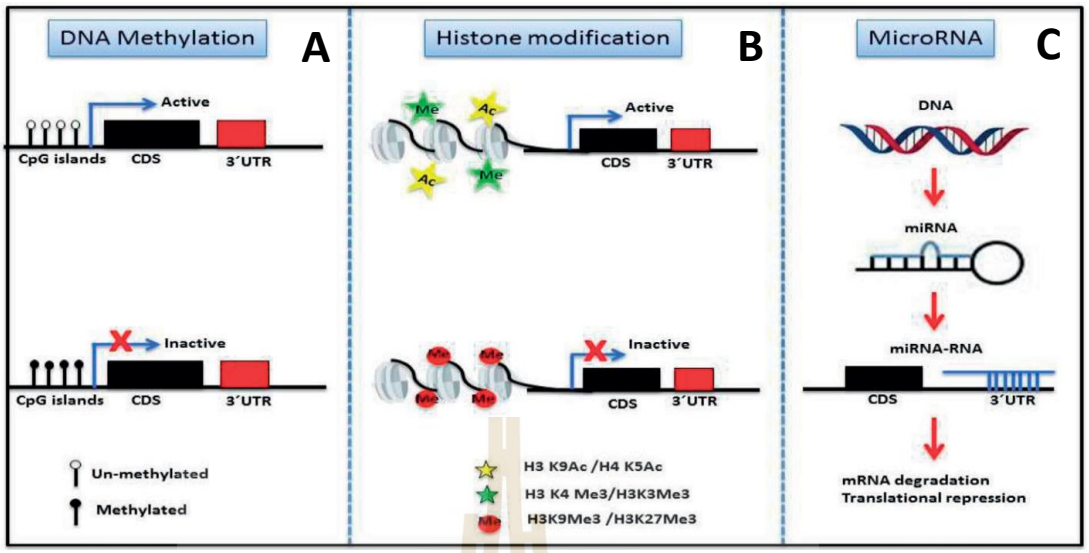

Figure 2.5 Epigenetic refer to changes of level gene expression without any change of the nucleotide (DNA sequences). Global DNA methylation at specific CpG regions (A). The modification of histones has two consequences on genes (B). The mechanism is influenced by microRNAs (miRNAs) (C). From Abukiwan and Berger. (2018).

Epigenetic modifications are available mechanisms effect from nutritional status could begin a metabolic remembrance by changing the chromatin structure and consequently the regulation of the genes, and hence their transcription (Jiménez-Chillarón et al., 2012). Naturally, changes in the epigenome (gene expression) are partly in response to natural impacts (environment and/or nutrient) which will subsequently lead to modification of metabolism. One of the research epigenetic modification effects from environmental and chemicals are altered DNA methylation, a covalent modification of cytosine nucleotides in a specific CG dinucleotide context and an important mechanism of gene regulation. In the higher vertebrate genome, DNA methylation occurs mainly at CpG (cytosine followed by a guanine)

dinucleotides. CpG-rich regions of 0.5-4 kb in length, known as CpG islands, are found in many house-keeping gene at promoters and are generally protected from methylation (Bird, 2002). It has been demonstrated that DNA methylations (hypo/hyper global methylation) at specific sites can affect regulation of gene level. While there are examples that do not seem to support such correlations, in several instances methylations at critical sites of genes have been shown to lead to their inhibit the activation (Langer et al., 1984). In many genes, at specific.

5' $C^{m}CGG$ 3' critical sites; these sites have not been recognized. This decline may explain apparent discrepancies. The biochemical mechanisms by which site specific DNA methylations (5' CmCGG 3') lead to transcriptional inactivation are less certain. It is credible that specific DNA methylations can cause or stabilize structural alterations of DNA (Malendel et al., 2016). There is limited information concerning epigenetic modifications at global genome and target gene levels mediated by nutritional status or by carbohydrates feeding and their physiological change (i.e., hyperglycaemia) in fish. Study has reported global DNA hypomethylation in the livers of trout fed a high level of carbohydrate without prospecting for histone mark changes at glucose metabolism-related gene loci (Craig and Moon, 2013). Dietary high level of dietary carbohydrates could affect the epigenetic landscape affected global hypomethylation of DNA in Rainbow trout (Marandel et al., 2016) and Zebrafish (Sanchez et al., 2015; Williams et al., 2008). Indeed, the catabolic capacities and global hepatic epigenome are modified in juvenile rainbow trout fed different vitamin levels at first feeding stage period of life (Panserat et al., 2017). In the present study we therefore first assessed in tilapia whether and how nutritional programming status

(glucose injection combination with dietary carbohydrate challenging) affected global epigenome modifications by targeting DNA methylation at specific 5' $C^{m}CGG$ 3' site.

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CHAPTER III

MOLECULAR RESPONSES OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*) TO DIFFERENT LEVELS OF DIETARY CARBOHYDRATES

3.1 Abstract

The objective of the study was to test for the first time the molecular adaptation of the glucose metabolism to this fish species know to be a good user of carbohydrates. In this way, Nile tilapia were fed with 3 different levels of carbohydrates 0% (CHO-F) 30% (CHO-M) and 50% (CHO-H) dextrin. After 45 days and 90 days of feeding we analyzed the plasma parameters, zootechnical performance and the mRNA levels for genes involved in glycolysis, gluconeogenesis, lipogenesis and glucose transport in liver and muscle. The best growth performance was found for fish fed M-CHO diet, the fish fed with the H-CHO diets showing the worst growth performance. Increased of hepatic and muscle glycogen, hepatic somatic index and plasma metabolites (glucose, triglycerides and cholesterol) was linked to the increased of dietary carbohydrates. However, no hyperglycemia, no change of body compositions was found showing that dietary carbohydrates are efficiently used as an energy source in tilapia. Moreover, in contrast to the dietary protein-linked decreased of amino acid catabolic mRNA levels, no clear molecular adaptation for glycolysis, gluconeogenesis, lipogenesis in liver and glycolysis in muscle was detected except

higher mRNA levels for glucose transporter in muscle. Our data suggest that these metabolic pathways at a molecular level are not the main actors explaining the efficient use of glucose in tilapia. Statement of relevance: Our study aimed at characterizing for the first time the molecular effects of increased dietary carbohydrates (dextrin) from 0% up to 50% in Nile tilapia during 45 and 90 days. Our study confirmed that Nile tilapia can use high level of carbohydrates without any deregulation of glucose homeostasis. However, no strong regulations of expression for genes involved in glucose metabolism in liver and muscle-which could explain the reasons for an efficient use of carbohydrate by this fish species-were detected.

Key words: Nile tilapia, Dietary dextrin, Gene expression, Glucose-metabolism

3.2 Introduction

Global production of Nile tilapia (*Oreochromis niloticus*) has dramatically increased year by year to provide staple protein source for world demand of fish products (FAO, 2014). Although the entire life cycle of tilapia culture has been well conducted, researches in various areas of tilapia are still needed in order to increase the productivity of tilapia with low production costs. Development of tilapia farming with low cost would contribute to achieve price stability or decrease price of tilapia products, thereby expanding global tilapia market. Since feed generally is the largest cost item (accounting for 40%-60% of the production cost), intensive researches on low-cost feeds present a challenge.

Among nutrients, carbohydrates are included in fish feed as much as possible because they are considered as the less expensive sources of substances for growth. In addition, carbohydrates could have a protein sparing effect for growth. Nile tilapia is

an omnivorous grazer, which can efficiently utilize carbohydrates as the main energy source (Kamalam et al., 2017). It was demonstrated that juvenile tilapia could utilize 46% dietary starch in feed containing 29% protein and an energy/protein ratio of 37.9 kJ g^{-1} without growth retardation although feed containing 22% starch was optimal (Wang et al., 2005). The protein- sparing effects of carbohydrates (starch or dextrin) were demonstrated when dietary protein level was suboptimal (Shiau and Peng, 1993). For intensive production, commercial diets generally contain nitrogen free extract up to 40% which provide energy source and pellet binder. Therefore, tilapia is a fish which has a high adaptive capacity to deal with varying levels of carbohydrate intake.

In previous studies, metabolic responses of the carnivorous trout which has the limited ability to use efficiently carbohydrates at a metabolic level were demonstrated (<20% in the diet) (Kamalam et al., 2017; Polakof et al., 2012). Indeed, in rainbow trout, it was suspected that a combination of atypical molecular regulation of metabolic pathways could explain the low dietary carbohydrate use: absence of molecular inhibition of hepatic gluconeogenesis (Panserat et al., 2000, 2001; Marandel et al., 2015), low molecular induction of hepatic lipogenesis (Panserat et al., 2009), futile cycle between glucose and glucose-6-phosphate in liver (Seiliez et al., 2011; Marandel et al., 2015), no molecular regulation of glucose transport and glycolysis in muscle (Polakof et al., 2012). Generally, digestion and metabolism of dietary carbohydrate vary among fish species following their feeding habits (Kamalam et al., 2017). Better use of dietary carbohydrates are observed with omnivorous fish (common carp, gibel carp, Nile tilapia) and herbivorous fish (grass carp) as shown previously (Panserat et al., 2002a, 2002b; Figueiredo-Silva et al., 2013, Li et al., 2013 as examples). However, while metabolic responses to carbohydrate in carnivorous

rainbow trout were intensively investigated at a molecular level, the molecular regulation of the metabolic pathways related to varying levels of dietary carbohydrate in non-carnivorous fish is limited. Indeed, increased of lipogenic and decreased of neoglucogenic enzyme activity could account for a better use of carbohydrates in tilapia (Figueiredo-Silva et al., 2013). Therefore, tilapia would be a good model for investigation of the molecular responses of the metabolism to different levels of dietary carbohydrates.

For a comparative study on metabolic responses to carbohydrate intake between carnivorous fish (rainbow trout) and omnivorous fish, juvenile tilapia was used in this study. In this way, tilapia were fed with three semi-purified diets containing 3 different levels of carbohydrates (0% 30% and 50% of dextrin). Fish were fed with these 3 diets during 90 days. Analysis of glucose metabolism known to be atypically regulated in rainbow trout at a molecular level in liver and muscle was performed at a molecular level after 45 and 90 days of rearing respectively.

3.3 Materials and methods

3.3.1 Experimental design and diet formulation

The experimental design was completely randomized with three treatment diets, each of which was replicated five times to test the validity of the conclusions. To test the effect of different carbohydrate level, the experimental diets included carbohydrate-free (CHO-F), carbohydrate-moderate (CHO-M) and carbohydrate-high (CHO-H) diets. Table 3.1 shows the ingredients of three semi-purified diets (CHO-F, CHO-M, CHO-H) and the nutritive contents (moisture, crude protein, crude fat, and ash content) as determined following standard AOAC methods (1990). All test

ingredients were obtained from commercial companies. All experimental diets were stored at -20°C until use.

3.3.2 Experimental fish and fish culture

The Nile tilapia used in this study were all male fish which were cultured at the Suranaree University of Technology Farm (SUT Farm; Nakhon Ratchasima, Thailand). Since this study aimed to investigate the metabolic responses of Nile tilapia when it ingested different dietary carbohydrate levels, Nile tilapia at the adulthood (160-170 g) which normally has high ability to utilize carbohydrate as energy source were used. Fifteen cement ponds (2×2×1 m3) (i.e., five replicates of three treatments) were used for the experiment. Ten fish were randomly distributed into each cement pond containing water (depth 0.7 m) under continuous aeration. In addition, a flow-through water change system was implemented by replacing one-third of the water in each pond with dechlorinated water every other week. To acclimatize the Nile tilapia to the experimental conditions, the fish were gradually changed feeding from commercial diets (30% crude protein; 4% crude fat) to each experimental diet. After fish accepted each experimental diet for 2 weeks, the experiment began. Each fish was weighed individually. There were no significant differences in initial body weight ($P>0.05$). Throughout the experimental period, the fish were handfed ad libitum twice daily (9:00 and 16:00), and feed intake by replicate was recorded each week. To assess growth performance, fish were weighed individually at the end of 45 and 90 days of experimental period. Air and water temperatures were measured daily and were 31-35°C and 27-28°C, respectively. Dissolved oxygen (DO) content and pH were measured weekly using a DO meter and

pH meter, and values were within acceptable ranges of 4.68-6.84 mg L^{-1} and 7.19-8.54, respectively. No mortality was recorded in this study.

3.3.3 Fish sampling and blood collection

At the end of the experimental period (45 and 90 days), fish were not fed for 5 h before being sampled (5 h after last meal correspond to the peak of posprandial glycemia in tilapia fed with these diets, data not shown). Two fish from each diet replicate (n= 10 fish per dietary condition) were sampling and anesthetized with 2-phenoxyethanol (0.2%). Blood samples were collected from the caudal vein using a hypodermic syringe, mixed with K_2EDTA (at 1.5 mg mL^{-1} blood) as an anticoagulant, and kept on ice until plasma collection. Plasma was collected by centrifugation of the K_2EDTA-treated blood at 9,000g for 10 min at 4°C and stored at -80°C for blood chemistry analysis. After blood sampling, liver and muscle were sampled and kept at -80°C for RNA extraction. A portion of fish flesh were minced and frozen for proximate chemical analysis according to AOAC (1990). In addition, one fish was randomly taken from each replication tank, minced and frozen for analysis of the proximate chemical composition of whole body according to AOAC (1990) (n=5 fish per dietary condition).

3.3.4 Blood chemistry analysis

Plasma metabolites were analyzed with the 10 fish per dietary conditions. Plasma glucose was analyzed using the GOD-PAP method (Trinder, 1969). Serum triglyceride content was measured using the glycerol-3-phosphate oxidase‑sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (GPO-ESPAS) method described by Bucolo and David (1973). Plasma cholesterol was quantitatively analyzed using the cholesterol oxidase-phenol+aminophenazone (CHOD PAP) technique described by Flegg

Table 3.1 Ingredients and chemical composition (g kg^{-1}) of the experimental diets.

Ingredients	**CHO-F**	**CHO-M**	**CHO-H**
Casein	820	570	320
Gelatin	70	70	70
Soybean oil	60	60	60
Dextrin	0	250	500
Dicalcium phosphate	10	10	10
Premix[a]	30	30	30
Vitamin C	10	10	10
Proximate composition (g kg^{-1}dry weight)			
Dry Matter	885.2	881.5	930.5
Protein	630.7	456.4	271.8
Fat	62.3	61.2	61.1
Fiber	3.0	3.0	2.7
Ash	40.5	37.4	31.7
NFE[b]	148.7	323.6	563.2
total energy (kJ/g)	16.5	15.9	16.3

[a] Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1}diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5,000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12 0.00005 mg; vitamin C, 1 g; vitamin D3, 1,000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g

[b] Nitrogen-free extract = dry matter - (crude protein + crude lipid + crude fibre + ash)

(1973). Plasma protein contents were determined using the Biuret method (Gornall et al., 1949). Plasma albumin content was quantitatively estimated using the bromocresol green method (Doumas et al., 1997). Plasma urea nitrogen (BUN) content was measured using a modified indophenol colorimetric method (Weatherburn, 1967).

3.3.5 Total RNA extraction and relative quantification of mRNA

Liver and muscle of five fish per nutritional condition (1 per tank) were analyzed. Total RNAs from were extracted from tissues (liver and muscle) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. RNA was quantified by Nano Drop (Thermo Fisher, USA) and the quality was verified on %1 agarose gel. Relative gene expression was determined by quantitative real-time RT-PCR on RNA extracted from tissues-muscle and liver (adults). An amount of 1 µg of total RNA was chose for cDNA synthesis. SuperScript III RNAseH-Reverse transcriptase kit (Invitrogen) with random primers (Promega, Charbonniéres, France) was used according to the manufacturer's protocol to synthesize cDNA (n =6 for each nutritional condition). The primer sequences used in the real-time RT-PCR assays as well as the protocol conditions of the were defined here for the time using the complete cDNA sequences of the ENSEMBL data base following (http://www.ensembl.org/Oreochromis_niloticus/Info/Index?db=core; g=ENSONIG00000017100; r=GL669455-831287.1:658579; t=ENSONIT(00000021583, except for the *ef1α* gene reference (Yang et al., 2013) (Table 3.2). Glucose metabolism in liver were studied at glycolytic 2 (glucokinase-*gck*; phosphofrucokinase-*Pfklr* and pyruvate kinase-*Pklr*), and gluconeogenic (glucose--6phosphatase-*G6pca1-G6pca2* and phosphoenopyruvate carboxykinase-cytosolic *Pck1* and mitochondrial *Pck* (*2* levels. Glucose use in muscle were analyzed by measuring mRNAs levels of glucose

transporter (*Glut* (*4*and glycolysis) hexokinase II-*Hk2*, phosphofructokinase-*Pfkma* and pyruvate kinase-*Pkma*). Lipogenic capacities (fatty acid synthase-*Fasn* and glucose--6phosphate dehydrogenase-*G6pd*) as well as the enzymes involved in amino acid catabolism were also studied using specific primers (Table 3.2). All the PCR products were sequenced to check the quality of the product. For real-time RT-PCR assays of transcripts of metabolic genes, the Roche Light cycler 480 system was used (Roche Diagnostics, Neuillysur-Seine, France). The assays were performed using a reaction mix of6 µl per sample, each of which contained 2 µl of diluted cDNA template, 0.12 µl of each primer (10 µM), 3µl Light Cycler 480SYBR® Green I Master mix and 0.76 µl DNAse/RNAse free water (5 Prime GmbH, Hamburg, Germany). The PCR protocol was initiated at 95°C for10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at 95°C; 40 s at 60-64°C), according to the primer set used. Melting curves were systematically monitored (temperature gradient at 1.1°C/10 s from 65 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicate of reverse transcription and PCR amplification, respectively) and negative controls (reverse transcriptase- and cDNA template-free samples, respectively). For the analysis of mRNA levels, relative quantification of target gene expression was performed using the ΔCT method (Pfaffl, 2001). The relative gene expression of ef1 was used for the normalization of measured mRNA in liver and muscle, as its relative expression did not significantly change over samplings (data not shown). In all cases, PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.2.

3.3.6 Glycogen analysis

Liver and muscle of five fish per nutritional condition (1 per tank) were analyzed for glycogen. Liver and muscle glycogen were determined according to Kirchner et al. (2003).

3.3.7 Data analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS for Windows (Release 10) (SPSS Inc., Chicago, IL, USA). When significant differences were found among the groups, Duncan's multiple range tests were used to rank the groups. Throughout the experiment, effects and differences were declared to be significant at $P<0.05$.

3.4 Results

The growth performances of Nile tilapia fed the experimental diets are shown in Table 3. After the 45-day feeding period, compared to fish fed the CHO-F and CHO-M diets, fish fed the CHO-H had lower growth response as reflected by the following parameters i.e. final body weight, ADG and SGR ($P<0.05$). Fish fed the CHO-H diet had also the highest FCR and FI ($P<0.05$). By the 90-day feeding period, the growth response was higher in fish fed on CHO-M, followed by fish fed on CHO-F, and then the fish fed on CHO-H. Again, fish fed the CHO-H diet had the highest FCR and FI ($P<0.05$). By the 45-day feeding period, no significant differences for the HSI have been observed. By contrast, fish fed the CHO-H diet had the highest HSI ($P<0.05$). The condition factor appeared to be similar among treatment diets. All along the experimental period, no mortality was observed among experimental groups.

Table 3.2 List of tilapia primers used for qRT-PCRs.

Genes	5/′3 ′forward primer	5/′3 ′reverse primer	Access number
Reference gene			
*ef1**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	AB075952
Liver metabolism			
gck	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC	XM003451020
pfklr	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC	XM003447353
pklr	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA	XM005472622
g6pca1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA	XM003448671
g6pca2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA	XM013273429
pck1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA	XM003448375
pck2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT	XM019354843
fasn	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT	XM013276809
g6pd	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT	XM013275693
asat	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC	XM003451918

Table 3.2 (Continues).

Genes	5/′3 ′forward primer	5/′3 ′reverse primer	Access number
alat	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG	XM005476466
gdh	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC	XM003457465
Muscle metabolism			
glut4	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG	JN900493
hk2	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC	XM003448615
pfkma	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG	XM019349871
pkma	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA	XM005447626

*From Yang et al. (2013).

Table 3.3 Growth performance of Nile tilapia fed experimental diets (mean±SD, *n*=5).

	Final weight (g)	ADG (g day^{-1})	SGR (%)	FCR (% day^{-1})	FI (g day^{-1})	HSI (%)	Condition factor
45 days							
CHO-F	311.35±9.54^{a}	4.90±0.12^{a}	2.12±0.06^{a}	1.20±0.02^{b}	5.68±0.04^{b}	2.83±0.17	1.92±0.05
CHO-M	324.16±10.23^{a}	5.21±0.23^{a}	2.18±0.10^{a}	1.17±0.02^{b}	5.83±0.12^{b}	2.82±0.37	1.97±0.08
CHO-H	279.87±11.49^{b}	3.66±0.32^{b}	1.65±0.09^{b}	1.87±0.11^{a}	6.50±0.28^{a}	3.42±0.88	1.95±0.09
90 days							
CHO-F	494.26±15.53^{b}	3.58±0.32^{b}	1.19±0.10^{a}	1.96±0.23^{b}	6.59±0.07^{c}	1.52±0.24^{b}	2.06±0.15
CHO-M	567.36±17.51^{a}	4.25±0.32^{a}	1.30±0.07^{a}	1.78±0.18^{b}	6.96±0.13^{b}	1.42±0.23^{b}	2.01±0.04
CHO-H	449.46±18.71^{c}	3.10±0.33^{c}	1.04±0.07^{b}	2.83±0.31^{a}	7.37±0.18^{a}	2.02±0.36^{a}	1.95±0.10

Means with different superscripts in each column differ significantly from each other (P<0.05). Average daily gain (ADG) = (final body weight-initial body weight)/experimental days; Specific growth rate (SGR) = 100 x [(ln final body weight – ln initial body weight)/experimental days]; Feed conversion ratio (FCR) = dry feed fed/wet weight gain; Hepatosomatic index = 100 x (liver weight/body weight); Feed intake (FI) = dry feed fed/experimental days; Condition factor =100 × (final body weight/body length^{3}).

The effects of varying dietary carbohydrate level on the proximate chemical composition in muscle and whole body were presented in Tables 3.4-3.5 Through the experimental period, there were no significant differences in the nutritive composition in muscle including moisture, crude protein, crude fat and ash (Table 3.4). In addition, the proximate chemical composition of Nile tilapia whole body appeared to be similar among the experimental groups (Table 3.5).

Table 3.6 showed the examined blood chemistry parameters including glucose, triglyceride, cholesterol, albumin, total protein and BUN of Nile tilapia fed different dietary carbohydrate levels. The results showed that, through the experimental period, plasma glucose, triglyceride and cholesterol positively responded to the dietary carbohydrate inclusion levels ($P<0.05$) In contrast, plasma albumin, protein and BUN showed the inverse trend with the carbohydrate contents in experimental feed ($P<0.05$)

We then analyzed mRNAs levels of key metabolic genes in liver (Table 3.7) and in muscle (Table 3.8) of tilapia after 45 days and 90 days of feeding. We chose also the peak of postprandial glycemia for samplings ie 5 h after the last meal in Tilapia. In liver, we analyzed mRNA levels of genes encoding for proteins related to amino acid catabolism. Indeed, we observed that alat and gdh mRNA levels decreased significantly 45 days after feeding and asat at 90 days after feeding in relation to the decreased of dietary proteins (Table 3.7). Except for the gck gene at 45 days after feeding wich is higher expressed with inceasing levels of dietary carbohydrates, we detected no significant variation of mRNA levels for genes coding for proteins involved in hepatic glycolysis (Table 3.7). For the gluconeogenic pathway (Table 3.7) and the lipogenic pathway (Table 3.7), there was no significant effect of all the dietary

treatments on mRNA levels for the target enzymes. In muscle (Table 3.8), except for the glut4 at 45 days after feeding, there was no significant effect of dietary carbohydrates intake on mRNA levels encoding for proteins related to glycolysis.

We also measured the level of glycogen in liver and muscle after 45 days and 90 days of feeding (Figure. 3.1). In liver, higher glycogen are found in relation to the increasing of the level of dietary carbohydrates after 45 days and 90 days of feeding. In muscle, the significant increase of glycogen in relation to the increase of dietary carbohydrates is only observed at 90 days after feeding.

Table 3.4 Whole body composition of Nile tilapia fed experimental diets (mean±SD, *n*=5).

	Moisture (%)	**Crude Protein (%)**	**Crude Fat (%)**	**Crude Ash (%)**
Initial				
CHO-F	77.5±2.5	10.5±0.6	3.4±0.4	3.8±0.2
CHO-M	78.3±2.3	10.9±1.4	3.4±0.4	3.7±0.5
CHO-H	79.1±1.1	11.0±0.7	3.7±0.1	3.6±0.2
45 days				
CHO-F	72.1±0.6	12.4±1.4	6.4±1.0	3.9±0.5
CHO-M	72.1±1.1	12.6±0.8	6.1±1.3	3.8±1.0
CHO-H	71.9±0.6	13.1±1.0	5.9±0.5	3.8±0.8
90 days				
CHO-F	73.8±2.0	12.4±0.9	6.7±1.0	4.1±0.7
CHO-M	73.4±2.4	12.6±0.4	6.3±1.3	4.1±0.6
CHO-H	72.2±2.7	13.1±0.5	6.1±0.7	4.1±0.9

Table 3.5 Muscle of Nile tilapia fed experimental diets (mean±SD, *n*=5).

	Moisture (%)	**Crude Protein (%)**	**Crude Fat (%)**	**Crude Ash (%)**
Initial				
CHO-F	79.9±1.4	11.6±1.5	2.3±0.2	1.5±0.3
CHO-M	79.2±2.1	13.3±0.5	2.3±0.2	1.6±0.4
CHO-H	78.5±1.9	12.7±0.8	2.1±0.2	1.5±0.2
45 days				
CHO-F	77.5±1.7	14.3±0.6	3.4±0.4	1.6±0.2
CHO-M	76.8±2.9	14.5±0.6	3.5±0.5	1.6±0.4
CHO-H	77.0±0.7	14.6±1.2	3.4±0.5	1.8±0.3
90 days				
CHO-F	77.2±0.3	15.9±0.7	3.6±0.7	1.4±0.1
CHO-M	78.9±1.9	16.6±1.8	3.4±0.8	1.4±0.5
CHO-H	77.6±1.4	15.7±0.7	3.9±1.3	1.5±0.3

Table 3.6 Blood chemistry of Nile tilapia fed experimental diets (mean±SD, *n*=5).

	Glucose (mM)	**Triglyceride (mM)**	**Cholesterol (mM)**	**Albumin (g/L)**	**Total protein (g/L)**	**BUN[1] (mM)**
45 days						
CHO-F	2.61±0.12^{c}	1.55±0.04^{c}	2.90±0.15^{c}	8.42±0.92	36.84±2.63^{a}	0.90±0.079^{a}
CHO-M	3.18±0.20^{b}	1.72±0.08^{b}	3.09±0.16^{b}	7.78±0.35	34.65±2.33^{ab}	0.76±0.05^{b}
CHO-H	3.86±0.25^{a}	1.94±0.05^{a}	3.49±0.18^{a}	7.54±0.99	32.61±2.24^{c}	0.66±0.07^{b}
90 days						
CHO-F	2.77±0.06^{c}	1.64±0.06^{c}	2.05±0.13^{c}	11.99±0.69^{a}	37.42±3.32	0.80±0.02^{a}
CHO-M	3.83±0.11^{b}	1.75±0.05^{b}	2.33±0.07^{b}	10.93±0.85^{ab}	35.75±2.61	0.75±0.04^{b}
CHO-H	5.16±0.08^{a}	1.95±0.07^{a}	2.83±0.14^{a}	9.96±0.95^{b}	33.10±2.29	0.61±0.03^{c}

BUN[1] :Blood Urea Nitrogen.

Means with different superscripts in each column differ significantly from each other (P<0.05).

Table 3.7 Molecular effects on hepatic metabolism of diets with no (0%; CHO-F), intermediary (30%; CHO-M) and high levels (50%; CHO-H) of carbohydrates in Nile tilapia. Genes involved in glycolysis (Gck Pfk, Pk), gluconeogenesis (G6pca1 and G6pca2, Pck1 and Pck2), lipogenesis (Fasn and G6pd) and amino acid catabolism (Alat, Asat, Gdh), were analyzed. Relative abundance of mRNA were performed on total RNA extracted from liver, sampled 5 h after the last meal, following 45 days or 90 days of rearing. Data represent means (A.U.: aribitrary units) ± SD (n =5). Statistical differences in mRNA levels (normalised by the reference Ef1α gene) between means were evaluated by one-way anova statistical test (R-software): different letters mean that the target gene is differentially expressed between the 3 different dietary groups ($P<0.05$). n.d: not detectable.

	gck	***pfklr***	***pklr***	***G6pca1***	***G6pca2***	***Pck1***	***Pck2***	***fasn***	***G6pd***	***alat***	***asat***	***gdh***
45 days												
CHO-F	n.d.^{b}	1.4±0.7	1.1±0.3	1.1±0.8	1.2±0.6	0.3±0.3	3.4±3.4	1.5±1.2	0.8±0.6	2.0±0.2^{a}	1.4±1.1	1.3±0.2^{a}
CHO-M	0.5±0.8^{b}	1.0±0.4	1.1±1.1	1.0±0.5	1.5±1.2	1.1±2.0	1.1±0.6	0.7±0.7	0.7±0.6	1.7±0.5^{a,b}	1.1±1.2	1.4±0.1^{a}
CHO-H	2.2±0.9^{a}	1.4±0.4	0.6±0.2	0.6±0.4	0.4±0.3	0.2±0.2	0.9±0.7	0.8±0.8	0.8±0.4	1.1±0.4^{b}	0.8±0.6	0.9±0.1^{b}
90 days												
CHO-F	2.4±1.2	1.7±0.6	1.0±0.5	1.4±0.5	1.3±0.2	3.1±3.4	1.2±0.5	1.7±1.6	0.7±0.7	1.2±0.4	2.0±0.8^{a}	1.2±0.4
CHO-M	3.1±1.9	2.5±1.3	1.7±0.5	1.1±0.6	1.6±0.7	1.4±1.4	2.1±1.9	2.8±1.1	2.0±0.7	0.8±0.2	1.4±0.3^{a}	1.2±0.2
CHO-H	2.5±0.4	1.6±0.7	1.0±0.6	1.2±0.6	1.2±0.5	0.5±0.5	1.0±0.4	1.3±0.7	0.8±0.2	1.1±0.1	0.5±0.4^{b}	1.1±0.4

Table 3.8 Molecular effects on muscle metabolism of diets with no (0%; CHO-F), intermediary (30%; CHO-M) and high levels (50%; CHO-H) of carbohydrates in Nile tilapia. Genes involved in glucose transport (*Glut4*) and glycolysis (*Hk2, Pfk, Pk*) were analyzed. Relative abundance of mRNA were performed on total RNA extracted from muscle, sampled 5 h after the last meal, following 45 days or 90 days of rearing. Data represent means (A.U.: aribitrary units)±SD (n=5). Statistical differences in mRNA levels (normalised by the reference Ef1α gene) between means were evaluated by one-way anova statistical test (R-software): different letters mean that the target gene is differentially expressed between the 3 different dietary groups (P<0.05).

	glut4	***hk2***	***pfkma***	***pkma***
45 days				
CHO-F	0.3±0.1[b]	1.1±0.2	0.6±0.2	0.8±0.2
CHO-M	0.6±0.3[a,b]	0.7±0.3	1.1±0.5	0.8±0.3
CHO-H	1.1±0.6[a]	1.1±0.3	1.2±0.4	0.9±0.3
90 days				
CHO-F	0.8±0.4	1.1±0.3	0.8±0.5	0.9±0.3
CHO-M	1.0±0.3	1.0±0.3	0.9±0.3	1.0±0.4
CHO-H	1.0±0.6	1.2±0.3	0.9±0.5	1.1±0.3

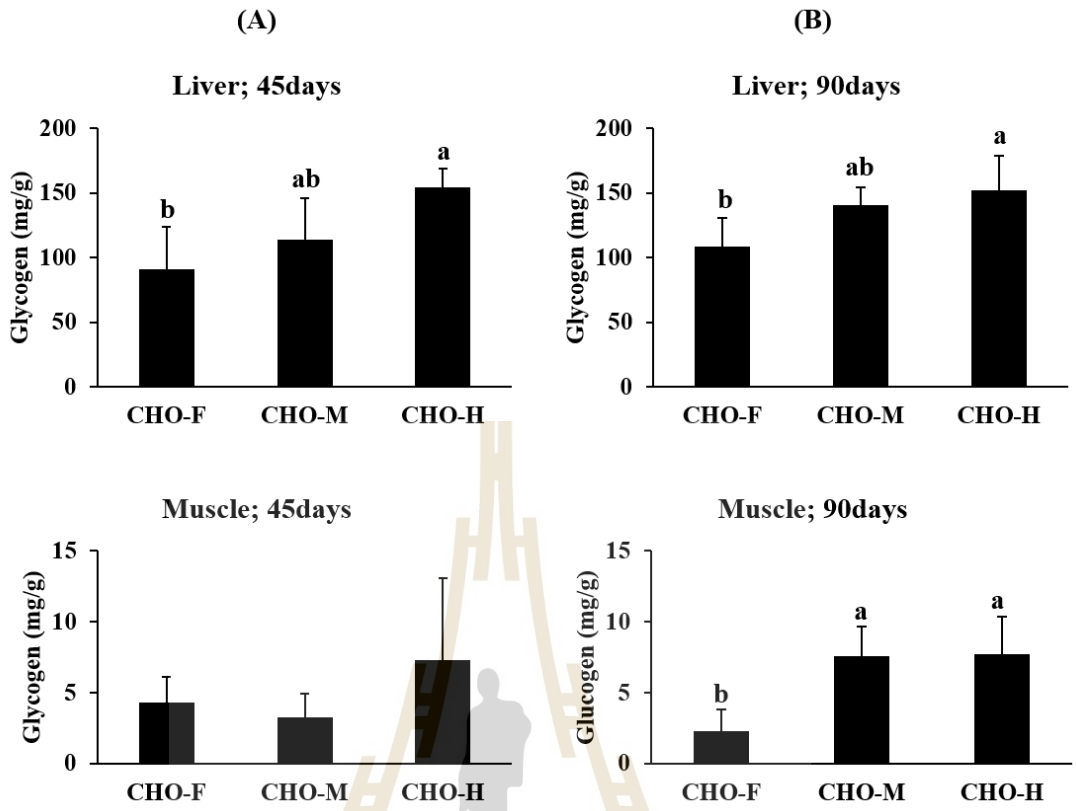

Fig. 3. 1 Glycogen levels after intake of diets with no (%0; CHO-F), intermediary (%30; CHO-M) and high levels (%50; CHO-H) of carbohydrates in liver and muscle of Nile tilapia. Glycogen was performed on muscle and liver, sampled 5 h after the last meal, following45 days (A) and 90 days (B) of rearing. Data represent means±SD (n=5 individual per dietary groups). Statistical differences in glycogen between samples were evaluated in group means by one-way Anova statistical test (R-software): different letters mean that the target gene is differentially expressed between the different dietary groups ($P<0.05$)

3.5 Discussion

3.5.1 Growth performance was decreased in tilapia fed either high or low levels of dietary protein/carbohydrate ratios

This study showed that fish fed the CHO-M diet (456.4 g kg^{-1} crude protein and 323.6 g kg^{-1} NFE) had highest growth response whereas the lowest growth performance was found in fish fed the CHO-H diet (271.8 g kg^{-1} crude protein and 563.2 g kg^{-1} NFE). Differences in growth responses among experimental groups would be mainly due to the protein contents in experimental diets. The protein requirement for optimum growth of tilapia during grow-out stage ranges from 250 up to 350 g kg^{-1} (Bhujel, 2001). However, for commercial tilapia production, cost-effective diets containing 250-300 g kg^{-1} crude proteins have been commonly used. Although the protein content in CHO-H was within the range of protein requirement for Nile tilapia, our finding indicated inadequate protein content in the CHO-H for maximal growth of Nile tilapia during grow-out stage. High FCR due to high feed intake was observed in fish fed the CHO-H diet. Our observation suggests also that fish fed CHO-H diet eat more in order to gain adequate amino acids levels for a better growth performance. Indeed, juvenile Nile tilapia achieved similar growth performance when feeding diets containing starch (163.5-322.0 g kg^{-1}) to protein (344.0-258.0 g kg^{-1}) ratios while the reduction of growth performance was observed when fish a diet containing 401.3 g kg^{-1} starch and 214.2 g kg^{-1} protein (Azaza et al., 2015).

Moreover, our results showed also that fish fed the CHO-F diet (630.7 g kg^{-1} crude protein and 148.5 g kg^{-1} NFE) had significantly lower growth response compared to that of CHO-M diet, indicating that excess protein content could also

affect growth performance. Indeed, growth of juvenile tilapia fed lower dietary protein/energy ratio was better than for those fed higher dietary protein/energy ratio (Wang et al., 2005; Li et al., 2013). Excess of dietary protein resulting in the reduction of growth performance was demonstrated in several fish species such as black sea bream (Sparus macrocephalus) and Nibea diacanthus (Zhang et al., 2010; Li et al., 2016a, 2016b). The excess of dietary protein would result in the loss of energy for deamination of amino acids toward protein catabolism.

3.5.2 Whole body composition was not affected between tilapia fed with different levels of dietary protein/carbohydrate ratios

Our results showed that the proximate chemical composition of whole body and muscle of Nile tilapia appeared to be similar among the experimental groups at 45 and 90 days of feeding, demonstrating that varying dietary carbohydrate and protein contents (with the similar energy content) did not significantly modulate the body nutrients contents in Nile tilapia. These findings indicated that there might be anabolism and catabolism processes to stabilize the proximate composition in whole body and muscle in Nile tilapia during grow-out stage. By contrast, previous study showed that significant effects of dietary carbohydrate and protein level on whole body composition were observed in juvenile tilapia and hybrid tilapia (Wang et al., 2005). We cannot exclude that the source of carbohydrates (dextrin versus starch) can be one of the reason to explain the differences between studies.

3.5.3 Plasma metabolites reflect the efficient use of dietary carbohydrates in tilapia

The present study investigated the effect of different dietary carbohydrate levels on blood intermediary metabolites which were sampling during

the peak of glycemic response after 45 and 90 days of feeding. Irrespective to the duration of the feeding period, the results showed that an increase in dietary carbohydrate level led to elevation in plasma glucose but without going up to hyperglycemia (no more than 5 mM), confirming that Nile tilapia efficiently utilized high levels of dextrin as carbohydrate source (Lin et al., 1995; Lee et al., 2003) in contrast to rainbow trout (Polakof et al., 2012). When fish ingested CHO-H, higher plasma cholesterol and triglyceride were also observed, as already found in other fish species fed with carbohydrates (Castro et al., 2015; Kamalam et al., 2012, 2013). High BUN was also found in fish fed CHO-F which was probably generated by catabolism of excess of dietary amino acids. These metabolic deaminations toward protein catabolism could be in part the cause of the growth retardation in fish fed the CHO-F diet.

3.5.4 Liver and muscle metabolic responses to increased dietary carbohydrates

Highest HSI and levels of hepatic and muscle glycogen in fish fed the CHO-H diet showed unambiguously that liver and muscle responds well at a metabolic level to dietary carbohydrates, similar to previous reports in juvenile tilapia and hybrid tilapia (Wang et al., 2005; Azaza et al., 2015) and other fish species including eel, trout, striped bass and European sea bass (Suárez et al., 2002; Rawles et al., 2008; Moreira et al., 2008; Enes et al., 2010). We could expect molecular regulations for many of the actors involved in glucose utilization in Tilapia as previously observed at least at a biochemical and enzymatic level (Gaye-Siessegger et al., 2006; Chen et al., 2013, 2017; Figueiredo-Silva et al., 2013). However, there was no regulation at mRNA levels for any of the glucose metabolic actors (glycolysis, gluconeogenesis) whereas hepatic amino acid catabolism mRNA levels were clearly

dependent of the quantity of dietary proteins. It was not expected because increased lipogenic and decreased gluconeogenic enzyme activity were observed in tilapia fed increased carbohydrates (Figueiredo-Silva et al., 2013): however, even though interesting, this comparison is limited due to some differences between the two studies (i) the sources of carbohydrates (starch vs dextrin), (ii) the content of dietary lipids (not isolipidic vs isolipidic diets); and (iii) the type of analysis (enyzmatic vs molecular). In muscle there was also no (up) regulation of mRNA levels for genes encoding proteins involved in glycolysis. We can conclude that the liver and muscle did not react in response to dietary carbohydrates at a mRNA levels even though the first steps of glucose utilization mediated by the hepatic glucokinase enzyme (gck gene) and by the muscle Glut4 glucose transporter (glut4 gene) were clearly higher in fish fed the CHO-H diet, suggesting an adaptation of these key metabolic reactions but only after 45 days of feeding. Finally, the absence of such regulations in fish fed 90 days could suggest that the adaptation in the long term of the tilapia to carbohydrates does not need persistent molecular adaptation of glucose use in liver and muscle.

3.6 Conclusion

The objective of the study was to understand the reasons for a higher metabolic use of carbohydrates in omnivorous fish than in carnivorous fish. However, in tilapia fed high carbohydrate diet (even with 50% of carbohydrates), no down regulation of hepatic gluconeogenesis, no induction of hepatic lipogenesis and of muscle glycolysis have been detected at a molecular level to explain the absence of hyperglycemia in tilapia. We must note here that diets for tilapia are low in lipids (5%) in comparison with rainbow trout aquafeeds (15-20%); this could be one of the main reasons

knowing that high levels of lipids induced glucose intolerance in rainbow trout and that Senegalese sole fed also with a low lipid diets had a better glucose tolerance. Finally, we cannot exclude also that (i) higher glycogen storing capacities in tilapia fed CHO-H diet in liver and muscle was sufficient for this fish species to control correctly glucose homeostasis or/and (ii) there is high potential to use glucose as an energy source in different tissues (such as brain or heart) in tilapia (not tested in this study).

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CHAPTER IV

NUTRITIONAL PROGRAMMING AT EMBRYONIC STAGES OF FISH GLUCOSE INJECTION INTO YOLK POSITIVELY MODULATES INTERMEDIARY METABOLISM AND GROWTH PERFORMANCE IN JUVENILE NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

4.1 Abstract

The aim of this study was to explore for the first time in omnivorous fish the concept of nutritional programming. A nutritional stimulus was accomplished by microinjecting 2 M glucose into yolk reserves during the alevin stage in Nile tilapia (*Oreochromis niloticus*). At the molecular level in fry, at 1 week post-injection, glucose stimuli were associated with the up-regulation of genes involved in glycolysis (*pklr, hk1, hk2,* and *pkma*), glucose transport (*glut4*) pathways and down-regulation of genes related to gluconeogenesis (*g6pca1, g6pca2,* and *pck1*) and amino acid catabolism (*asat, alat*) ($P<0.05$), demonstrating that the larvae well received the glucose stimulus at a molecular level. Moreover, 20 weeks after glucose injection, early glucose stimuli were always linked to permanent effects in juvenile fish, as reflected by a higher level of glycolytic enzymes [*gck, hk1* and *hk2* at both mRNA and enzymatic levels and pyruvate kinase

(PK) activity]. Finally, the effects of the glucose stimulus history were also examined in fish fed with two different dietary carbohydrate/protein levels (medium-carbohydrate diet, CHO-M; high-carbohydrate diet, CHO-H) in juvenile fish (during weeks 20-24). As expected, the CHO-H diet induced the expression of glycolytic and lipogenic genes (*gck, pklr,hk1, hk2, fpkma, fasn,* and *g6pd*) and suppressed the expression of gluconeogenic and amino acid catabolism genes (*g6pca1, pck1, pck2, asat, alat,* and *gdh*). Nevertheless, the early glucose stimulus led to persistent up-regulation of glycolytic enzymes (*gck, pklr, hk1,* and *hk2*) at both the mRNA and enzyme activity levels and glucose transporter *glut4* as well as lower gluconeogenic *pck1* gene expression ($P<0.05$). More interestingly, the early glucose stimulus was associated with a better growth performance of juvenile fish irrespective of the diets. These permanent changes were associated with DNA hypomethylation in the liver and muscles, suggesting the existence of epigenetic mechanisms at the origin of programming. In conclusion, for the first time in tilapia, early glucose stimuli were found to be clearly associated with a positive metabolic programming effect later in life, improving the growth performance of the fish.

Key words: Nile tilapia; Nutritional programming; glucose injection; gene expression; glucose metabolism

4.2 Introduction

Fish nutrition is one of the most important perspectives for developing sustainable fish farming, which has become an important food-producing sector for global food security. In order to improve fish nutrition, it is scientifically challenging to not only search for potential alternative feed ingredients and feed supplementation but also perform research into understanding fish metabolism and how it can be modulated.

Recently, several hypothesis-driven scientific approaches for fish nutrition have been demonstrated (Panserat et al., 2019). Nutritional programming is an issue that needs to be explored to examine how it modulates metabolism in fish (Geurden et al., 2007; Rocha et al., 2014, 2015; Fang et al., 2014). Metabolic programming is defined as the long-term consequences of environmental events or stimuli during early development that exert permanent effects on metabolism and physiology later in life (Lucas, 1998; Symond et al., 2009). Persistence of long-term effects of environmental stimuli was proposed to be linked with epigenetics, which might be transmissible from one cell generation to another (Panserat et al., 2017; Gravery and Robert, 2017; Veron et al., 2018). The concept of nutritional programming might offer a potential application in fish nutrition for the goal of modifying fish metabolism for efficient use of alternative feed, such as plant-based ingredients, high-carbohydrate diet and low-fish-oil/fishmeal-containing diet (Geurden et al., 2013; Rocha et al., 2014, 2015; Lozzarotto et al., 2016; Clarkson et al., 2017). In order to test whether nutritional programming exists, several factors should be explored, including the types of the early environmental/nutritional stimuli, testing of different developmental windows for applying the stimulus and the types of challenges during adult stage (Geurden et al., 2007, 2014; Mennigen et al., 2013; Rocha et al., 2014, 2015, 2016a,b; Fang et al., 2014; Gong et al., 2015; Marandel et al., 2016a,b).

In aquaculture, digestible dietary carbohydrates are among the less expensive sources of energy, and they are generally incorporated in fish diets as much as possible to produce low-cost aquafeed. As a better understanding of nutritional regulation of carbohydrate metabolism in fish would enable the efficient use of carbohydrates as an energy source to spare proteins for growth, nutritional regulation of glucose metabolism

has been intensively studied in various fish species in the last decades (Panserat et al., 2000, 2001, 2009; Polakof et al., 2012; Seiliez et al., 2017; Marandel et al., 2015; Kamalam et al., 2017; Boonanuntanasarn et al., 2018a,b). In order to improve the metabolic use of carbohydrates in carnivorous fish, generally known to be poor users of this nutrient, the concept of nutritional programming for carbohydrate metabolism has recently been demonstrated in rainbow trouts (Mennigen et al., 2013; Geurden et al., 2007, 2014; Marandel et al., 2016a,b) sturgeon (Gong et al., 2015) and gilthead seabream (Rocha et al., 2016a,b). However, data on nutritional programming in omnivorous and herbivorous fish, known to be good carbohydrate users, are limited to model fish species such as zebrafish (Ribas and Piferrer, 2014). Therefore, testing the nutritional programming of glucose metabolism in omnivorous and herbivorous fish would provide, for the first time, scientific evidence for comparative studies among fish species.

Nile tilapia (*Oreochromis niloticus*) is an omnivorous fish species that can use high levels of dietary carbohydrates (Shiau and Peng, 1993; Wang et al., 2005; Kamalam et al., 2017). Our previous findings unambiguously demonstrated adapted molecular responses of glucose metabolism to carbohydrate intake even during a long-term feeding trial (Boonanuntanasarn et al., 2018a,b). Indeed, high-dietary-carbohydrate intake led to increased hepatic glycogen and muscle glycolysis (higher PK enzyme expression) and was associated with reduced gluconeogenic capacities (lower expression of genes for glucose-6-phosphatase and phosphoenolpyruvate carboxykinase enzymes). Hence, investigating the potential effects of nutritional programming on glucose metabolism in Nile tilapia would expand our knowledge of carbohydrate nutrition in Nile tilapia, which is the second most cultured fish after the common carp.

In the present study, nutritional programming of glucose metabolism in tilapia was achieved using an early glucose stimulus in the alevin stage. Glucose was micro-injected into sac-fry larvae, and the effects of the glucose stimuli on the growth performance in alevins and juvenile fish were determined. In addition, molecular and enzymatic analyses of hepatic and muscle enzymes of glucose metabolism, plasma metabolites and tissue compositions were performed in juvenile fish before and after a final dietary challenge with a high-carbohydrate diet (67%) compared to a medium-rich-carbohydrate diet (37%). Finally, because one of the main possible mechanisms underlying programming effects is epigenetics (Marandel et al., 2016a; Panserat et al., 2017), we also analysed the global DNA methylation in the liver and muscles of fish depending on the glucose (injection) history and high-carbohydrate challenging diets.

4.3 Materials and methods

4.3.1 Fish for the Experiment, Experimental Design and Diet Formulation

All experimental protocols were approved by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval no. A-18/2562). The Nile tilapia alevins used in this study were obtained from brood stock obtained from a farm at Suranaree University of Technology, Nakhon Ratchasima, Thailand. Nile tilapia brood stock (0.8-1.2 kg) were cultured in an earthen pond (800 m^2) and fed with a commercial feed (30% crude protein (CP)+4% crude fat (CF) at 3% body weight) at 9:00 and 16:30 daily. A schematic view of the experimental design is shown in Figure 4.1. In order to investigate the effects of glucose micro-injection on the survival rate and glucose content, the experimental design was completely randomised with three treatments non-injected control, injection of a solution of 0.85% NaCl (saline)

and injection of a solution of 2 M glucose- on six replicates (family). To investigate the effects of glucose stimuli on carbohydrate metabolism in the long term, two groups of fish (six replicates) from either the saline (0.85%) or the 2 M glucose injection group were reared for 20 weeks. For the dietary challenge, a 2×2 factorial design with the two stimuli (0.85% NaCl and 2 M glucose) combined with two dietary carbohydrate levels (37% CHO-M and 67% CHO-H) was employed in a completely randomised design using six replicates (cages). Table 4.1 presents the ingredients of the two diets and the proximate composition of the two experimental diets (i.e. the medium-carbohydrate diet [CHO-M] and the high-carbohydrate diet [CHO-H]). The diets were analysed for moisture, CP, CF and ash content according to the standard method of the Association of Official Analytical Chemists (AOAC) (1990).

Table 4.1 Ingredients and chemical compositions (g kg^{-1}) of the challenging diets.

Ingredients	**CHO-M**	**CHO-H**
Fish meal	350	140
Soybean meal	300	60
Rice flour	150	700
Rice bran	180	30
Soybean oil	-	40
Dicalcium phosphate	-	10
Fish premix[a]	20	20
Proximate composition (***g kg^{-1} dry weight***)		
Dry matter	957.7	957.3
Protein	356.7	154.9
Fat	69.0	64.8
Fibre	28.9	8.6
Ash	129.2	60.3
NFE[b]	373.9	668.8
Gross energy (kJ g^{-1})	17.6	17.2

[a]Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1} diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5,000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12, 0.00005 mg; vitamin C, 1 g; vitamin D3, 1,000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g.

[b]Nitrogen-free extract = dry matter-(CP+crude lipid+crude fibre+ash).

Table 4.2 mRNA levels of genes involved in carbohydrate metabolism in the whole body of Nile tilapia (at 1 wpi) that were micro-injected with either 0.85% NaCl or 2 M glucose (mean±SD, $n = 6$).

Gene	0.85% NaCl	2 M glucose	*P*-value
Glycolysis			
gck	0.8±0.3	1.5±0.8	0.067
pfklr	1.1±0.2	0.9±0.3	0.156
pklr	0.7±0.2	1.0±0.3	0.030
Gluconeogenesis			
g6pca1	1.3±0.1	0.7±0.1	<0.001
g6pca2	1.3±0.1	0.5±0.1	<0.001
pck1	1.3±0.1	0.6±0.2	<0.001
pck2	0.8±0.4	1.1±0.4	0.317
Lipogenesis			
fasn	1.1±0.0	1.0±0.1	0.081
g6pd	0.9±0.1	0.9±0.1	0.207
Amino acid catabolism			
asat	1.2±0.1	0.7±0.1	<0.001
alat	1.0±0.1	0.9±0.0	0.001
gdh	1.3±0.6	0.8±0.4	0.146
Glucose transport and muscle metabolism			
glut4	0.7±0.2	1.0±0.3	0.022
hk1	1.0±0.1	1.1±0.1	0.005
hk2	0.9±0.0	1.1±0.1	0.011
pfkma	1.0±0.1	0.9±0.1	0.052
pfkmb	0.9±0.2	0.9±0.3	0.877
pkma	1.0±0.1	1.1±0.1	0.013

4.3.2 Alevin Collection and Microinjection Protocol and Fish Culture

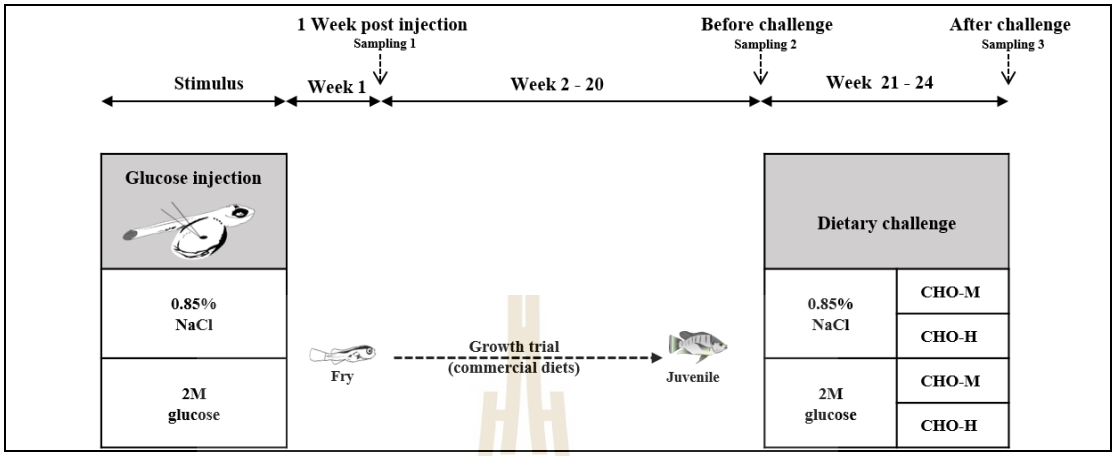

Figure 4.1 Experimental plan of glucose stimulus by injection (history) and dietary carbohydrate challenge test. Nile tilapia alevins were injected with either a saline (0.85% NaCl) or a glucose (2 M) solution into the yolk sac at the newly hatching stage. At 1 wpi, larvae were sampled to determine the body glucose and glycogen content and the expression of genes that are involved in carbohydrate metabolism. See Table 4.1 for the list of genes and their respective primers. Subsequently, the fish were cultured for a growth trial during 2-20 wpi. During the growth trial, commercial diets (during 2-8 wpi: 40% CP+8% CF; during 9-20 wpi: 32% CP+4% CF) were used. During 20-24 wpi, the fish were subjected to a challenge test with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Fish sampling was carried out before (20 wpi) and after (24 wpi) the challenge test.

A schematic view of the feeding plan is shown in Figure 4.1. Fertilised eggs at stages 11-12 (Fujimura and Okada, 2007) were collected from female Nile tilapia (six replications) and transferred to a hatching tray (20×30×5 cm^3) with flow-through freshwater at 27-29°C. After three days, the hatched larvae were selected for micro-injection. For normal-saline-injected and glucose-stimulated alevins (100 larvae/replication), 0.85% NaCl and 2M glucose (in 0.85% NaCl), respectively, were micro-injected into the yolk sac of the newly hatched larvae (stage 17) (Fujimura and Okada, 2007). A 0.5 mm diameter glass capillary (EG-400; Narishige, Tokyo, Japan) was used to make a needle to deliver 60 nL of normal saline and 2M glucose into the yolk sac of the newly hatched larvae (3-10 h after hatching). After injection, each group of micro-injected larvae were transferred to a hatching tray (20×30×5 cm^3) and reared with gentle aeration for one week. Non-injected control larvae were also reared to compare their survival rates at one week after injection (week post-injection, wpi). The glucose levels in the yolk reserves of all the experimental groups were determined. For that measure, the yolk sacs of 10 larvae were removed, pooled together, weighed and homogenised. The yolk homogenates were centrifuged at 3,000 rpm at 4°C for 5 min. The aqueous phase (the layer between the lipid and cell debris) was then transferred to a new tube. An equal volume of de-ionised water was mixed and used for glucose determination in accordance with Trinder's method (Trinder, 1969).

Experimental larvae (normal-saline-injected and glucose-stimulated alevins) were transferred to cages (40×40×60 cm^3) with aeration (six replicates). In this study, only male tilapia were used to avoid confounding effects linked to sex. During weeks 1-4, all experimental larvae were fed with a commercial powder feed (40% CP+8% CF) mixed with 17α-

methyltestosterone at 60 mg kg^{-1} five times daily (at 09:00 11:00 13:00 15:00 and 17:00) (Boonanantanasarn et al., 2018b). Subsequently, during weeks 5-20, the fish were transferred to cement ponds (2×2×0.8 m^3) and fed with a commercial feed (32% CP+4% CF) *ad libitum* twice daily (at 09:00 and 16:00). Fish mortality was monitored daily. In order to assess the growth performance, the fish were weighed and the feed intake was recorded every four weeks throughout the experimental period. Air and water temperatures were measured daily and were 18.8-27.6°C and 20.6-32.0°C, respectively. The dissolved oxygen (DO) content and pH were measured once a week using a DO metre and a pH meter, and the values were within acceptable ranges of 4.2-5.8 mg L^{-1} and 7.5-7.8, respectively.

During weeks 20-24, 10 fish from each replicate were randomly selected and subjected to a dietary challenge with two different levels of dietary carbohydrates (CHO-M and CHO-H; Table 1). The fish ($n = 10$/replication, six replications) were then transferred to culture in a cage (90×80×110 cm^3) for four weeks. Throughout the trial, the fish were fed twice daily (at 09:00 and 15:00). Fish mortality was monitored daily, and the growth performance was also recorded. Daily air and water temperatures were in range of 19.0-25.4°C and 16.4-23.0°C, respectively. The DO and pH were measured weekly, and their values were within acceptable ranges of 4.1-4.2 mg L^{-1} and 7.5-7.9, respectively.

4.3.3 Fish Sampling and Blood Collection

At week 1, pooled larvae (pool of three whole-body larvae/replicate tank) were randomly selected and immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction. At week 20, 5 h after feeding (i.e. the peak of glucose absorption in tilapia), five fish per tank were also sampled for plasma metabolites and chemical composition of the liver and muscles. Following anaesthesia, blood samples were collected from the caudal vein using a

hypodermic syringe and mixed with K_2EDTA (at 1.5 mg mL^{-1}). Plasma was collected after centrifugation at 3,000 ×g for 10 min at 4°C and stored at -80°C for metabolite analysis. Then, liver and muscle tissue samples were collected and frozen with liquid nitrogen and kept at -80°C for chemical composition analysis according to the AOAC. At week 24, fish sampling was performed as previously described for week 20.

4.3.4 Blood Metabolite Analysis

Plasma metabolites were analysed with 30 fish per experimental group (five fish/replication, $n = 30$ per experimental group). Plasma glucose was analysed according to the GOD-PAP method (Trinder, 1969). The triglyceride levels were determined using the glycerol-3-phosphate oxidase-sodium *N*-ethyl-*N*-(3-sulfopropyl)-*m*-anisidine method as described by Bucolo and David (1973). Blood urea nitrogen (BUN) content was measured using a modified indophenol colorimetric method (Weatherburn, 1967).

4.3.5 Total RNA Extraction and Relative Quantification of mRNAs

Relative gene expression was determined using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) of RNA extracted from whole-body larvae (week 1) (pool of three fish/replication; $n = 18$ per experimental group) and the liver and muscles (weeks 20 and 24) (two fish/replication; $n = 12$ per experimental group). Total RNA was extracted from tissue samples (week 1, pool of three larvae; weeks 20 and 24; 50 mg of liver and 100 mg of muscles) using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA was quantified by Nano Drop (Thermo Fisher, Madison, WI, USA), and the quality was verified on 1% agarose gel. A SuperScript III RNase H Reverse-Transcriptase Kit (Invitrogen) with random primers (Promega, Charbonnières, France) was used, following the manufacturer's protocol, to synthesise cDNA ($n = 12$ for each

treatment group). A sample of 1 μg of total RNA was used for cDNA synthesis, using 100 units of Super Script III enzyme and 40 units of RNase OUT enzyme. Reverse transcription was carried out in duplicate for each sample.

Supplementary Table 4.1 details the primer sequences used in the real-time RT-PCR assays (Boonanantanasarn et al., 2018a,b; Yang et al., 2013). Glucose metabolic gene expression in the liver was analysed, including glycolysis (glucokinase [GK], *gck*; phosphofructokinase, *pfklr*; PK, *pklr*) and gluconeogenesis (glucose-6-phosphatase, g*6pca1* and *g6pca2*; phosphoenolpyruvate carboxykinase cytosolic, *pck*1; mitochondria, *pck2*). Glucose use in muscles was analysed by measuring the mRNA levels of glucose transporter (*glut4*) and glycolysis (hexokinase [HK] I/II, *hk1* and *hk2*; phosphofructokinase, *pfkma* and *pfkmb*; PK, *pkma*). Lipogenic capacities (fatty acid synthase, *fasn*; glucose-6-phosphate dehydrogenase, *g6pd*) were examined. In addition, the enzymes involved in amino acid catabolism (glutamate dehydrogenase, *gdh*; alanine aminotransferase, *alat*; aspartate amino transferase, *asat*) were measured. A Roche Light Cycler 480 system was used (Roche Diagnostics, Neuilly-sur-Seine, France) for real-time RT-PCR assays of transcripts of metabolic genes. Assays were performed using a reaction mix of 6 μL per sample, each of which contained 2 μL of diluted cDNA template (1 : 40), 0.24 μL of each primer (10 μM), 3 μL of LightCycler 480 SYBR® Green I Master Mix (Roche Diagnostics) and 0.76 μL of DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany). The PCR protocol was initiated at 95°C for 10 min for the initial denaturation of the cDNA and hot-start Taq polymerase activation, followed by 45 cycles of a three-step amplification programme (15 s at 95°C, 10 s at 60-64°C [according to the primer set used] and 15 s at 72°C to extend the DNA).

Supplementary Table 4.1. List of tilapia primers used for qRT-PCR.

Genes	5′/3′ forward primer	5′/3′ reverse primer	Access number
Reference gene			
*ef1**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	AB075952
Liver metabolism			
gck	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC	XM003451020
pfklr	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC	XM003447353
pklr	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA	XM005472622
g6pca1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA	XM003448671
g6pca2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA	XM013273429
pck1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA	XM003448375
pck2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT	XM019354843
fasn	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT	XM013276809
g6pd	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT	XM013275693
asat	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC	XM003451918
alat	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG	XM005476466
gdh	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC	XM003457465
Muscle metabolism			
glut4	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG	JN900493
hk1	CGTCGCTTAGTCCCAGACTC	TGACTGTAGCGTCCTTGTGG	XM019360229
hk2	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC	XM003448615
pfkma	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG	XM019349871
pfkmb	TTTGTGCATGAGGGTTACCA	CACCTCCAATCACACACAGG	XM003441476
pkma	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA	XM005447626

*From Yang et al. (2013).

The melting curves were systematically monitored (temperature gradient at 1.1°C/s from 65 to 97°C, five acquisitions/1°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicates of RT and PCR amplification, respectively) and negative controls (reverse-transcriptase- and cDNA-template-free samples, respectively). For the analysis of the mRNA levels, relative

quantification of target gene expression was performed using the Roche Applied Science E-Method (Pfaffl, (2001) The relative gene expression of *ef*1α was used for the normalisation of the measured mRNA in each tissue, as its relative expression did not significantly change over the sampling process (data not shown). In all cases, PCR efficiency was measured from the slope of a standard curve using serial dilutions of cDNA. In all cases, the PCR efficiency values ranged between 1.8 and 2.0.

4.3.6 Chemical Composition and Glycogen Analysis

At weeks 20 and 24, chemical composition, including protein, fat and ash of the liver and muscles (five fish/replication, $n = 30$ per experimental group), was assessed according to the AOAC (1990). The liver (100 mg) and muscles (200 mg) were analysed for glycogen (two fish/replication, $n = 12$ per experimental group). The glycogen content was determined using a hydrolysis technique that was previously described by Good et al. (1933). Each sample was ground in 1 M HCl (VWR, USA). An aliquot was saved at this stage and neutralised by 5 M KOH (VWR) to measure the free glucose content in the samples. After 10 min of centrifugation at 10,000 ×g at 4°C, free glucose was measured using a plasma glucose kit (Glucose RTU; bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. The remaining ground tissue was boiled at 100°C for 2.5 h and then neutralised by 5 M KOH (VWR). After 10 min of centrifugation at 10,000 ×g at 4°C, total glucose (free glucose+glucose obtained from glycogen hydrolysis) was measured using the same kit as before. The glycogen content was evaluated by subtracting free glucose levels.

4.3.7 Enzymatic Assays

At weeks 20 and 24, muscles (200 mg) or livers (100 mg) were used to analyse enzyme activities. Tissue samples (from two fish/replication, total number of samples: 12) were

homogenised in seven volumes of ice-cold buffer at pH 7.4 (50 mmol L^{-1} Tris, 5 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA] and 2 mmol L^{-1} DTT) and a protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 10 min at 900 ×g at 4°C. Assays for GK (EC 2.7.1.2) and HK (EC 2.7.1.1) activity were performed on the recovered supernatants. For PK (EC 2.7.1.40) activity, additional centrifugation was performed (20 min at 10,000 ×g at 4°C), and the recovered supernatants were used for enzyme assays. The GK (high-K_M) and HK (low-K_M) enzymes were analysed as described by Panserat et al. (2000). The activity of the PK enzyme was also measured as previously described by Panserat et al. (2001). Each enzyme activity was measured in duplicate at 37°C following the variation of absorbance of nicotinamide adenine dinucleotide phosphate ($NADP^+$) at 340 nm. The reactions were started by the addition of a specific substrate; a Power Wave X (BioTek Instruments Winooski, VT, USA) plate reader was used. De-ionised water was used as a blank for each sample. Enzyme activity units, defined as micro-moles of substrate converted into product per minute at the assay temperature, were expressed per milligram of protein. Protein concentration was measured in duplicate, according to Bradford (1976), using a protein assay kit (Bio-Rad, Munich, Germany) with bovine serum albumin as a standard.

4.3.8 Analysis of Global DNA Methylation

For genomic DNA extraction, livers (50 mg) and muscles (100 mg) were digested in 1 mL of ice-cold extraction buffer (125 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulphate, 4 M urea and 10 mM Tris-HCl, pH 8.0) with 20 µg mL^{-1} proteinase K (P6556; Sigma-Aldrich), followed by incubation overnight at 37°C under agitation (250 rpm). Six replicates per condition were performed. After overnight digestion, an equal volume of phenol chloroform isoamyl alcohol (25:24:1) was added to each sample and mixed by inverting tubes. Samples

were then centrifuged for 15 min at 10,000 ×*g* at room temperature. Aqueous phases were then transferred into new tubes, and then 0.25 volume of 5 M NaCl was added, followed by two volumes of ice-cold absolute ethanol, and then incubated at -20°C for 15 min. After centrifugation at 10,000 ×*g* at 4°C for 15 min, DNA pellets were obtained, washed with 1 mL of 75% ice-cold ethanol and then re-centrifuged for 15 min at 10,000 ×*g* at 4°C. The pellets were then dried and re-suspended in 100 µL of DNase-free water. Samples were submitted to RNase treatment using 1 µg of RNase A (R4642; Sigma-Aldrich) and incubated for 1.30 h at 37°C under stirring (250 rpm). The quality of the genomic DNA was checked on 1% agarose gel, and quantification was carried out using NanoDrop (Thermo Fisher). The DNA global methylation pattern (C^mCGG methylation pattern) was analysed using the LUminometric Methylation Assay (LUMA) according to Karimi et al. (2006).

4.3.9 Data Analysis

All data were analysed using SPSS for Windows, version 10 (SPSS Inc., Chicago, IL, USA). In order to determine the effects of glucose micro-injection on the survival rate and glucose content, one-way analysis of variance (ANOVA) was performed. When significant differences were found among the groups, Tukey's method was used to rank the groups. In addition, an independent *t*-test analysis was conducted to evaluate the differences between the two groups at weeks 1 and 20: saline (0.85% NaCl) versus glucose (2 M glucose) stimuli. After the nutritional challenge (week 24), the statistical factors consisted of the analysis of the effects of the glucose stimulus history, dietary carbohydrate level and their interactions. Two-way factorial ANOVA was also carried out. When the interaction of the factors was statistically significant, one-way ANOVA following Tukey's range test was performed to rank

the treatment combination groups. Throughout the experiment, the effects and differences were declared to be significant when $P<0.05$.

4.4 Results

4.4.1 Direct Effects of Early Glucose Stimuli on Larval Survival and Expression of Glucose Metabolic Genes

Early glucose stimuli were performed using a micro-injection of either 2 M glucose or normal saline (0.85% NaCl) into the yolk sac of the alevins. Before glucose injection, the glucose levels in the yolk reserves of the control (non-injected fish) and normal-saline-injected yolks were 2.4-2.6 mg g^{-1} yolk. Glucose injection significantly increased the level of glucose in the yolk to 4.1-5.6 mg g^{-1} yolk (Figure 4.2A), indicating that injecting 2 M glucose is effective to overload the glucose content in yolk. Figure 4.2B demonstrated that injection 2 M glucose led to an increase in not only the glucose level, but also the glycogen content in fish larvae at 1 wpi.

The survival rates of the control, saline-injected and glucose-injected larvae at 1 wpi are shown in Figure 4.2C. Although micro-injection slightly reduced the survival rates of tilapia larvae, there were no significant differences in the survival rates between normal-saline-injected and glucose-injected larvae ($P<0.05$), indicating that this level of glucose stimulus does not cause detectable detrimental effects on fish. Note that no malformities were observed in any fish throughout the experimental period. As the yolk was completely absorbed one week after hatching, the effects of glucose stimuli on the expression of genes related to glucose metabolism were determined in larvae at 1 wpi. Our results showed significant up-regulation of genes involved in glycolysis in the liver and muscles, including hepatic *pklr* and muscular *hk1* and *hk2*

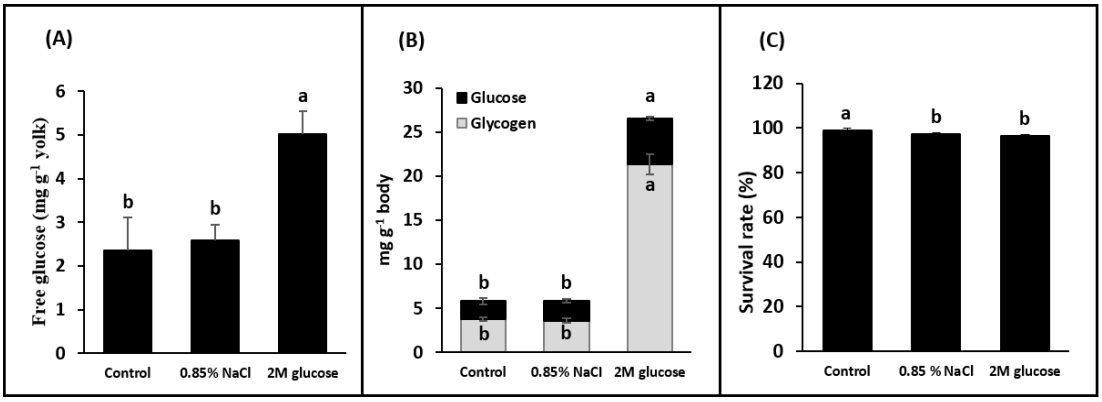

Figure 4.2 Glucose content in the yolk reserves of alevins and their subsequent bodily glucose and glycogen content. (A) Glucose levels in the yolk of alevins that were micro-injected with saline (0.85% NaCl) or glucose (2 M), as well as the control (non-injected alevin). Note that fish alevins were sampled for examination of the glucose level in the yolk sac immediately after injection. At 1 wpi, the glucose content and glycogen content were examined in the bodies of larvae injected with saline (0.85% NaCl) and glucose (2 M) and in the control (non-injected) larvae (B), and their survival rates were determined (C). Different letters in the bar graph indicate significant differences ($P<0.05$).

($P<0.05$) (Table 4.2). In addition, the *glut4* mRNA level was significantly increased in glucose-injected larvae. Moreover, down-regulation of gluconeogenesis and amino acid catabolism was observed for *g6pca1*, *g6pca2*, *pck1*, *asat* and *alat* genes ($P<0.05$). It can be noted that there were no significant differences in the transcripts of any detected genes related to lipogenesis ($P<0.05$). Overall, glucose stimuli showed direct significant effects on glucose metabolic pathways.

4.4.2 Long-term Effects of Early Glucose Stimuli on Growth Performance, Hepatic and Muscle Composition, Plasma Metabolites and Expression of Glucose Metabolic Genes in Juvenile Fish (20 weeks) Effect of temperature on dielectric properties

The growth performance of experimental fish until the juvenile stage is shown in Figure 4.3. Early glucose stimuli appeared to increase the growth performance of fish between weeks 12 and 16 ($P<0.05$). At week 20, by contrast, there was no more significant difference in growth between the glucose-injected fish and normal-saline-injected fish. Note that a low water temperature (19-23°C) occurred during the culture period of weeks 16-20, which was lower than the optimal temperature for growth of Nile tilapia (28-30°C) (Qiang et al., 2014). This low temperature resulted in lower feed intake and consequently reduced the growth response in all experimental groups.

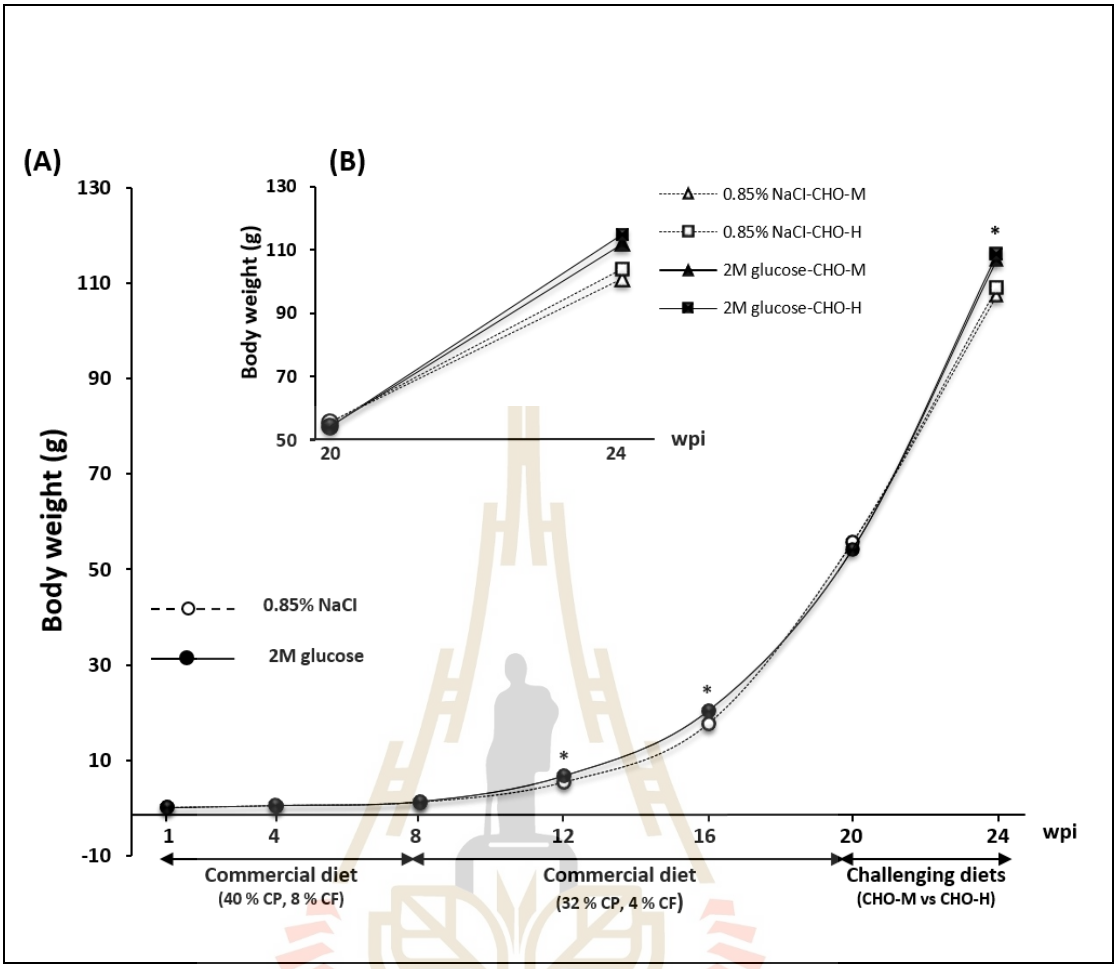

Figure 4.3 Growth response of fish injected with saline (0.85% NaCl) and glucose (2 M) during 1-24 wpi. Experimental fish were fed with commercial diets during 1-20 wpi. The asterisk indicates significant differences ($P<0.05$) between fish injected with saline (0.85% NaCl) and glucose (2 M) at 12 and 16 wpi. The fish were then subjected to a challenge test with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H) during weeks 21-24. Different growth responses were observed after the challenge test (24 wpi). (A) Whole experimental period. (B) Focus on the challenging period.

At week 20, early glucose stimuli led to an increase in the hepatic fat content in juvenile fish, while other chemical compositions, including protein, ash and glycogen, were not significantly different (Table 4.3). On the other hand, the hepatosomatic index (HSI) of both experimental fish appeared to be similar. While no significant differences between glucose-injected and normal-saline-injected fish were found in muscles for protein, fat and ash, early glucose injection led to increased glycogen content in the muscles ($P<0.05$) (Table 4.3). This study also evaluated the effects of early glucose stimuli on the levels of plasma metabolites, such as glucose, triglycerides and BUN (Table 4.3). The levels of these plasma metabolites were similar between the experimental groups.

The long-term effects of early glucose stimuli on the expression of genes related to glucose metabolism in muscles and livers were also shown in fish at 20 weeks (Table 4.4, Figs**.** 4.4). The results showed that early glucose stimuli led in the long term to higher expression of hepatic GK (both *gck* mRNA levels and enzyme activity) (Figure 4.4A) ($P<0.05$). Muscle HK1 and HK2 (both *hk1* and *hk2* mRNA levels and enzyme activity) (Figure 4.4E) and PK (enzyme activity) (Figure 4.4G) were also higher in fish with an early glucose stimulus history ($P<0.05$). However, we can observe that the expressions of other genes related in glucose metabolic pathways in the liver and muscles (*pfklr*, *pklr*, *g6pca1*, *g6pca2*, *pk1*, *pck2*, *fasn*, *g6pd*, *asat*, *alat*, *gdh*, *glut4*, *pfkma*, *pfkmb* and *pkma*) and PK (enzyme activity) (Figure 4.4C) were not significantly different ($P<0.05$).

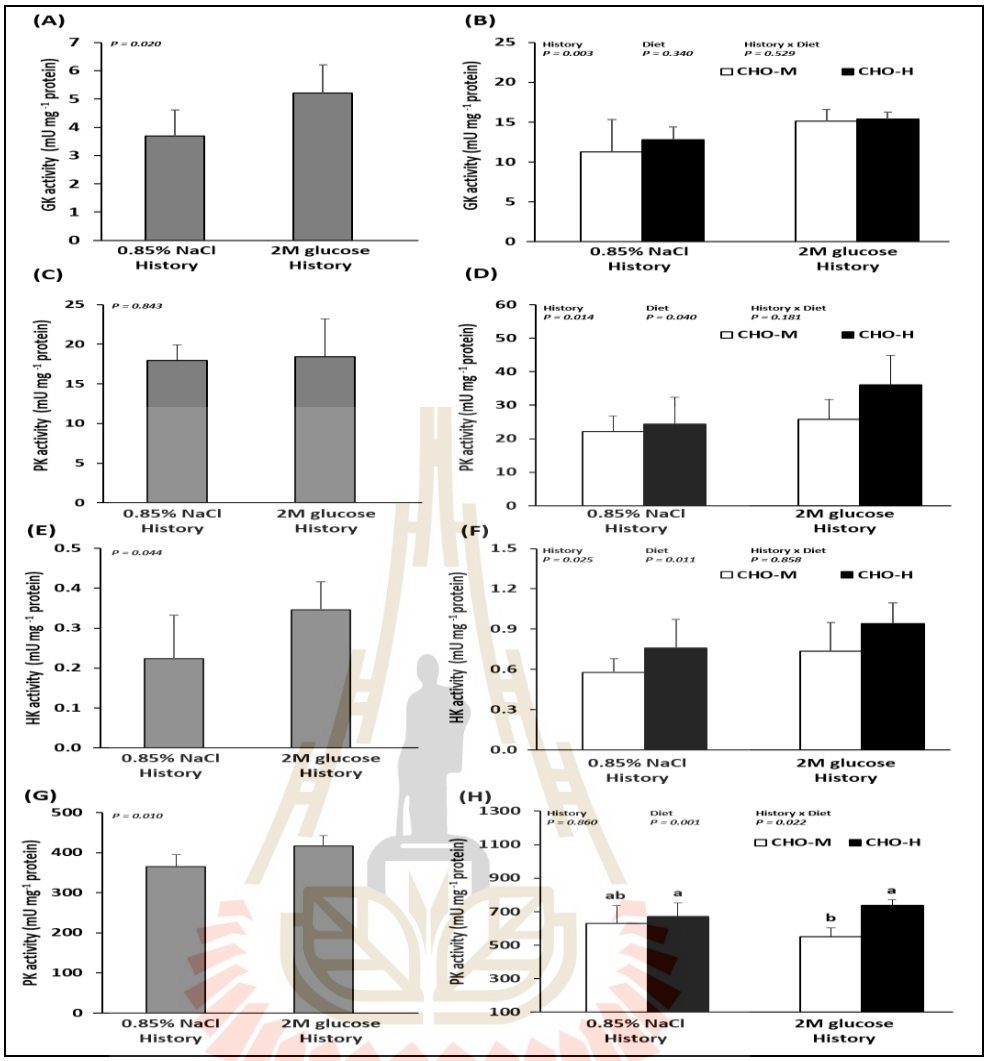

Figure 4.4 Enzyme activity (mU mg^{-1} protein) in the livers and muscles of Nile tilapia that were micro-injected with either 0.85% NaCl or 2 M glucose (history). GK (A, B) and PK (C, D), which are involved in glycolysis, were analysed in the liver samples. HK (E, F) and PK (G, H), which are involved in glycolysis, were analysed in the muscle tissue samples. At 21-24 wpi, the fish were subjected to a challenge test with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H).Before the challenge test, the fish were sampled to determine the effect of the glucose injection history on the GCK and PK activity in the liver (A, C) and the

HK and PK activity in the muscles (E, G).The asterisk in the bar graph indicates a significant difference (P<0.05). After the challenge test (24 wpi), the combination effects of glucose history and challenging diet on the GCK and PK activity in the liver (B, D) and the HK and PK activity in the muscles (F, H) were examined.Data are presented as the mean±standard deviation (SD) ($n = 6$). Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). When significant interaction effects were observed, one-way ANOVA following Tukey's range test was performed to rank the treatment combination groups. Different letters in the bar graph indicate significant differences (P<0.05).

4.4.3 Combination Long-term Effects of Early Glucose Stimuli and Four-week High-dietary-Carbohydrate Challenge in Fish at 24 Weeks

The effects of early glucose stimuli combined with a challenge with a high-carbohydrate diet were examined in normal-saline-injected and glucose-injected fish by feeding them CHO-M and CHO-H diets during weeks 21-24 (Figure 4.1). Table 4.4 shows that, irrespective of the diet, the glucose injection history led to a significant improvement in the growth performance in terms of the final body weight, average daily gain, specific growth rate and feed conversion ratio (FCR) (P<0.05). By contrast, no significant effects of the two diets on growth performance were observed (P<0.05).

The effects of glucose history as well as those of the two diets on the chemical composition in the liver and muscles are shown in Table 4.3. Early glucose injection stimuli together with dietary carbohydrates led to a decrease in the protein content in the liver (P<0.05)

Interaction effects were also observed, showing that the glucose-injected fish that were fed with CHO-H had the lowest hepatic protein level ($P<0.05$). Both early glucose stimuli and challenging diets had significant effects on the hepatic lipid and glycogen content ($P<0.05$). The glucose-injected fish that were fed with CHO-H had the highest liver fat and glycogen level ($P<0.05$). Dietary carbohydrates had an effect on the hepatosomatic index, and its interaction effect with glucose stimuli was found. Moreover, the glucose-injected fish fed with CHO-H had the lowest HSI ($P<0.05$). Regarding the muscle composition, both glucose history and dietary carbohydrates were observed to have a significant effect only for glycogen ($P<0.05$) (Table 4.3). Both glucose history and high dietary challenge resulted in an increase in the glycogen content in muscles ($P<0.05$). Again, the glucose-injected fish fed with CHO-H had the highest glycogen content in the muscles compared to other groups.

Glucose history had a significant effect on plasma glucose, triglycerides and BUN and was associated with an increase in plasma glucose and a decrease in BUN (Table 4.3) ($P<0.05$), but the challenging diets had no significant effect. Only the effects of diet on plasma triglycerides were observed (Table 4.3) ($P<0.05$).

Table 4.4 shows the effects of the glucose stimulus history and dietary challenge on the expression of glucose metabolic genes. The glucose stimulus history is associated with the up-regulation of *gck*, *pklr*, *hk1*, *hk2* and *glut4*; indeed, the expression of *gck*, *pklr*, *hk1* and *hk2* was higher at both mRNA and enzymatic levels ($P<0.05$) (Table 4.4, Figs**.** 4.4A, 4.4D and 4.4F). In addition, fish with a glucose history had lower *pck1* mRNA levels. Moreover, no significant differences were observed for *pfklr*, *g6paca1*, *g6pca2*, *pck2*, *fasn*, *g6pd*, *asat*, *alat*, *gdh*, *pfkma*, *pfkmb* and *pkma* ($P<0.05$). Fish fed with a high-carbohydrate diet exhibited higher expression of *gck* (mRNA level), *pk* (enzyme activity), *hk1* (mRNA level and enzyme activity)

and *hk2* (enzyme activity) ($P<0.05$) (Table 4.4, Figs. 4.4D, 4.4F and 4.4H). Furthermore, dietary CHO-H down-regulated all the examined genes in gluconeogenesis (except for *g6pca1*), lipogenesis and amino acid catabolism in the liver ($P<0.05$) (Table 4.4). It can be noticed that there was an interaction effect between the glucose stimuli and dietary carbohydrates for the *asat* and *pkma* genes, showing that glucose-injected fish fed with CHO-H had the lowest hepatic *asat* and highest muscle *pkma* gene expression (Table 4.4).

Because the mechanisms that can be at the origin of programming (hereby glucose history) could be mediated by epigenetic modifications, we investigated the effects of the glucose history (and also dietary carbohydrate diets) on global DNA methylation using a LUMA approach. Fish with a glucose stimulus history showed a lower methylation level at $C^{m}CGG$ in the liver and muscles compared to the control fish, whereas the dietary carbohydrate challenge had no effect on this parameter (Figure 4.5).

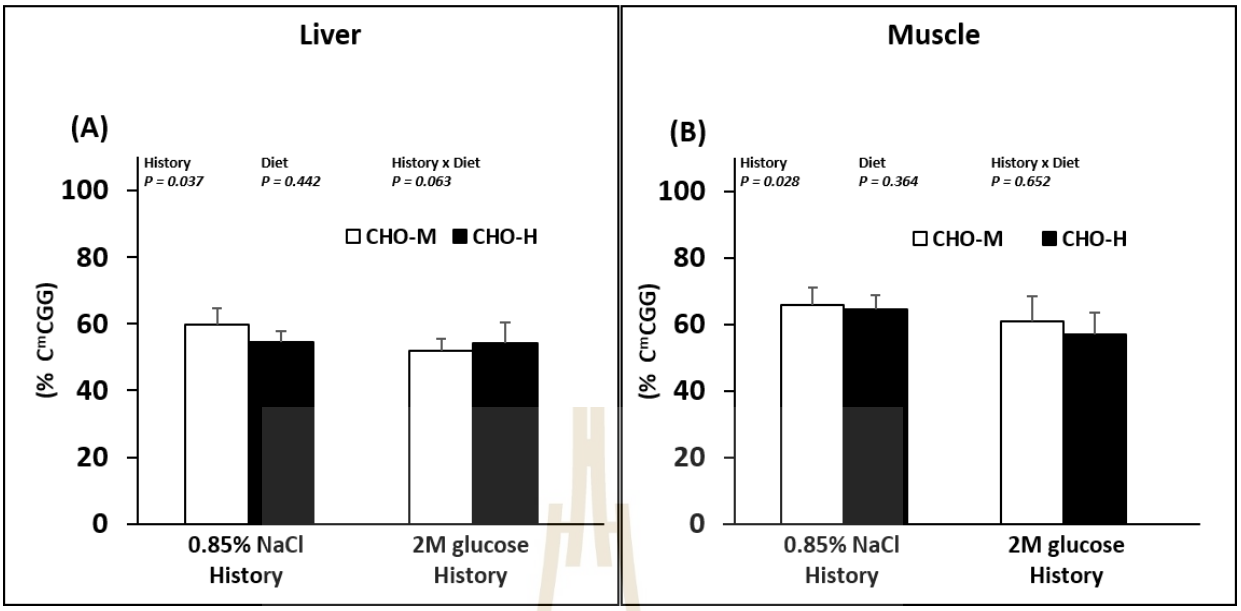

Figure 4.5 Effects of the glucose injection history and dietary carbohydrate challenge on the global DNA methylation in Nile tilapia. The levels of 5-methylcytosine (5-mC%: 5-mC/total DNA) in the DNA in the liver (A) and muscles (B) were measured in fish challenged with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Data are presented as the mean±SD ($n = 6$). Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History×Diet).

Table 4.3 Chemical composition in liver, muscles and carcass, glycogen content in the liver and muscles, HSI and plasma metabolites of fish injected with saline (0.85% NaCl) and glucose (2 M) before (20 wpi) and after (24 wpi) challenging with CHO-M and CHO-H for four weeks (mean ± SD, $n = 6$).

Composition ($g\ kg^{-1}$)	Before challenge			0.85% NaCl history		2 M glucose history		P-value[1]		
	0.85% NaCl history	2 M glucose history	P-value	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Liver at week 20				***Liver at week 24***						
Protein	102.5 ± 4.5	106.9 ± 5.0	0.134	106.5 ± 2.6^{a}	95.63 ± 3.5^{b}	99.5 ± 2.0^{b}	72.7 ± 1.7^{c}	<0.001	<0.001	<0.001
Fat	34.1 ± 3.7	39.4 ± 3.8	0.034	25.0 ± 1.0^{c}	27.0 ± 0.7^{b}	20.5 ± 0.9^{d}	28.9 ± 1.0^{a}	0.002	<0.001	<0.001
Ash	9.6 ± 1.7	10.8 ± 0.7	0.148	11.2 ± 0.3	10.8 ± 0.6	11.5 ± 0.8	10.6 ± 1.7	0.896	0.146	0.530
Glycogen ($mg\ g^{-1}$)	115.1 ± 9.7	108.8 ± 6.6	0.214	125.2 ± 24.5	207.8 ± 14.2	149.1 ± 34.3	236.4 ± 23.4	0.019	<0.001	0.818
HSI[2] (%)	1.7 ± 0.3	1.4 ± 0.3	0.086	3.0 ± 0.7^{ab}	3.1 ± 1.3^{ab}	4.2 ± 0.6^{a}	2.6 ± 0.4^{b}	0.299	0.038	0.022
Muscles at week 20				***Muscle at week 24***						
Protein	183.8 ± 2.0	184.4 ± 4.9	0.815	192.9 ± 7.4	192.4 ± 2.1	191.5 ± 1.8	189.2 ± 1.9	0.184	0.406	0.620
Fat	9.2 ± 0.6	9.1 ± 0.6	0.714	12.2 ± 0.8	12.3 ± 0.3	11.8 ± 0.9	12.4 ± 0.5	0.534	0.278	0.350
Ash	12.0 ± 0.2	11.9 ± 0.3	0.252	12.6 ± 0.3	12.7 ± 0.4	12.4 ± 0.1	12.5 ± 0.2	0.088	0.283	982.0

Table 4.3 (Continous).

Composition (g kg^{-1})	Before challenge			0.85% NaCl history		2 M glucose history		*P*-value[1]		
	0.85% NaCl history	2 M glucose history	*P*-value	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Glycogen (mg g^{-1})	4.0 ± 1.4	6.5 ± 1.4	0.010	3.7 ± 1.4	7.6 ± 2.6	5.8 ± 1.8	9.0 ± 1.9	0.048	<0.001	0.652
Carcass at week 20				***Carcass at week 20***						
Protein	164.7 ± 7.1	163.7 ± 6.3	0.805	178.1 ± 1.7^{a}	162.1 ± 2.4^{c}	178.6 ± 1.9^{a}	173.2 ± 1.9^{b}	<0.001	<0.001	<0.001
Fat	22.0 ± 1.4	21.2 ± 1.4	0.353	20.9 ± 2.0	22.0 ± 1.2	21.3 ± 1.2	20.5 ± 1.1	0.373	0.808	0.130
Ash	21.5 ± 0.9	21.2± 0.4	0.448	27.3 ± 2.8	27.8 ± 1.0	28.2 ± 2.0	27.5 ± 1.9	0.788	0.913	0.493
Plasma metabolites at week 20				***Plasma metabolites at week 24***						
Glucose (mM)	4.5 ± 0.5	4.6 ± 0.6	0.742	4.6 ± 0.4	4.7 ± 0.3	5.0 ± 0.6	5.5 ± 0.5	0.005	0.185	0.257
Triglycerides (mM)	1.5 ± 0.2	1.4 ± 0.4	0.451	2.3 ± 0.7	3.9 ± 0.3	3.0 ± 0.7	4.0 ± 0.6	0.086	<0.001	0.256
BUN[3] (mM)	1.03 ± 0.06	0.91 ± 0.20	0.206	0.94 ± 0.14	0.91 ± 0.08	0.85 ± 0.07	0.79 ± 0.10	0.020	0.302	0.828

[1]Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values for four combination groups (P<0.05)).

[2]Hepatosomatic index (HSI) = 100 × (liver weight (g)/body weight (g)).

Table 4.4. Growth performance of fish injected with saline (0.85% NaCl) and glucose (2 M) at 24 wpi after challenging with CHO-M and CHO-H for four weeks (mean ± SD, $n = 6$).

	0.85% NaCl history		2 M glucose history		*P*-value[a]		
	CHO-M	**CHO-H**	**CHO-M**	**CHO-H**	**History**	**Diet**	**Interaction**
Initial weight (g)	54.3 ± 0.7	54.7 ± 0.4	54.7 ± 0.6	55.1 ± 0.4	0.079	0.073	0.875
Final weight (g)	107.4 ± 2.0	108.9 ± 5.8	114.8 ± 5.5	116.0 ± 4.7	0.001	0.492	0.931
ADG[b] (g day^{-1})	1.8 ± 0.1	1.8 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	0.002	0.613	0.948
SGR[c] (% day^{-1})	2.3 ± 0.1	2.3 ± 0.2	2.5 ± 0.1	2.5 ± 0.1	0.002	0.787	0.976
FI[d] (g day^{-1})	2.4 ± 0.1	2.4 ± 0.1	2.5 ± 0.2	2.4 ± 0.0	0.655	0.459	0.459
FCR[e]	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.013	0.201	0.781
Survival rate (%)	98.3 ± 4.1	100.0 ± 0.0	96.7 ± 8.2	100.0 ± 0.0	0.660	0.195	0.660

[a]Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet).

[b]Average daily gain (ADG) = (final body weight-initial body weight)/experimental days.

[c]Specific growth rate (SGR) = 100 × [(ln final body weight-ln initial body weight)/experimental days].

[d]Feed intake (FI) = 100 x (dry feed intake/[(initial wet body mass C final wet body mass)/2])/days

[e]Feed conversation ratio (FCR) = dry feed fed/wet weight gain.

Table 4.5 mRNA levels of genes involved in carbohydrate metabolism in the liver and muscles of fish injected with saline (0.85% NaCl) and glucose (2 M) before (20 wpi) and after (24 wpi) challenging with CHO-M and CHO-H for four weeks (mean ± SD, $n = 6$).

Genes	Before challenge			0.85% NaCl history		2 M glucose history		*P*-value[1]		
	0.85% NaCl history	2 M glucose history	*P*-value	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Liver glycolysis at week 20				***Liver glycolysis at week 24***						
gck	0.4 ± 0.3	0.8 ± 0.2	0.027	0.5 ± 0.2	1.0 ± 0.3	1.3 ± 1.0	2.4 ± 0.8	0.001	0.010	0.329
pfklr	1.0 ± 1.0	0.9 ± 0.4	0.810	1.4 ± 0.9	1.8 ± 1.4	2.6 ± 2.2	2.0 ± 1.9	0.314	0.889	0.485
pklr	1.4 ± 1.4	0.8 ± 0.3	0.325	1.0 ± 0.2	1.1 ± 0.7	1.3 ± 0.5	1.6 ± 0.3	0.024	0.360	0.655
Liver gluconeogenesis at week 20						***Liver gluconeogenesis at week 24***				
g6pca1	1.3 ± 0.5	1.4 ± 0.8	0.626	1.3 ± 0.5	0.9 ± 0.4	1.1 ± 0.4	0.8 ± 0.1	0.313	0.059	0.715
g6pca2	0.9 ± 0.3	1.2 ± 0.4	0.103	1.6 ± 0.9	0.9 ± 0.4	1.4 ± 0.5	0.8 ± 0.5	0.856	0.003	0.477
pck1	0.6 ± 0.4	1.4 ± 1.3	0.230	1.5 ± 0.9	0.8 ± 0.5	0.8 ± 0.6	0.2 ± 0.1	0.016	0.010	0.779
pck2	0.7 ± 0.3	0.7 ± 0.6	0.938	2.9 ± 1.8	0.6 ± 0.6	1.7 ± 1.4	0.7 ± 0.4	0.244	0.003	0.209
Liver lipogenesis at week 20						***Liver lipogenesis at week 24***				
fasn	1.2 ± 1.0	1.0 ± 0.3	0.682	1.1 ± 0.8	1.3 ± 0.8	1.1 ± 0.8	2.3 ± 0.2	0.087	0.031	0.111
g6pd	0.9 ± 0.5	1.3 ± 0.7	0.328	0.9 ± 0.4	1.4 ± 0.7	0.9 ± 0.3	2.4 ± 1.2	0.077	0.007	0.211

Table 4.5 (Continous).

Genes	Before challenge			0.85% NaCl history		2 M glucose history		*P*-value[1]		
	0.85% NaCl history	2 M glucose history	*P*-value	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Liver amino acid catabolism at week 20				***Liver amino acid catabolism at week 24***						
asat	0.9 ± 0.5	1.3 ± 0.5	0.266	1.4 ± 0.5^{b}	0.4 ± 0.2^{c}	2.1 ± 0.5^{a}	0.2 ± 0.1^{c}	0.119	<0.001	0.010
alat	0.9 ± 0.4	1.1 ± 0.2	0.280	2.3 ± 1.6	1.1 ± 0.7	2.7 ± 1.3	1.0 ± 0.4	0.810	0.005	0.651
gdh	1.0 ± 0.5	1.2 ± 0.2	0.470	1.7 ± 0.5	1.2 ± 0.4	1.7 ± 0.7	1.3 ± 0.3	0.937	0.039	0.904
Glucose transport and muscle metabolism at week 20				***Glucose transport and muscle metabolism at week 24***						
glut4	0.8 ± 0.4	1.2 ± 0.3	0.071	0.6 ± 0.4	0.8 ± 0.1	1.1 ± 0.3	1.1 ± 0.4	0.005	0.327	0.631
hk1	0.8 ± 0.3	1.2 ± 0.3	0.047	0.7 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	0.019	0.018	0.656
hk2	0.7 ± 0.3	1.1 ± 0.3	0.030	0.8 ± 0.2	0.9 ± 0.4	1.1 ± 0.3	1.2 ± 0.1	0.009	0.225	0.752
pfkma	0.8 ± 0.4	1.2 ± 0.7	0.262	1.0 ± 1.0	0.7 ± 0.2	0.7 ± 0.3	0.9 ± 0.5	0.853	0.825	0.305
pfkmb	1.1 ± 0.2	0.8 ± 0.3	0.165	1.2 ± 0.6	0.9 ± 0.3	1.0 ± 0.3	1.1 ± 0.2	0.971	0.407	0.237
pkma	0.8 ± 0.3	1.0 ± 0.4	0.617	0.8 ± 0.1^{b}	0.8 ± 0.2^{b}	0.7 ± 0.1^{b}	1.1 ± 0.2^{a}	0.205	0.002	0.015

[1]Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values for four combination groups (P<0.05).

4.5 Discussion

Recently, it has been revealed that early nutritional programming exerts several modulating effects on metabolic processes in juvenile/adult fish, making this a promising approach for fish nutrition (Panserat et al., 2019). Particularly, the effects of nutritional programming on carbohydrate metabolism have been intensively studied in aquaculture fish with a low capacity to efficiently use carbohydrates (Geurden et al., 2007, 2014; Rocha et al., 2014, 2015, 2016a,b; Fang et al., 2014; Gong et al., 2015; Liang et al., 2017). Among vertebrates, fish exhibit various feeding habits. Therefore, in order to provide a general and comparative concept of nutritional programming in fish, a deeper investigation of the effects of nutritional programming in omnivorous fish, which are capable of using carbohydrates as a primary energy source, is required. Nile tilapia have been known to be a good user of dietary carbohydrates, and their ability to adapt to a high-carbohydrate dietary level has been well demonstrated (Shiau and Peng, 1993; Wang et al., 2005; Azaza et al., 2015; Gaye-Siessegger et al., 2006; Boonanuntanasarn et al., 2018a, b). During very early development, nutrient intake of fish is relied only on their yolk reserves, and ontogenic development of digestive tract and their relative organs are ongoing (Morrison et al., 2001). Indeed, the prenatal embryonal stage was demonstrated to be an early developmental stage for efficient nutritional programming in mammals (reviewed by Langley-Evans, 2009). This is why we chose the larval stage to inject glucose into the yolk reserves during the very early development stage. Our findings demonstrated for the first time the existence of nutritional programming in Nile tilapia, a model of omnivorous fish.

4.5.1 Direct Effects of Early Glucose Stimuli on the Survival and Glucose Metabolic Gene Expression of Alevins

Nutritional intervention at early developmental stages is an important factor for recording the programming stimuli that contribute to persistent effects during the later lifespan (Lucas, 1998). Up till now, most nutritional interventions to induce nutritional programming have been conducted during the first feeding stage in fry (Geurden et al., 2007, 2014; Rocha et al., 2016a, b; Fang et al., 2014; Gong et al., 2015; Liang et al., 2017). In the present study, we conducted an early nutritional stimulus through the micro-injection of glucose into the yolk sacs of Nile tilapia alevins at stage 17 (Fujimura and Okada, 2007). Our results showed that the direct survival and growth rates of fish injected with 2 M glucose or with the saline solution were similar, suggesting that injecting 2 M glucose did not cause any negative effects on the fish. In this study, an increase in the glucose level in the yolk revealed the successful of glucose receive in fish alevins, which also led to an increase in the glycogen and glucose content in glucose-injected larvae at 1 wpi. These findings demonstrated that the injected glucose was efficiently extracted from the yolk to the animal and subsequently converted into glycogen deposition in the larvae.

The effects of early glucose stimuli on the glucose-metabolism-related gene expression were assessed in larvae at 1 wpi, which is assumed to be the stage of almost complete absorption of the injected glucose from the vitellus to the larvae. Our results showed that, at the transcriptional level, glucose stimuli during the early alevin stage exerted the expected effects on carbohydrate-metabolism-related pathways (Geurden et al., 2014). Indeed, the increase in the expression of hepatic *pklr* and muscular *hk1*, *hk2* and *pkma* together with *glut4* (mRNA) implies that the fish

received well the glucose stimulus at the molecular level. In the same way, the glucose stimuli down-regulated the expression of several enzymes related to gluconeogenesis (*g6pca1*, *g6pca2* and *pck1*) and amino acid catabolism (*asat*, *alat*) as expected (Geurden et al., 2014). There are few reports describing the effects of hyperglucidic stimuli via glucose injection into the yolk sac. Similar to our findings, in zebrafish, micro-injection of glucose into yolk at 0.2 dpf (30% epiboly stage) resulted in the down-regulation of gluconeogenic genes (*g6pca1*, *pck 2*) in larvae (Rocha et al., 2014, 2015). However, the glucose stimuli suppressed the expression of the muscular glycolytic gene (*pkma*), whereas other genes in the hepatic and muscular glycolysis pathways remained unchanged, which is different from the tilapia in the present study. Our data suggest strong direct effects of glucose injection on the regulation of glucose metabolism. It was also possible to test the existence of metabolic programming 20 weeks and 24 weeks after the early stimuli.

4.5.2 Long-term Effects of Early Glucose Stimuli on Growth Performance and Glucose Metabolism in Juvenile Fish Fed with a Commercial Diet

In the present study, the glucose history during the alevin stage led to an increase in the growth performance of Nile tilapia until the fingerling stage (a significant improvement was observed at 12-16 wpi). Subsequently, normal-saline-injected fish showed catch-up growth (at 20 wpi) when compared to hyperglucidic stimuli fish. This is similar to what was previously observed regarding the maternal effects in fish: it was demonstrated that maternal/parental effects resulted in faster growth only during a short period of the early life of the descendants, and compensatory mechanisms were exhibited later (Heath et al., 1999; Donelson et al., 2009).

Moreover, our data demonstrated the long-term effects of glucose overload in the yolk sac on several metabolic actors in the liver and muscles of juvenile fish. Indeed, this study showed that the early glucose stimulus during the early alevin stage persisted up to 20 weeks in the carbohydrate-metabolism-related pathways (even though these changes were less pronounced than those observed directly after glucose injection). Indeed, our findings revealed that glucose stimuli led to the induction of glycolysis pathways in the liver (*gck*, both mRNA level and enzyme activity) and muscles (both mRNA level of *hk1* and *hk2* and their enzyme activity), as well as pk activity in the long term. In addition, a higher muscle glycogen content in glucose-injected fish was detected, confirming that the whole glucose metabolism process was modified in juvenile tilapia by the early glucose stimulus. It must also be noted that although there were no variations in lipogenic gene mRNA levels, there was an increase in the juvenile hepatic fat content of glucose-injected fish. Combined together, early glucose stimuli exerted long-term effects on carbohydrate (and probably lipid) metabolism in juvenile fish and demonstrated clear metabolic programming in tilapia for the first time.

Finally, the higher expression of the glycolytic pathway suggests that the early glucose stimulus led to an increase in the use of glucose as an energy source in Nile tilapia (even though tilapia are well known to be a better user of dietary carbohydrates compared to carnivorous fish) (Schrama et al., 2012, 2018; Polakof et al., 2012; Boonanuntanasarn et al., 2018a,b). Programming of glucose metabolism seems to be associated with a better growth performance between 12 and 16 wpi, suggesting that the early glucose injection could be associated with a better use of carbohydrates, leading to a higher dietary protein-sparing effect.

4.5.3 Long-term Effects of Early Glucose Injection on Growth Performance and Glucose Metabolism in Juvenile Fish when Fed with Different Levels of Carbohydrates

Our data clearly showed a better growth performance in fish previously injected with glucose after challenging with the two diets (weeks 21-24), demonstrating that early glucose stimuli promoted protein sparing by dietary carbohydrates in the juvenile stage, as suggested also during the 12-16 wpi stage (see above). These findings suggested that hyperglucidic stimuli during the alevin stage have positive effects on the growth performance later in life. By contrast, glucose injection into the yolk sac of zebrafish (*Danio rerio*) was not associated with a better growth performance in juvenile fish (Rocha et al., 2014, 2015), suggesting that the effects of glucose injection depend on the fish species.

The combination effects of early hyperglucidic stimuli and high-carbohydrate diet on the biochemical composition, liver size, muscle composition and plasma metabolites were shown to be an important line of evidence to reveal the existence of nutritional programming. First, high-carbohydrate diets exerted expected effects on the chemical composition of the liver and muscles: (1) reduced protein in the liver, (2) increased fat in the liver and (3) elevated glycogen in the liver and muscles. Indeed, the effects of high-carbohydrate dietary intake were similar to those in previous reports (Azaza et al., 2015; Wang et al., 2017; Boonanuntanasarn et al., 2018b). Combined with the effects of early glucose stimuli, our results suggested that the hyperglucidic stimulus history modulated the effects of high-carbohydrate diet on the liver composition: early glucose stimuli and high-carbohydrate diets synergistically reduced hepatic proteins, increased hepatic lipids and increased glycogen in the liver

and muscles. Moreover, regarding the plasma metabolites, as expected, there was an increase in plasma triglycerides (in relation to the higher fat content of the tissues); however, in contrast to previous studies (Boonanuntanasarn et al., 2018a,b), no variations in glycaemia or BUN were detected with carbohydrate intake. While the effect of the glucose stimulus history on plasma metabolites was not observed in fish at 20 wpi, after the dietary challenge, there was an increase in glycaemia (even though at a relatively low level, i.e. 5.5 mM maximum) and a decrease in BUN in glucose-injected fish. Lower levels of plasma BUN in glucose-injected fish could indicate lower amino acid catabolism, which can be related to a better growth performance in these fish. Taken together, the glucose stimulus history exerted modulating effects on biochemical parameters (nutrient composition, plasma metabolites) later in life. It would be very interesting if these metabolic effects can be related to specific regulation of enzymes, in particular at the molecular level, as expected in the context of programming (Sydmond et al., 2009).

First, as shown in previous studies (Boonanuntanasarn et al., 2018a,b), our results showed that the increase of dietary carbohydrates and the concomitant decrease of dietary proteins are associated with (1) an increase in *gck*/*hk1* (glucose phosphorylation) and *fasn-g6pd* (lipogenesis) and (2) a decrease in *g6pca1*, *pck1* and *pck2* (gluconeogenesis) and in *asat*, *alat* and *gdh* (amino acid catabolism). Moreover, the high glycolytic activity for PK (in the liver and muscles) and HK (muscle) confirmed the good adaptation of tilapia to carbohydrates (Figueiredo-Silva et al., 2013; Wang et al., 2005, 2017; Boonanuntanasarn et al., 2018a,b). The most important and original point here is that early glucose stimuli appeared to synergistically influence liver glycolysis and gluconeogenesis and muscle glucose metabolism.

Indeed, glycolytic enzymes (*gck* expression and its enzyme activity, *pk* expression and its enzyme activity) as well as muscle *glut4* (mRNA) and *hk1*/*2* (mRNA and enzyme activity) were higher in glucose-injected tilapia. In zebrafish injected with glucose in the yolk, fish at 1 dpf fed with a high-carbohydrate diet showed up-regulated metabolic marker genes in muscle glycolysis (*hk1* and *6pfk*) and down-regulated gluconeogenesis as in tilapia, whereas almost no more effects were observed in juvenile fish (Rocha et al., 2014, 2015). Combined together, these findings strongly suggest that alevins receiving glucose injection could remember it and subsequently influence (positively) glucose metabolism in juvenile fish.

Overall, our findings revealed the existence of nutritional programming of metabolism in juvenile tilapia. We, therefore, investigated whether the modulation of metabolism could be linked to a modification in the global epigenetic landscape which is a potential mechanism for long-lasting modulation of metabolic gene expression (Sydmond et al., 2009). Carbohydrate diets did not exert an effect on the global DNA methylation in the liver and muscles. Dietary carbohydrate intake was previously associated with total DNA hypomethylation in both the liver and muscles of rainbow trouts (Craig and Moon, 2013; Marandel et al., 2016a) when compared with fish fed without carbohydrates both in the short (four days of nutrition; Marandel et al., 2016a) and the long (eight weeks of nutrition; Craig and Moon, 2013) term. However, compared to the present study, in which we searched for an estimation of the global DNA methylation only at $C^{m}CGG$ sites using the LUMA technique, these authors evaluated the genomic DNA methylation level in an exhaustive way using an enzyme-linked immunoscorbent assay technique. Thus, regarding our results, we could not exclude the fact that DNA methylation may occur at non-CCGG sites (i.e.

other CpG sites or non-CpG sites), sites not monitored with the LUMA assay. Moreover, previous studies were performed on rainbow trouts, whose metabolism and physiology are quite different from those of Nile tilapia. DNA methylation/ demethylation pathways may, thus, differ between these two species, leading to different results. On the other hand, interestingly, in juvenile fish with early glucose injection, we found global $C^{m}CGG$ hypomethylation in the liver and muscles compared to non-injected fish. Our data suggest that early nutritional stimuli could have a long-term effect on the DNA methylation of the genome of tilapia, as observed previously in juvenile rainbow trouts after first feeding with high levels of vitamins (Panserat et al., 2017).

4.6 Conclusion

In conclusion, early glucose stimuli in the larval stage led to an impressive improved growth performance in Nile tilapia. This could be associated with permanent up-regulation of glycolysis and glucose transporters and down-regulation of gluconeogenesis and amino acid catabolism in glucose-injected fish, suggesting a better use of carbohydrates as an energy source and a protein-sparing effect. The glucose stimulus history is more pronounced when the fish are challenged with medium- and high-carbohydrate diets. The mechanisms at the origin of this programming could be due to at least an epigenetic mechanism, as revealed by the global DNA hypomethylation in the liver and muscles of juvenile fish.

4.7 Acknowledgements

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CHAPTER V

GLUCOSE INJECTION INTO THE YOLK INFLUENCES INTERMEDIARY METABOLISM IN ADULT NILE TILAPIA (*OREOCHROMIS NILOTICUS*) WITHOUT ANY CONSEQUENCES ON GROWTH PERFORMANCE

5.1 Abstract

Nutritional programming concept has been proposed to be applied in fish nutrition to improve use of new diets in aquaculture. This study aimed to investigate the long-term effects of early glucose injection on intermediary metabolism in adult Nile tilapia (*Oreochromis niloticus*). A nutritional stimulus history was performed by direct microinjection of 0.85% NaCl or 2 M glucose into yolk sac in Nile tilapia as previously shown (Kumkhung et al., 2020). In adult tilapia (32 week after first feeding and fed with a commercial diet), we investigated the effects of the early carbohydrate intake on growth performance, blood metabolites, chemical composition in liver and muscle, expression of genes involving in glucose transport and metabolism (glycolysis and gluconeogenesis) and related pathways (amino acid catabolism and lipogenesis). Our results showed that the early glucose injection had no effect on growth performance in adult fish and few significant effects on other parameters (higher muscle glycogen and muscle glycolytic *pfkma* gene expression). Moreover, to deeper test a potential metabolic programming due to the glucose injection, fish were fed with

two different dietary carbohydrate/protein diets (medium-carbohydrate diet, CHO-M; high-carbohydrate diet, CHO-H) between week 33 and 37. As expected, the CHO-H diet led to a decrease in growth performance, higher glycemia and triglyceridemia, higher glycogen and lipid in liver, as well as down-regulation of gluconeogenesis and amino acid catabolism gene expressions. More interestingly, early glucose injection exhibited no significant effect on the growth performance but enhanced capacities for lipogenesis, glycolysis and gluconeogenesis, in particular in fish fed CHO-H diet. Thus, the nutritional programming of adult tilapia linked to the glucose injection into yolk of alevins is possible even though less intense than in juvenile fish.

Key words: Nile tilapia; Nutritional programming; glucose injection; gene expression; glucose metabolism

5.2 Introduction

Nutritional programming have been considered to be a plausibility process at the origin of human metabolic disorders in adulthood (reviewed in Lang-Evans, 2009; Vickers and Sloboda, 2012). This concept has been extensively studied in different animal models such as rat, mouse and sheep (reviewed in Langley-Evans, 2009; Orozco-Solı´s et al., 2010; Gopalakrishnan et al., 2005). In general, the effects of early environmental stimuli may drive permanent changes in physiologic and metabolic pathways in later life. Although several mechanisms have been proposed to underlie nutritional programming including changes in cell number and cell type, hormonal actions, impaired mitochondria functions and epigenetics, the precise mechanisms are still poorly understood (for review see Symonds et al., 2009).

In aquaculture, the concept of nutritional programming has been recently applied in fish nutrition research to investigate if early environmental stimuli can modulate metabolic pathways for better growth and nutrient use in adult fish (for review: Hou et al., 2019; Panserat et al., 2019). This concept was tested using different developmental stages in fish: broodstock, first feeding and embryo-larvae stages (Izquierdo et al., 2015 Lazzarotto et al., 2016; Turkmen et al., 2017; Geurden et al., 2007, 2014; Mennigen et al., 2013; Fang et al., 2014; Marandel et al., 2016; Song et al., 2019; Zambonino-Infante et al., 2019; Vagner et al., 2007, 2009).

Nutritional programming strategies have been successfully used to explore the possibility of modulating some metabolic pathways for the efficient utilisation of alternative feed ingredients in offspring's through specific broodstock nutrition (Izquierdo et al., 2015; Turkmen et al., 2017; Lazzarotto et al., 2015, 2016). In addition, tests at first feeding with a high-carbohydrate diet revealed permanent modifications of carbohydrate metabolism in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*), and these findings varied depending on fish species, stimulus periods, method and challenging conditions (Geurden et al., 2007, 2014; Rocha et al., 2016a,b; Song et al., 2019). Moreover, non-nutritional stimulus, such as a hyperglucidic stimulus applied in larvae through direct glucose injection into yolk reserve in zebrafish (*Danio rerio*), have lead to demonstrate the existence of permament effects of this early stimulus on carbohydrate metabolic pathways in juvenile (Rocha et al., 2014, 2015). Overall, further investigations on the domain of nutritional programming using different fish species are required to underlie this concept in fish for further application in fish nutrition.

Nile tilapia (*Oreochromis niloticus*) is an economically important fish, and its aquaculture is the second largest one for freshwater fish after carps (FAO, 2018 in tilapia nutrition has intensively facilitated cost-effective diet with proper quality (Ng and Romano, 2013). Nile tilapia is an omnivorous grazer that can efficiently utilise dietary carbohydrates as the main energy source (Kamalam et al., 2017); therefore, metabolic responses of Nile tilapia to dietary carbohydrates have been intensively investigated (Boonanuntanasarn et al., 2018a,b; Azaza et al., 2013; Wang et al., 2005). However, increasing the level of dietary carbohydrates (more than 40%) is always an objective in this fish species (ref). The use of early stimulus with glucose injection into the yolk to improve glucose use in tilapia juvenile has been recently hypothesised (Kumkhong et al., 2020). Indeed, early injection of glucose into the yolk of alevin tilapia (10 mg) was associated with strong, persistent and notable positive molecular effects on several carbohydrate-related pathways including the increase of glucose transport and glycolysis and the decrease of gluconeogenesis and amino acid catabolism in juvenile tilapia (Kumkhong et al., 2020). Moreover, this glucose injection during alevin stage promoted growth and modulated severals plasma matabolites and hepatic and muscular nutrient compositions, suggesting that the early glucose injection was linked to highly significant protein sparing effects during juvenile stage (Kumkhong et al., 2020). In order to continue our study, we investigated now if the effects of the early glucose injection into yolk found previously at the alevin stage (Kumkhong et al., 2020) could be maintained later in life, *i.e*. in the adult fish. For this purpose, this study evaluated if the effects on growth performance and metabolism of the early hygerglucidic stimulus could be also

detected in adult tilapia (300 g) when the adult fish were fed with different carbohydrates levels: 67% versus 35%.

5.3 Materials and methods

5.3.1 Experimental design, experimental fish, microinjection and fish culture and challenging diet formulation

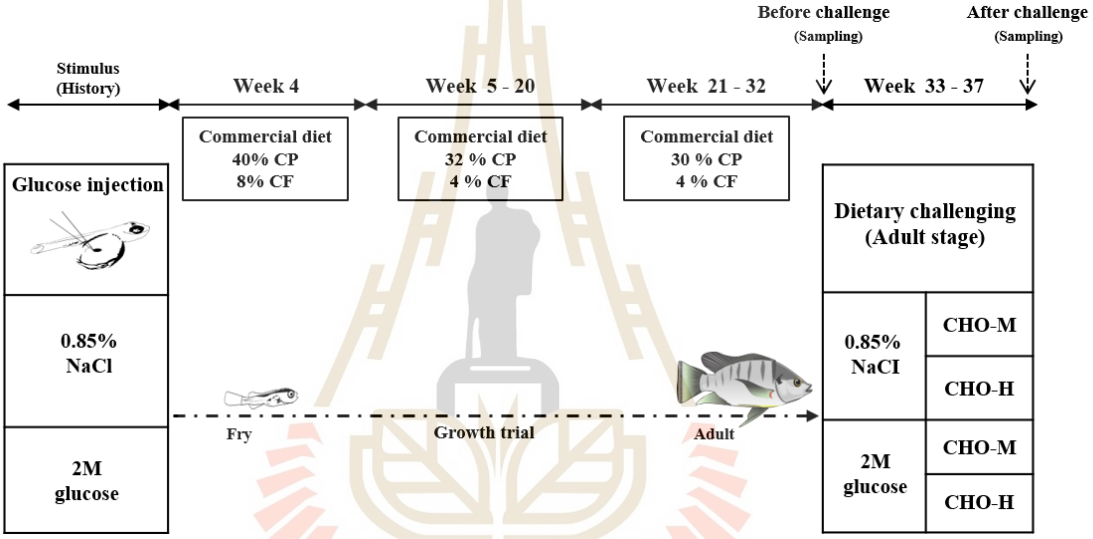

Figure 5.1 Schematic of experimental plan of glucose injection stimulus (history) and dietary carbohydrate challenge test. Glucose stimulus was performed by injecting of either a saline (0.85% NaCl) or a glucose (2 M) solution into the yolk sac of the newly hatching larvae. Subsequently, the injected larvae were cultured for a growth trial through 32 weeks (adulthood). Through growth trial, commercial diets were used to feed the experimental tilapia following commercial practices. During week 33-37, the adult tilapia was subjected to a challenge test with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates,

CHO-H). Fish sampling was performed at before (week 32) and after (week 37) the challenge test.

Figure 5.1 showed the schematic view of the experimental design. The experimental design was completely randomised with two treatment stimuli-injections of 0.85% NaCl and 2 M glucose, each of which included six replicates (family as previously described (Kumkhong et al., 2020). Two groups of fish were reared in cement ponds for 32 weeks. Subsequently, combination effects of high-glucose stimuli history and dietary carbohydrate challenging were evaluated during week 33-37. A 2*2 factorial design with the two stimuli and two dietary carbohydrate levels was employ in a completely randomised design with six replicates (cages).

All experimental protocols were approved by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval no. A-18/2562). The Nile tilapia broodstock (0.8-1.2 kg) were reared in an earthen pond (800 m^2) at University farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The broodstock were fed with a commercial diet at 3% body weight at 9:00 and 16:30 daily.

For microinjection, fertilised eggs were collected from the mouth of female (six replications) and reared in a hatching tray (20×3×5 cm^3) with flow-through water (27°C-29°C) for 3 days. The hatched larvae (stage 17; Fujimura and Okada, 2007) were microinjected with either saline or glucose, as described previously (Kumkhong et al., 2020). The microinjected embryos were reared in the hatching tray with gentle aeration for 1 week. Subsequently, fish (n = 70/replication) were reared in the cement ponds (2×2×0.8 m^3) for 32 weeks. During week 33-37, twelve fish were randomly selected from the cement pond and distributed into two

cages (80×90×110 m^3) (n = 6/cage) for subjecting to challenging diet test (Figure 4.1). The temperature of water (27.0°C-28.7°C) and air (30.0°C-36.0°C) was determined daily. Dissolved Oxygen (DO) and pH were measured weekly, and a DO level of 3.71±0.1 mgL^{-1} and a pH of 7.58±0.2 were acceptable. The growth and feed intake parameters were evaluated at the beginning and end of challenge period and calculated as described previously (Kumkhong et al., 2020). Fish mortality was monitored daily throughout the experiment period.

5.3.2 Fish sampling, blood collection and proximate analysis

After week 32 (before challenging), fish were randomly sampled for hepatic and muscle chemical composition (a pool of 3 fish/replication pond) analysis. In addition, 3 fish of each replication pond were randomly selected for blood metabolites (n = 3/replication pond), and the liver and muscle were collected from 2 of them for analysis of metabolic related gene expression, enzyme activities and glycogen contents. After week 37 (after challenging), the same number of sampling fish were collected from each replication cage.

For sampling procedure, at 5 h after feeding, fish were anaesthetised with clove oil (40 mg L^{-1}). Blood samples were collected from the caudal vein using a hypodermic syringe and transferred into a tube containing 1.0% (v/v) of 15% ethylenediaminetetraacetic acid (EDTA). After bleeding, the liver and muscle were dissected and kept at -0°C until gene expression, enzyme and glycogen analyses. The hepatosomatic index (HSI) was determined. Plasma was collected by centrifugation of the EDTA blood at 3000g for 10 min at 4°C and stored at -80°C until analysis. Moreover, for analysis of chemical composition of liver and muscle, fish were also anaesthetised with clove oil. Fish were dissected to collect liver and muscle.

Table 5.1 Ingredients and proximate compositions (g kg^{-1}) of the challenging diets.

Ingredients	**CHO-M**	**CHO-H**
Fish meal	350	140
Soybean meal	300	60
Rice flour	150	700
Rice bran	180	30
Soybean oil	-	40
Di-calcium phosphate	-	10
Fish premix [a]	20	20
Proximate composition (g kg^{-1} dry weight)		
Dry matter	957.7	957.3
Protein	356.7	154.9
Fat	69.0	64.8
Fibre	28.9	8.6
Ash	129.2	60.3
NFE[b]	373.9	668.8
Gross energy (kJ g^{-1})	17.6	17.2

[a] Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1}diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5,000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12, 0.00005 mg; vitamin C, 1 g; vitamin D3, 1,000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g

[b] Nitrogen-free extract = Dry matter-(crude protein+crude lipid+crude fibre+ash).

5.3.3 Blood chemical analysis and chemical composition of liver and muscle

Plasma metabolite analyses (3 fish/replication) including glucose, triglyceride and blood urea nitrogen (BUN) were performed. Plasma glucose was determined according to Trinder's method (Barham and Trinder, 1969). Triglyceride levels were measured using glycerol-3 phosphate oxidase-sodium N-ethyl-N-(-3 sulfopropyl)m-anisidine (GPO-ESPAS) method (Bucolo and David, 1973). BUN was evaluated using modified indophenol colorimetric method (weather burn, 1976). The proximate chemical analysis including protein, lipid and ash were performed according AOAC (1990) methodology. Glycogen content in liver (100 mg) and muscle (200 mg) was analysed according a hydrolysis technique described by Good et al. (1993) with modification (Kumkhong et al., 2020).

5.3.4 Total RNA extraction and relative quantification of mRNA

Table 5.2 List of the tilapia primers used for qRT-PCRs.

Genes	**5′/3′ forward primer**	**5′/3′ reverse primer**	**NCBI Access number**
Reference gene			
*ef1**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	AB075952
In Liver			
gck	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC	XM_003451020
pfklr	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC	XM003447353
pklr	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA	XM005472622
g6pca1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA	XM003448671
g6pca2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA	XM013273429
pck1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA	XM003448375
pck2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT	XM019354843
fasn	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT	XM013276809

Table 5.2 (Continues).

Genes	5′/3′ forward primer	5′/3′ reverse primer	NCBI Access number
g6pd	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT	XM013275693
asat	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC	XM003451918
alat	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG	XM005476466
gdh	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC	XM003457465
In Muscle			
glut4	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG	JN900493
hk1	CGTCGCTTAGTCCCAGACTC	TGACTGTAGCGTCCTTGTGG	XM019360229
hk2	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC	XM003448615
pfkma	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG	XM019349871
pfkmb	TTTGTGCATGAGGGTTACCA	CACCTCCAATCACACACAGG	XM003441476
pkma	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA	XM005447626

*: from the study by Yang et al. 2013.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was conducted for relative quantification of mRNA of carbohydrate metabolic related genes in liver and muscle (2 fish per replication pond or cage; n = 12 per experimental group). Total RNA was extracted from liver (50 mg) and muscle (100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quantity and quality of total RNA were evaluated by NanoDrop (Thermo Fisher, Madison, WI, USA) and 1% agarose gel electrophoresis, respectively. One microgram of total RNA was used for cDNA synthesis using a Super Script III RNAseH- Reverse transcriptase kit (Invitrogen) with random primers (Promega, Charbonniéres, France), following the manufacturer's protocol. Reverse transcription reaction was performed in duplicate for each sample. Table 2 showed the primer sequences used in the real-time RT-PCR analysis. Glucose metabolic gene expression in the liver was measured, including glycolysis (glucokinase [GK], *gck*; phosphofructokinase, *pfklr*; PK, *pklr*) and gluconeogenesis

(glucose-6-phosphatase, g*6pca1* and *g6pca2*; phosphoenolpyruvate carboxykinase cytosolic, *pck*1; mitochondria, *pck2*). Glucose use in muscles was evaluated by determing the mRNA levels of glucose transporter (*glut4*) and glycolysis (hexokinase [HK] I/II, *hk1* and *hk2*; phosphofructokinase, *pfkma* and *pfkmb*; PK, *pkma*). Examination of the expression of lipogenic capacities included fatty acid synthase (*fasn*) and glucose-6-phosphate dehydrogenase (*g6pd*). This study also examined the expression of the enzymes involved in amino acid catabolism (glutamate dehydrogenase, *gdh*; alanine aminotransferase, *alat*; aspartate amino transferase, *asat*). For real-time RT-PCR assays, a Roche LightCycler 480 system was used (Roche Diagnostics, Neuilly-sur-Seine, France). Assays were performed using the condition as described by Kumkhong et al. (2020). Each PCR assay included replicate samples; therefore, duplicates of reverse transcription and PCR amplification were conducted to analyse the mRNA level. Negative controls were reverse transcriptase- and cDNA-template-free samples, respectively. Relative quantification of target gene expression was conducted using the Roche Applied Science E-Method according to Pfaffl (2001). The relative gene expression of *ef*1α was used for the normalisation of the measured mRNA because its relative expression did not significantly change over experimental groups (data not shown). In all cases, PCR efficiency was analysed from the slope of a standard curve using serial dilutions of cDNA, and the PCR efficiency values ranged between 1.8 and 2.0.

5.3.5 Enzymatic assays

Muscles (200 mg) or livers (100 mg) were used to analyse enzyme activities. Tissue samples (from 2 fish/replication) were homogenised in seven volumes of ice-cold buffer (50 mmol L^{-1} Tris, 5 mmol L^{-1} EDTA and 2 mmol L^{-1}

DTT, pH 7.4) and a protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO, USA) and subsequently centrifuged for 10 min at 900 ×*g* at 4°C. Assays for GK (EC 2.7.1.2) and HK (EC 2.7.1.1) activity were performed using supernatants. For PK (EC 2.7.1.40) activity, additional centrifugation was performed for 20 min at 10,000 ×*g* at 4°C), and the supernatants were used for enzyme assays. The GK (high-K_M) and HK (low-K_M) enzymes were analysed as described by Panserat et al. (2000). The activity of the PK enzyme was also measured as previously described by Panserat et al. (2001). Each enzyme activity was determined in duplicate at 37°C following the variation of absorbance of nicotinamide adenine dinucleotide phosphate ($NADP^+$) at 340 nm. The reactions were started by the addition of a specific substrate, and a PowerWave X (BioTek Instruments Winooski, VT, USA) plate reader was used. De-ionised water was used as a blank for each sample. Enzyme activity units, defined as micro-moles of substrate converted into product per minute at the assay temperature, were calculated as per milligram of protein. Protein concentration was measured in duplicate, according to Bradford (1976), using a protein assay kit (Bio-Rad, Munich, Germany) with bovine serum albumin as a standard.

5.3.6 Data analysis

All data were analysed using SPSS for Windows, version 10 (SPSS Inc., Chicago, IL, USA). An independent *t*-test was used to evaluate the differences between the two stimuli groups-saline (0.85% NaCl) versus glucose (2 M glucose) before the dietary challenge. After the nutritional challenge (week 37), the statistical factors included the analysis of the effects of the glucose stimulus history, dietary carbohydrate level and their interactions. Two-way factorial ANOVA was performed. When the significant interaction of the factors was observed, one-way ANOVA

following Tukey's range test was performed to rank the treatment combination groups. In this study, the effects and differences were declared to be significant when $P<0.05$.

5.4 Results

All results about the direct effects of the early glucose injection have been shown in a first study (Kumkhong et al, 2020). A clear direct effect on the early glucose injection on metabolism and growth performance has been previously described (Kumkhong et al, 2020) showing the success of the early stimulus.

Through experimental period (32 weeks), the survival rate was high in both groups having received or not the glucose injection at larvae stage, ranging from 97% to 99%. During the challenging period (week 33-37), there were no significant differences in the survival rate among experimental groups (Table 5.3). There was no significant difference in growth performance between the glucose-injected fish and normal-saline-injected fish (Week 32). When both experimental fish were subjected to challenge with different dietary carbohydrate amount, again, no significant effect of glucose injection stimuli history on growth performance was observed (Table 5.3). However, effects of different dietary carbohydrate levels on growth response were observed. As expected, adult tilapia fed with CHO-M had significantly higher final weight, ADG, SGR and FCR (Table 5.3).

The effects of early glucose stimuli history on blood metabolites including glucose, triglycerides and BUN (Table 5.4). There were no significant differences in these blood metabolite parameters between the glucose-injected fish and saline-injected fish (Week 32). When these fish were confronted with different dietary

carbohydrate level. The effects of glucose history were detectable for BUN ($P<0.05$). Glucose-injected fish had lower BUN compared with normal-saline-injected fish. As expected, tilapia fed on CHO-H had higher glucose and triglyceride but lower BUN when compared to that fed with CHO-M ($P<0.05$) (Table 5.4).

Table 5.5 shows the effects of glucose stimuli history on chemical composition in liver and muscle and HSI. At week 32, the hepatic composition of protein, fat, ash and glycogen and HSI appeared to be similar between the glucose-injected fish and saline-injected fish ($P>0.05$). However, the significant differences in hepatic protein and fat were observed when fish were challenged with different dietary carbohydrate levels. The glucose-injected fish had higher fat and lower protein contents in liver when compared with normal-saline-injected fish ($P<0.05$). In addition, as expected, high-carbohydrate diet led to decreased protein content and increased fat and glycogen composition in liver ($P<0.05$). significant interactions were observed on protein, fat and HSI which demonstrated that glucose-injected fish fed on CHO-H had highest hepatic fat and HSI and lowest protein contents (Table 5.5). Glucose stimuli history significantly affected glycogen content in muscle at both before (Week 32) and after (Week 37) dietary carbohydrate challenging ($P<0.05$) (Table 5.5). Moreover, significant effect of carbohydrate challenging diet was observed in muscle protein content ($P<0.05$) (Table 5.5). Indeed, an interaction effect was detected which showed that glucose-injected fish fed on CHO-H had lowest protein composition in muscle ($P < 0.05$).

The long-term effects of early glucose stimuli on the mRNA levels of genes related to glucose metabolism in muscles and livers were demonstrated in adult tilapia at 32 weeks (Table 5.6). Early glucose stimuli led to decrease mRNA level of *pfkma*

(P<0.05), whereas the other genes remained unchanged. Indeed, hepatic Gk and Pk as well as muscle Hk and Pk enzymatic activities appeared to be similar between glucose-injected fish and normal-saline injected fish (P>0.05) (Figure. 5.2A,C and Figure. 5.3A,C). At week 37, combination effects of glucose stimuli history and dietary carbohydrate were observed when fish were confronted with high-carbohydrate diet (Table 5.6). The results showed that early glucose stimuli led to elevated hepatic *gck*, *g6pca1*, *asat*, *hk2* and decreased *pkma* mRNA levels (P<0.05) (Table 5.6), whereas other carbohydrate metabolism related genes (*pfklr*, *pklr*, *g6pca2*, *pck1*, *pck2*, *fasn*, *g6pd*, *alat*, *gdh*, *glut4*, *hk1*, *pfkma*, *pfkmb*) remained unchanged (P> 0.05). In addition, up-regulation of *gck* and *hk* by glucose stimuli was observed in their enzymatic activities (P<0.05) (Figure. 5.2B and Figure. 5.3B). The significant effects of dietary carbohydrate level were also observed in several genes involving in gluconeogenesis and amino acid catabolism. Expectedly, CHO-H led to down-regulation of *g6pca2*, *pck1*, *pck2* and *asat* (P<0.05) (Table 5.6). Note that interactive effects between glucose stimuli and dietary carbohydrates were observed for the expression of *g6pca2*, *fasn*, *alat*, *glut4*, *pfkma* and *pkama* as well as hepatic and muscle Pk activities (P<0.05). According to interaction effects, the highest up-regulation of *fasn* as well as hepatic and muscle Pk enzymatic activities were observed in glucose-injected fish fed with CHO-H, whereas the highest *alat* mRNA was observed in glucose-injected fish fed with CHO-M (P<0.05) (Table 5.6 and Figure. 5.2D, Figure. 5.3D).

Table 5.3 Growth performance of adult tilapia injected with either saline (0.85% NaCl) of glucose (2 M) before (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (mean ± SD, $n = 6$).

	Before challenge			0.85 %NaCl History		2M glucose History		*P-value*[1]		
Parameter	0.85% NaCl History	2M glucose History	*P*-value	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Initial weight (g)	164.3 ± 2.1	163.3 ± 1.3	0.949	222.1 ± 1.5	223.2 ± 2.0	224.6 ± 3.6	223.6 ± 1.8	0.141	0.963	0.286
Final weight (g)	222.7 ± 2.4	222.6 ± 1.9	0.229	305.8 ± 4.8	283.6 ± 3.2	308.4 ± 4.5	286.4 ± 2.8	0.112	<0.001	0.958
ADG[2] (g day^{-1})	2.3 ± 0.1	2.4 ± 0.0	0.257	2.8 ± 0.2	2.1 ± 0.1	2.8 ± 0.2	2.1 ± 0.1	0.990	<0001	0.949
SGR[3] (% day^{-1})	1.2 ± 0.0	1.2 ± 0.0	0.131	1.1 ± 0.1	0.8 ± 0.0	1.1 ± 0.1	0.8 ± 0.0	0.752	<0.001	0.861
FI[4] (g day^{-1})	2.8 ± 0.8	2.9 ± 1.4	0.412	2.8 ± 0.3	2.5 ± 0.4	2.7 ± 0.2	2.6 ± 0.3	0.963	0.118	0.389
FCR[5]	1.4 ± 0.0	1.4 ± 0.0	0.115	1.0 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	1.3 ± 0.1	0.926	<0.001	0.424
Survival rate (%)	97.3 ± 0.8	96.5 ± 0.7	0.111	97.2 ± 6.8	94.4 ± 8.6	97.2 ± 6.8	97.2 ± 6.8	0.646	0.646	0.646

[1]Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet).

[2]Average daily gain (ADG) = (final body weight-initial body weight)/experimental days.

[3]Specific growth rate (SGR) = 100 × [(ln final body weight-ln initial body weight)/experimental days].

[4]Feed intake (FI) = dry feed fed/experimental days.

[5]Feed conversion ratio (FCR) = dry feed fed/wet weight gain.

Table 5.4 Blood metabolite of adult tilapia injected with saline (0.85% NaCl) and glucose (2 M) at before (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (mean ± SD, $n = 6$).

Blood chemistry	Before challenge			0.85 % NaCl History		2M glucose History		*P*-value[1]		
	0.85% NaCl History	2M glucose History	*P*-value	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Glucose (mM)	6.3 ± 0.9	6.3 ± 0.9	0.866	5.9 ± 0.5	6.9 ± 0.7	5.6 ± 0.4	7.1 ± 1.0	0.876	<0.001	0.254
Triglyceride (mM)	2.5 ± 0.5	2.2 ± 1.0	0.562	2.6 ± 0.5^{ab}	3.0 ± 0.3^{a}	2.1 ± 0.4^{b}	3.2 ± 0.4^{a}	0.348	<0.001	0.027
BUN (mM)	0.9 ± 0.0	0.9 ± 0.0	0.718	1.1 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	<0.001	<0.001	0.219

[1]Two-way ANOVA was used to determine the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values among four combination groups (P<0.05).

Table 5.5. Chemical composition and glycogen content in the liver and muscles and HSI of adult tilapia injected with saline (0.85% NaCl) and glucose (2 M) at before (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (mean ± SD, $n = 6$).

Proximate compostion (g kg^{-1})	Before challenge			0.85 % NaCl History		2M glucose History		*P*-value[1]		
	0.85% NaCl History	2M glucose History	*P*-value	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Liver										
Protein	93.2 ± 2.1	94.1 ± 2.7	0.522	126.3 ± 1.6^{a}	113.5 ± 1.7^{b}	96.3 ± 2.4^{c}	93.9 ± 1.6^{c}	<0.001	<0.001	<0.001
Fat	26.2 ± 2.5	25.5 ± 1.4	0.593	29.9 ± 2.3^{c}	41.3 ± 2.6^{b}	29.9 ± 2.8^{c}	62.8 ± 2.4^{a}	<0.001	<0.001	<0.001
Ash	11.6 ± 0.3	11.5 ± 0.3	0.592	13.3 ± 0.8	13.4 ± 0.3	13.5 ± 0.4	13.5 ± 0.6	0.425	0.715	0.715
Glycogen (mg/g)	126.6 ± 7.8	130.3 ± 5.2	0.352	156.2 ± 25.3	217.3 ± 49.5	149.3 ± 55.7	218.5 ± 69.1	0.894	0.006	0.852
HSI* (%)	4.2 ± 0.3	4.1 ± 0.3	0.367	3.9 ± 0.2^{c}	5.2 ± 0.3^{b}	3.2 ± 0.2^{d}	5.7 ± 0.2^{a}	0.536	<0.001	<0.001
Muscle										
Protein	181.6 ± 5.5	183.5 ± 2.4	0.449	183.78 ± 2.4^{a}	173.3 ± 2.4^{c}	179.7 ± 3.1^{ab}	176.1 ± 4.3^{bc}	0.603	<0.001	0.014
Fat	20.0 ± 0.7	19.9 ± 0.8	0.799	15.5 ± 1.3	14.8 ± 1.5	13.5 ± 1.1	14.8 ± 2.2	0.136	0.623	0.130
Ash	12.5 ± 0.1	12.3 ± 0.1	0.005	13.5 ± 0.3	13.5 ± 0.8	13.2 ± 0.8	13.3 ± 0.5	0.385	0.721	0.871
Glycogen (mg/g)	2.5 ± 0.6	3.4 ± 0.5	0.024	2.7 ± 1.8	3.7 ± 1.6	4.2 ± 1.9	5.5 ± 0.9	0.022	0.091	0.783

[1]Two-way ANOVA was used to determine the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values among four combination groups (P<0.05).

Table 5.6 mRNA levels of genes involved in carbohydrate metabolism in the liver and muscles of adult tilapia injected with saline (0.85% NaCl) and glucose (2 M) at before (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (mean ± SD, $n = 6$)

mRNA level	Before challenge			0.85 %NaCl History		2M glucose History		*P-value*[1]		
	0.85% NaCl History	2M glucose History	*P-value*	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interaction
Liver glycolysis										
gck	0.6 ± 0.5	2.4 ± 2.3	0.115	0.7 ± 0.1	1.0 ± 0.8	1.3 ± 0.9	1.7 ± 0.8	0.039	0.233	0.890
pfklr	1.0 ± 0.4	0.9 ± 0.3	0.722	1.1 ± 0.4	0.8 ± 0.4	1.2± 0.5	0.8 ± 0.4	0.872	0.059	0.923
pklr	1.2 ± 0.8	0.8 ± 0.5	0.282	0.8 ± 0.5	1.0 ± 0.8	0.8 ± 0.4	0.9 ± 0.6	0.903	0.649	0.925
Liver gluconeogenesis										
g6pca1	1.9 ± 1.2	0.9 ± 0.7	0.102	0.7 ± 0.3	0.8 ± 0.4	1.3 ± 0.5	1.1 ± 0.8	0.042	0.721	0.555
g6pca2	1.5 ± 1.2	0.5 ± 0.3	0.100	0.8 ± 0.5^{b}	0.6 ± 0.5^{b}	1.7± 0.6^{a}	0.6 ± 0.2^{b}	0.064	0.003	0.044
pck1	2.0 ± 1.7	0.3 ± 0.2	0.063	0.3 ± 0.4^{ab}	0.1 ± 0.0^{b}	0.7 ± 0.6^{a}	nd	0.200	0.003	0.116
pck2	1.1 ± 0.5	0.8 ± 0.1	0.185	0.9 ± 0.8	0.4 ± 0.3	1.2 ± 1.0	0.5 ± 0.4	0.567	0.035	0.622
Liver lipogenesis										
fasn	1.0 ± 0.6	0.9 ± 0.5	0.775	1.0 ± 0.5^{ab}	0.6 ± 0.4^{b}	0.6 ± 0.4^{b}	1.7 ± 1.1^{a}	0.231	0.202	0.011
g6pd	0.9 ± 0.6	0.9 ± 0.3	0.951	0.8 ± 0.6	1.0 ± 0.9	1.4 ± 0.7	0.9 ± 0.4	0.432	0.625	0.191
Liver acid catabolism										
asat	1.7 ± 1.1	0.9 ± 0.4	0.172	0.7 ± 0.5	0.4 ± 0.2	1.6 ± 0.9	0.5 ± 0.4	0.049	0.004	0.109
alat	1.2 ± 0.7	1.1 ± 0.1	0.880	0.7 ± 0.3^{b}	0.9 ± 0.5^{ab}	1.3 ± 0.2^{a}	0.8 ± 0.2^{ab}	0.100	0.156	0.034
gdh	1.3 ± 0.9	1.0 ± 0.3	0.548	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.3	0.855	0.866	0.989
Glucose transport and muscle metabolism										
glut4	0.9 ± 0.5	0.9 ± 0.3	0.984	0.3 ± 0.1	1.3 ± 0.8	1.0 ± 0.8	0.8 ± 0.8	0.625	0.166	0.037
hk1	1.1 ± 0.4	1.0 ± 0.4	0.929	1.1 ± 0.5	0.9 ± 0.5	1.0 ± 0.6	0.8 ± 0.5	0.688	0.388	0.852
hk2	1.0 ± 0.4	1.1 ± 0.4	0.935	0.8 ± 0.2	0.8 ± 0.4	1.0 ± 0.5	1.2 ± 0.3	0.046	0.330	0.597
pfkma	1.3 ± 0.9	0.3 ± 0.2	0.032	1.4 ± 1.3	0.4 ± 0.3	0.6 ± 0.4	0.7 ± 0.5	0.592	0.213	0.048
pfkmb	0.9 ± 0.2	1.1 ± 0.2	0.270	1.0 ± 0.5	0.8 ± 0.3	0.9 ± 0.2	0.7 ± 0.2	0.678	0.088	0.962
pkma	0.9 ± 0.2	0.9 ± 0.2	0.591	1.7 ± 0.6^{a}	1.0 ± 0.5^{ab}	0.6 ± 0.4^{b}	0.8 ± 0.5^{b}	0.006	0.199	0.033

[1]Two-way ANOVA was used to determine the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values among four combination groups (P<0.05).

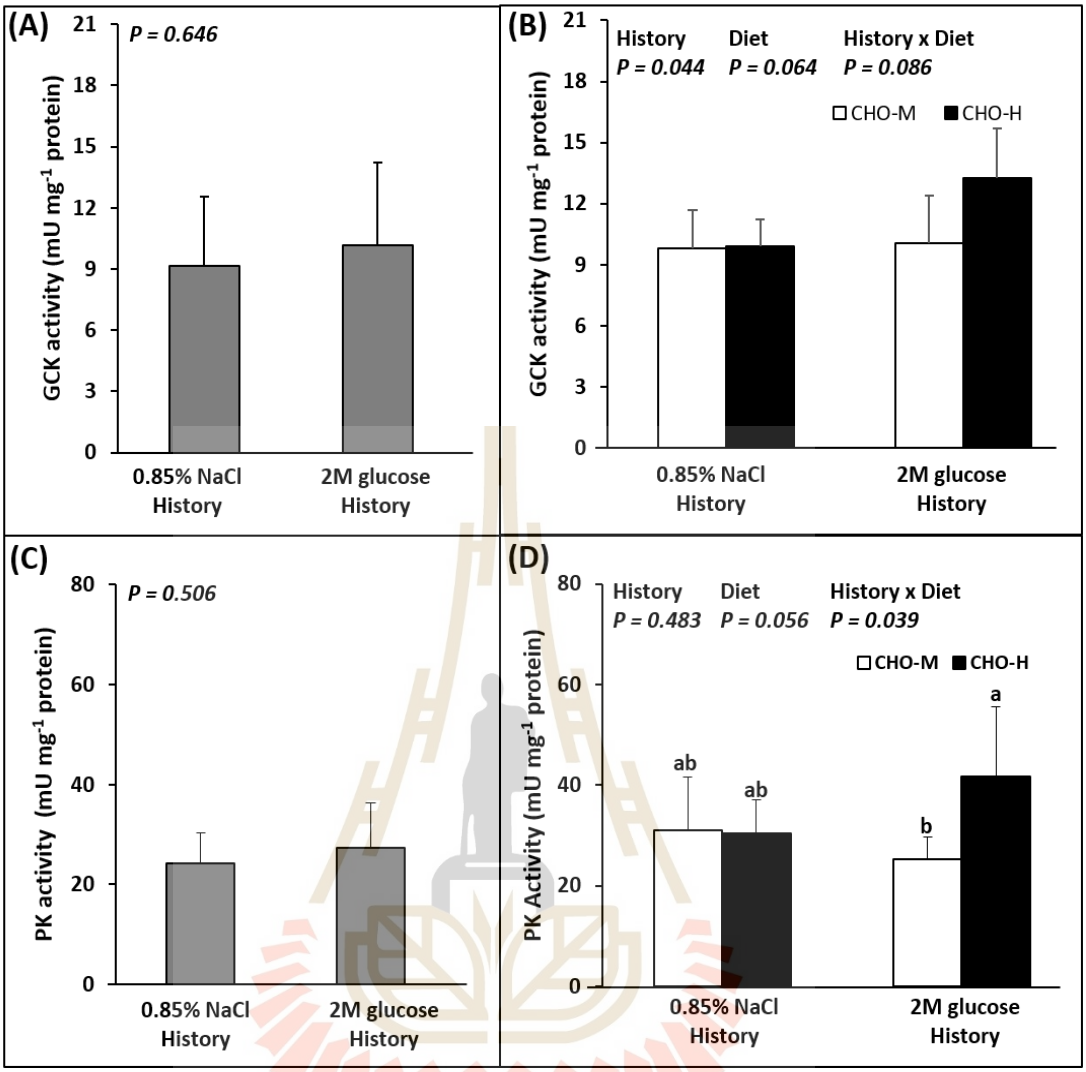

Figure 5.2 Enzyme activity (mU mg^{-1} protein) in the livers of adult tilapia that were microinjected with either 0.85% NaCl or 2 M glucose (history). Enzymes that are involved in hepatic glycolysis included GK (A,B) and PK (C,D). At week 32, enzymes were analysed to evaluate the effect of glucose stimuli on enzyme activities (A,C). During week 33-37, fish were subjected to challenge with different dietary carbohydrate diets (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Data are presented as the mean ± standard deviation (SD) ($n = 6$). Independent t-test was conducted to determine the effects of glucose injection before challenging.

Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History×Diet). When significant interaction effects were observed, one-way ANOVA following Tukey's range test was performed to rank the treatment combination groups. Different letters in the bar graph indicate significant differences ($P<0.05$).

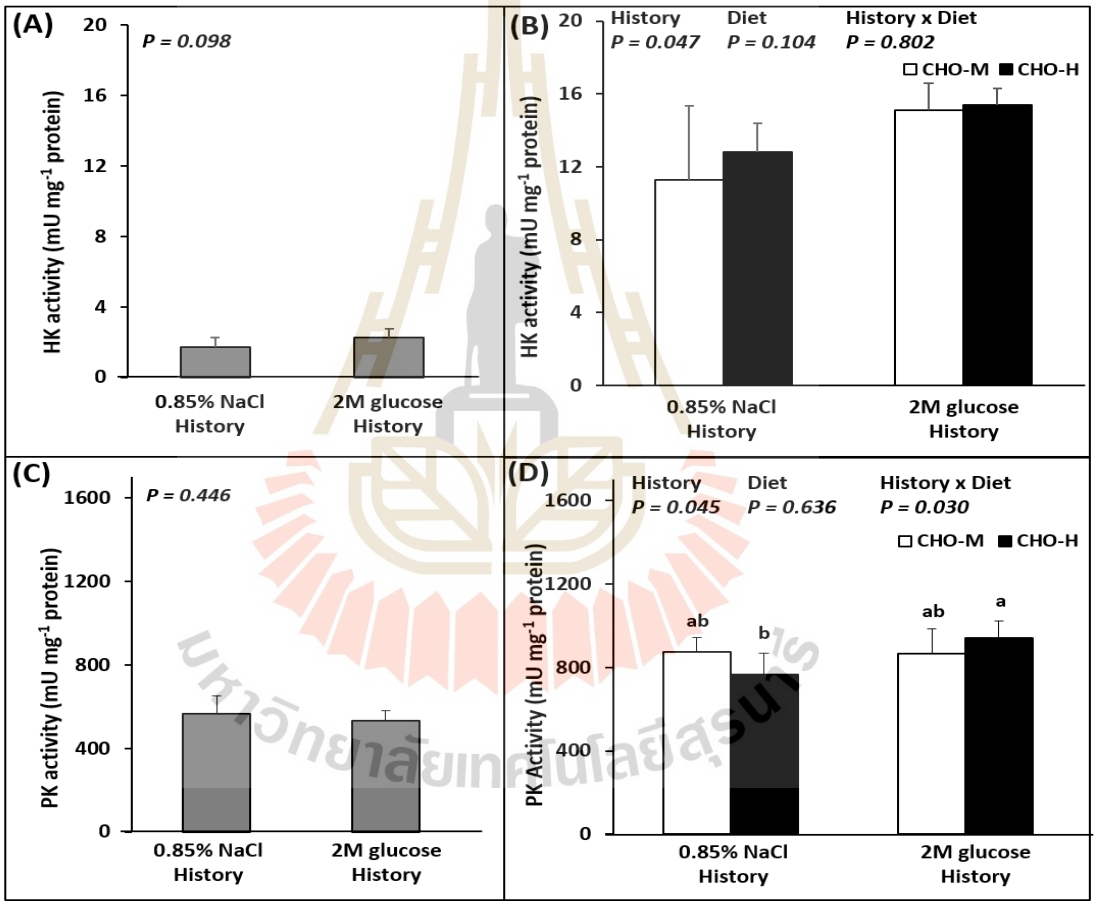

Figure 5.3 Enzyme activity (mU mg^{-1} protein) in the muscles of adult tilapia that were microinjected with either 0.85% NaCl or 2 M glucose (history). Enzymes that are involved in hepatic glycolysis included HK (A, B) and PK (C,D). At week 32, enzymes were analysed to evaluate the effect of glucose stimuli on enzyme activities (A, C). During week 33-37, fish

were subjected to challenge with different dietary carbohydrate diets (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Data are presented as the mean ± standard deviation (SD) ($n = 6$). Independent t-test was conducted to determine the effects of glucose injection before challenging. Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History×Diet). When significant interaction effects were observed, one-way ANOVA following Tukey's range test was performed to rank the treatment combination groups. Different letters in the bar graph indicate significant differences ($P<0.05$).

5.5 Discussion

The nutritional programming has been claimed to be a new strategy in fish nutrition for tailoring fish metabolism. Glucose injection into yolk reserve would be a direct method for fish larvae to experience a high-glucose stimulus. Recently, a direct glucose injection into yolk reserve was shown to be effective for permanent modification of glucose metabolism in juvenile zebrafish and juvenile Nile tilapia later in life (Rocha et al., 2015, 2016; Kumkhong et al., 2020). Kumkhong et al. (2020) studied the successfull programming up to the juvenile stage. The present study apports new information in adult Nile tilapia by researching the effects of direct high-glucose injection into yolk reserve on growth performance, blood metabolite (glucose, triglyceride, BUN), liver and muscular composition as well as glucose metabolism.

5.5.1 No long-term effects of the glucose injection on the growth performance and postprandial plasma metabolite profiles in adult Nile tilapia

Our present results showed no significant effects of glucose stimulus history on growth performance in adult tilapia (up to the week 33). Concomitantly, glucose injection into yolk sac (2.2 M) in zebrafish embryo during 30% epiboly stage did not change growth performance during juvenile stage (Rocha et al., 2014, 2015). This is in contrast to positive effects of early glucose injection on growth performance observed in fingerling/juvenile tilapia (16 weeks of age) (Kumkhong et al., 2020). Regarding the dietary challenge with the two diets (after the 33 week), as expected, medium-carbohydrate (35%)/medium-protein (35%) diet led to a better growth performance in adult tilapia compared to fish fed the high-carbohydrate (67%)/low protein (15%) diet, as previously observed (Boonanuntanasarn et al., 2018a,b; Kumkhong et al., 2020). On the other hand, our results did not show any significant effects of the glucose injection on the growth performance in adult fish in contrast to what has been observed previously in the juveniles (Khumkhong et al., 2020). Taken together, the long-term effects of the glucose injection found during the juvenile stage seem to be erased in the adult stage.

Whereas the effect of the CHO-H diet led to increased glucose and triglyceride and decreased BUN in adult tilapia as previously observed in (Boonanuntanasarn et al. 2018a,b), our results showed that early glucose stimuli had no significant effects on plasma metabolites in adult tilapia, in contrast to what was observed previously (Khumkhong et al., 2020). As for the growth performance, our findings suggest that the nutritional programming observed in juvenile fish due to the

early glucose injection seems to decrease later in life. More investigations about intermediary metabolism at molecular and enzymatic levels are thus needed to clarify whether the early glucose injection could have permament effects later in life.

5.5.2 A permanent effect of the early glucose injection on the metabolism in adult Nile tilapia in particular when fed with high levels of carbohydrates.

Before the challenge, even though there were no significant effects on hepatic chemical composition and HSI in adulthood an increase of muscle glycogen linked to the early glucose injection was found. These results were similar to the findings in juvenile stage (Kumkhong et al. 2020). Nevertheless, after the dietary challenge, the effects of early glucose injection were revealed.Indeed, glucose stimulus history led to lower levels of protein content, higher levels of lipid in liver and increased muscle glycogen, suggesting that the glucose experience maybe associated with modification of metabolism later in adult life as it has been previously observed in juvenile tilapia (Kunkhong et al., 2020). In this study, as shown previously (Boonanuntanasarn et al. 2018a,b), fish fed with high-carbohydrate diet had (1) a decrease of hepatic and muscle protein levels, (2) an increase of fat content in the liver, (3) an increase of hepatic glycogen and (4) higher liver size confirming the effects of the dietary carbohydrates on the intermediary metabolism (Polakof et al., 2012; Kamalam et al., 2017). Finally, early glucose injection and final high-carbohydrate intake acts in interaction for synergistically decreasing proteins in liver and muscle as well as increasing lipid contents and liver size. Taken together, these findings strongly suggest the existence of a programming due to the early glucose injection in Nile tilapia. It was thus important to check if these effects could be linked

to specific mechanisms on intermediary metabolism at molecular and enzymatic levels.

To determine whether glucose stimulus at alevin stage had long-term effects on glucose metabolic pathways, we first evaluated the mRNA levels of several genes involved in hepatic glycolysis, gluconeogenesis, lipogenesis and amino acid catabolism together with muscular glycolysis and glucose transporter just before the dietary challenge phase. Our results showed that, except *pfkma*, injection of glucose into yolk reserve had no significant long-term effects on the expression of glucose metabolism-related genes. The enzyme activities of Gck and Pk in liver and Hk and Pk in muscle confirmed these absence of changes. These data are different from our previous report in juvenile tilapia by Kumkong et al. (2020) which demonstrated that microinjection of glucose into yolk reserves had direct significant effects on up-regulation of hepatic *gck* and muscle *hk1* and *hk2*, as well as their activities. Taken together, our data suggest that the programming effects observed in juveniles (Kumkhong et al., 2020) seem to be erase later in life (adult stage). Because the effects on glucose metabolism could be potentially higher in adult fish fed with high carbohydrates diets, we analyse later the effect of two challenged diets in adult tilapia.

Secondly, Nile tilapia were fed with either a medium level of carbohdyrates or with high levels of carbohydrates for 5 weeks. In adult tilapia, high dietary carbohydrate ($\geq$12 weeks) influenced expression of several genes related to carbohydrate metabolism. Indeed, in fish fed with carbohydrates, we observed higher levels of lipogenic *fasn* and *g6pd* mRNAs and muscle glycolysis (*hk2* and *pkma*) as well as lower levels of gluconeogenic *g6pca2*, *pck1*, *pck2* and *asat (*amino acid catabolism*)* as previously observed *(*Boonanuntanasarn 2018a,b). On the other hand,

we found that the early glucose injection was associated with the induction of glycolytic enzymes (hepatic *gck* and muscle *hk2*, mRNA and enzymatic activities; and muscle Pk activity). Moreover, hepatic PK activity of adult glucose-injected tilapia is higher but only on fish fed high carbohydrates. Our study demonstrated that early glucose injection induced gluconeogenesis, lipogenesis and amino acid catabolism (*asat* and *alat*) in tilapia adulthood fed with carbohydrates. It must be noted that interactions for the hepatic genes *g6pca*2, *fasn*, *alat* and muscle *glut*4, *pfkma* and *pkma* were observed. Compared to the previous study at the juvenile stage (Khumkhong et al., 2020), there is still some metabolic programming in adult tilapia at molecular and enzymatic levels but globally lower enzymes activities are affected and also at a lower statistical significance (the *p* values are just below to the 0.05). Finally, the present findings would suggest that early glucose injection modulated carbohydrate metabolism in the long term with possible induction of glycolysis in liver and muscle, as welle as gluconeogenesis, amino acid catabolism and lipogenesis.

5.6 Conclusion

In conclusion, the long-term effects (on glucose and lipid metabolism) of early glucose injection into yolk were revealed mainly when adult tilapia were fed with a high-carbohydrate diet. However, the nutritional programming was weaker than to what we found previously in juvenile tilapia. Early glucose stimuli history appeared to promote glycolysis in liver and muscle, as well as gluconeogenesis, lipogenesis and amino acid catabolism in adult tilapia.

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CHAPTER VI

EARLY FEEDING WITH HYPERGLUCIDIC DIET DURING FRY STAGE EXERTS LONG-TERM POSITIVE EFFECTS ON NUTRIENT METABOLISM AND GROWTH PERFORMANCE IN ADULT TILAPIA (*OREOCHROMIS NILOTICUS*)

6.1 Abstract

This study aimed to investigate nutritional programming of carbohydrate metabolism in Nile tilapia. Early nutritional intervention stimulus was achieved by feeding fry with high-protein/low-carbohydrate (HP/LC) or low-protein/high-carbohydrate (LP/HC) diet since first feeding for 4 weeks, and the effect of nutritional stimulus on carbohydrate and its related metabolism was evaluated through the adult stage. Our findings indicated that at week 1, LP/HC diet-fed fry had lower levels of mRNA for genes coding gluconeogenesis and amino acid catabolism and higher levels of hk2 ($P<0.05$). As expected, in adult tilapia, although LP/HC diet-fed fish had poorer growth (end of stimulus), the fish showed compensatory growth. There were permanent effects of early high-carbohydrate intake on several parameters including 1) modulating hepatic composition, 2) increased muscle glycogen, 3) lower levels of enzymes involved in amino acid catabolism, and 3) higher levels of enzymes in glycolysis. Finally, HP/LC diet- and LP/HC diet-fed fish were challenged with

different dietary carbohydrate levels. Irrespective of challenging diets, the early high-carbohydrate stimulus had significant effects on adult tilapia by 1) promoting utilization of glucose, which had protein-sparing effects for better growth, 2) inducting lipogenesis, and 3) decreasing amino acid catabolism. Taken together, for the first time, we demonstrated that early high-carbohydrate feeding was effective for positive nutritional programing of metabolism in Nile tilapia (an omnivorous fish). It led to the improvement of growth performance in adult fish associated with early feeding, which is linked to a better ability to use glucose, to induce lipogenesis, and to suppress amino acid catabolism.

Key words: Nile tilapia; nutrient programming; growth performance; early feeding; gene expression; glucose metabolism

6.2 Introduction

Fish nutrition is vital for the development of a sustainable aquaculture (FAO, 2018). Recent studies in fish nutrition have focused on exploring the potential use of new feed ingredients and on developing a novel concept (nutritional programming) to modify specific metabolic functions for better use of new aquafeeds (Panserat et al., 2019). Nutritional programming is based on the fact that early feeding may have long-term impact on metabolic processes in later life (Lucas, 1998; Fernandez-Twinn and Ozanne, 2013). In fish, the nutritional programming has been tested to investigate the possibility of early nutritional history to be applied for tailoring metabolic pathways for better use of diets (Panserat et al., 2019; Garden et al., 2013; Lazzarotto et al., 2015; Izquierdo et al., 2015; Clarkson et al., 2017; Hou and Fuiman, 2019).

Although carbohydrates are not an essential nutrient in fish, they are incorporated into the diets, to produce low-cost diet, as well as diets with lower level of dietary proteins. The metabolic use of dietary carbohydrates has been intensively investigated in reared fish. For example, nutritional factors affecting carbohydrate metabolism were demonstrated in a number of fish species with distinct feeding habits from the carnivorous fish with low capacity to use dietary carbohydrates up to herbivorous and omnivorous fish with a high level of capacity to use dietary carbohydrates (Panserat et al., 2000, 2001 2009; Polakof et al., 2012; Marandel et al., 2015; Seiliez et al., 2017; Kamalam et al., 2016; Boonanuntanasarn et al., 2018a, 2018b). To improve the metabolic use of dietary carbohydrates, the nutritional programming concept for carbohydrate metabolism has been conducted recently in different fish species. Indeed, the nutritional programming concept has been successfully demonstrated using the first-feeding stage in carnivorous fish including rainbow trout (*Oncorhynchus mykiss*) (Geurden et al., 2007, 2014), Siberian sturgeon (*Acipenser baerii*) (Gong et al.,2015; Liang et al., 2017), sea bass (*Dicentrarchus labrax)* (Zambonino-Infante et al., 2019), and gilthead seabream (*Sparus aurata*) (Rocha et al., 2016a,b). However, the nutritional programming concept in omnivorous fish is still limited to few studies on zebrafish (*Danio rerio*) Fang et al., 2014; Rocha et al., 2014, 2015) and only one study on Nile tilapia (*Oreochromis niloticus*) (Kumkhong et al., 2020).

Nile tilapia (Oreochromis niloticus) is an omnivorous fish that is able to use efficiently high levels of dietary carbohydrates (Kamalam et al., 2016; Shiau and Peng ,1993; Wang et al., 2005). Numerous studies in nutrition have been conducted on Nile tilapia since it is an economically important freshwater fish. Indeed, all over the world, Nile

tilapia is the second most important cultured fish after the common carp (FAO, 2018). As a model of omnivorous fish, nutritional regulation of carbohydrate metabolism was demonstrated in previous studies that revealed that high intake of dietary carbohydrates (up to 40%) was associated with better growth performance and down-regulation of gluconeogenesis and an increase of glycogen and lipid deposits in liver and muscle (Boonanuntanasarn et al., 2018b), in contrast to carnivorous fish (Kamalam et al., 2016). Because early feeding with a high level of carbohydrates can have positive long-term effects on growth performance and glucose metabolism (Geurden et al., 2007, 2014; Rocha et al., 2016a; Fang et al., 2014; Rocha et al., 2014, 2015; Liu et al., 2017; Song et al., 2019), it is important to test this hypothesis of direct and long-term impacts of early feeding with high levels of carbohydrates in an omnivorous fish species (i.e., the Nile tilapia). Thus, for the first time, we aimed to evaluate direct effects of high-carbohydrate diet by measuring the mRNA levels of genes involved in intermediary metabolism in tilapia fry. The long-term effects of the nutritional history (early high-carbohydrate intake at first-feeding) were determined up to the adulthood stage by an analysis of intermediary metabolism at molecular and enzymatic levels in the liver and the muscle, determination of plasma metabolites, and tissue compositions before and after a final dietary challenge with a high-carbohydrate diet.

6.3 Materials and methods

6.3.1 Experimental fish and diets, experimental design, and fish culture

All experimental protocols about fish were approved by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval no. A-18/2562). Nile tilapia fry (*O. niloticus*) used in this study were

obtained from a brood stock that was cultured at the University Farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Nile tilapia brood stock (0.8-1.2 kg) was cultured in an earthen pond (800 m2) and fed with a commercial feed (30% crude protein (CP)+4% crude fat (CF) at 3% body weight) at 9:00 and 16:30 daily. Table 6.1 details the ingredients of the two diets for the early feeding stage, i.e., high-protein low-carbohydrate (HP/LC) and low-protein high-carbohydrate (LP/HC) diets and for the two diets for the final challenge, i.e., the medium-carbohydrate diet (CHO-M; 37% of carbohydrates) and the high-carbohydrate diet (CHO-H; 67% of carbohydrates). The proximate composition including moisture, CP, CF, crude fiber and ash, as well as gross energy, was analyzed according to the standard method of the Association of Official Analytical Chemists (AOAC,1990) (Table 6.1). To prevent the confounding effect associated with sex dimorphism, HP/LC and LP/HC diets were supplemented with 17 α-methyltestosterone at 60 mg kg^{-1} (Boonanantanasarn et al., 2018b) Currently, all male tilapia, which were produced using feeding fish fry 17MT, have been commercially cultured for global consumption and several reports have revealed no accumulation of 17MT in fish flesh at harvesting size (Rima et al., 2017).

An illustrative view of the experimental design is shown in Figure 6.1. To investigate the effects of the early high-carbohydrate diet (namely the “stimulus” phase), a completely randomized design with the two first-feeding diets (HP/LC and LP/HC diets) was employed using six replicates (cages). In total, 1,200 fry (9-10 mg) were randomly distributed into 12 cages (40×40×60 cm^3). To exclude the possible effects of environment during the stimulus phase, 12 cages were located in 1 cement pond (2×2×0.8 m^3) (six replicates; 100 fry/replicate) and an HP/LC or LP/HC diet was

fed for 4 weeks daily at 09.00, 11.00, 13.00, 15.00, and 17.00 as described previously (Boonanuntanasarn et al., 2018b).

Table 6.1 Ingredients and chemical composition (g kg^{-1}) of the 2 stimulis diets (diets at first feeding) and for the 2 challenge diets (diets for the final challenge)

Ingredients	**Stimuli diets**		**Challenge diets**	
	HP/LC	**LP/HC**	**CHO-M**	**CHO-H**
Fish meal	860	360	350	140
Soybean meal	-	-	300	60
Rice flour	100	560	150	700
Rice bran	-	-	180	30
Soybean oil	20	60	-	40
Fish premix[a]	20	20	-	10
Di-calcium phosphate	-	-	20	20
Proximate composition (g kg^{-1} dry weight)				
Dry matter	957.8	956.6	957.7	957.3
Protein	492.6	247.1	356.7	154.9
Fat	96.9	99.2	69.0	64.8
Fiber	5.8	4.8	28.9	8.6
Ash	238.1	108.1	129.2	60.3
NFE[b]	166.7	540.9	374.0	668.8
Gross energy (kJ g^{-1})	17.8	18.6	17.6	17.2

[a]Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1} diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5,000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12, 0.00005 mg; vitamin C, 1 g; vitamin D3, 1,000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g.

[b]Nitrogen-free extract = dry matter-(CP+crude lipid+crude fibre+ash).

Both diets were well accepted by the fish for 4 weeks. Subsequently, both experimental fish were continually cultured in cement ponds and fed with a commercial diet ad libitum twice daily (at 09:00 and 16:00) up to week 36, and growth performance was determined. During weeks 37-41, for the dietary "challenge" phase, a 2×2 factorial design, with the early dietary stimuli (HP/LC and LP/HC diets, namely HP/LC "history" and LP/HC "history") combined with two dietary carbohydrate levels for the dietary challenge (CHO-M and CHO-H), was employed in a completely randomized design using six replicates (cages). Twelve fish from each cement pond replicate were randomly distributed into 2 cages (80×90×110 cm^3) (n = 6/cage) and fed with either CHO-M or CHO-H. Growth performance was determined. Throughout the experimental period, dead fish were recorded daily. All 30 experimental fish were cultured under a 12/12 h light/dark cycle in a hatchery. Water and air temperature were determined daily, which were in ranges of 27.5-28.6°C and 30.0-36.0°C, respectively. Dissolved oxygen (DO) and pH were recorded weekly using a DO meter and a pH meter, respectively. DO levels of 4.2±0.4 mg L^{-1} (average±SD) and pH of 7.4±0.2 (average±SD), which were acceptable ranges, were found.

Nile tilapia fry were fed with either a High-protein and Low-carbohydrate (HP/LC) or a Low-protein and High-carbohydrate (LP/HC) at the first feeding stage for 4 weeks. At week 1, fry was sampled to examine the expression of genes that are involved in carbohydrate and its related metabolism. See Table 1 for the list of genes and their respective primers. At the end of stimulus (4 weeks), fry were sampled to determine the glycogen and triglyceride contents and the expression of genes that are involved in carbohydrate and its related metabolism. Subsequently, the fish were cultured for a growth trial during 5-36 week post stimulus. During the

growth trial, commercial diets (week 5-8, 40% CP+8% CF; week 9-20, 32% CP+4% CF); week 21-36, 30% CP+4% CF) were used for feeding. Before a challenge test (week 36), fish were sampled to examine the effects of stimulus history. During week 37-1, the fish were subjected to a challenge test with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Fish sampling was carried out before (week 36) and after (week 41) the challenge test for determination of blood metabolites, muscle and hepatic chemical compostion, and the expression of metabolic genes.

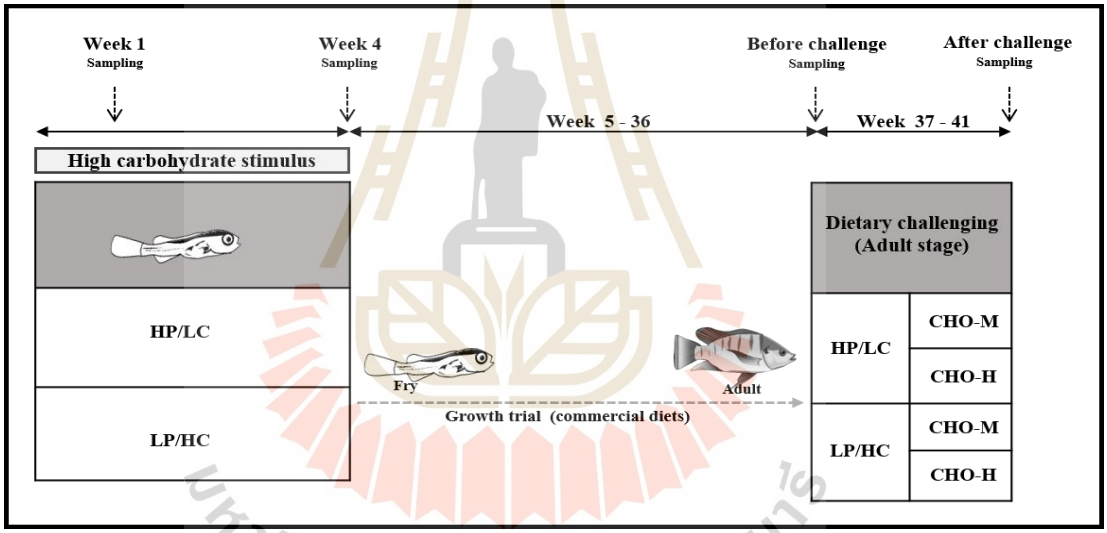

Figure 6.1 Experimental plan for the high-carbohydrate stimulus (history) and the dietary carbohydrate challenge test.

6.3.2 Fish sampling and blood collections

At week 1 of early dietary stimulus, a pool of three fish per replication (total=6 replicates) was sampled for analysis of metabolic gene expression. At the end of early dietary stimulus (4 weeks of feeding), whole bodies of fry (1 fish/replicate; total=6 replications) were collected to examine levels of glycogen and triglyceride

(TG) as well as some metabolic gene expression. For sampling, at 4 h after the last meal (corresponding to the peak of postprandial glycemia in tilapia; 4-5 h after meal), fish were euthanasized by exposing them to freezing cold water.

At week 36 (before the dietary challenge), six fish per pond were sampled for analysis of blood metabolites and metabolic gene expression and chemical composition in the liver and the muscle. At 5 h after the last meal (corresponding to the peak of postprandial glycemia in tilapia), fish were anesthetized with 10% clove oil. Subsequently, blood samples were collected from the caudal vein using a hypodermic syringe and mixed with K_2EDTA (at 1.5 mg /mL^{-1}). Plasma was collected after centrifugation at 3,000×*g* for 10 min at 4°C and stored at -80°C for plasma metabolite determination. Then, liver and muscle tissue samples were taken and frozen in liquid nitrogen and kept at -80°C for metabolic gene expression and chemical composition analysis according to the (AOAC, 1990).

At week 41, to investigate the interactions between early dietary stimuli and the final dietary challenge, fish (2 fish/cage) were collected for analysis of metabolic gene expression, and other fish (3 fish/cage) were selected for analysis of blood metabolites and chemical composition in the liver and the muscle, as described previously for the sampling at week 36.

6.3.3 Blood metabolite analysis

Plasma metabolites including glucose, triglyceride and blood urea nitrogen (BUN) were determined. Plasma glucose was analyzed in duplicate according Trinder's method (Trinder, 1969). The triglyceride levels were also determined in duplicate using the glycerol-3-phosphate oxidase-sodium *N*-ethyl-*N*-(3-sulfopropyl)-*m*-anisidine method, as described by Bucolo and David (Bucolo,1973).

BUN content was measured in duplicate using a modified indophenol colorimetric method (Weatherburn, 1967).

6.3.4 Chemical composition, glycogen and triglyceride analysis

At the end of the early dietary stimulus (week 4), fish was sampled for glycogen and triglyceride determination. At weeks 36 (before the challenge) and 41 (after the challenge), the chemical contents in liver and muscle included moisture, CP, CF, and ash, according to AOAC (1990) (AOAC, 1990). In addition, the hepatic and muscular glycogen contents were determined. The glycogen content was measured using a hydrolysis method with modification (Good et al., 1993). The sample was ground in 1 M HCl. An aliquot was obtained, neutralized by 5 M KOH and subsequently centrifuged at 10,000×g at 4°C for 10 min to measure the free glucose content. Free glucose was measured using a plasma glucose kit (Glucose RTU; bioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions. The other aliquot was boiled at 100°C for 2.5 h and then neutralized by 5 M KOH. After 10 min of centrifugation at 10,000×g at 4°C, total glucose (free glucose + glucose obtained from hydrolysis of glycogen) was analyzed using the glucose kit (Glucose RTU). The glycogen content was calculated by subtracting free glucose levels. To analyze triglyceride (TG), whole bodies of fry were homogenized in liquid nitrogen, and 100 mg of the sample was homogenized again in 1 mL of 5% Igepal in a deionized water solution using a Dounce homogenizer. Samples were heated at 90°C in a water bath for 5 minutes and subsequently cooled down to room temperature. Then, the heated step was repeated. Subsequently, centrifugation was performed at 10,000 g, 4°C for 10 minutes to remove any insoluble material, and supernatants were

collected and diluted with deionized water. TG was measured using a TG plasma kit (Sobioda, Montbonnot, France) following the manufacturer's instructions.

6.3.5 Total RNA extraction and relative quantification of mRNAs

Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to measure relative gene expression of intermediary metabolism (glucose, lipid, and amino acid metabolism). Samples used for total RNA preparation included whole bodies of fry (week 1, pool of 3 fish/replication, n = 18 per experimental group; week 4, 2 fish/replication, n = 12 per experimental group), liver (50 mg) and muscle (100 mg) (weeks 36 and 41, 2 fish/replication, n = 12 per experimental group). Total RNAs were extracted from tissue samples using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The extracted RNA was quantified by NanoDrop (Thermo Fisher, Madison, WI, USA) and verified on a 1% agarose gel. To synthesize cDNA, a SuperScript III RNase H Reverse-Transcriptase Kit (Invitrogen) with random primers (Promega, Charbonnières, France) was used with a sample of 1 µg of total RNA (duplicate for each sample, n = 12 for each treatment group), 100 units of SuperScript III enzyme and 40 units of an RNase OUT enzyme, following the manufacturer's protocol.

Table 6.2 presents the primer sequences used in the RT-PCR assays of each metabolic gene (Boonanuntanasarn et al., 2018a,b; Geurden et al., 2007). The expression of glucose metabolic genes in the liver was measured, including glycolysis (glucokinase, *gck*; phosphofructokinase, *pfklr*; pyruvate kinase, *pklr*) and gluconeogenesis (glucose-6-phosphatase1 and 2, g*6pca1* and *g6pca2*; phosphoenolpyruvate carboxykinase cytosolic, *pck*1; mitochondria, *pck2*). Glucose metabolism in muscles was analyzed by measuring the mRNA levels of glucose transporter (*glut4*) and glycolysis (hexokinase

Table 6.2 List of the primers used for the qRT-PCRs.

Genes	5/′3 ′forward primer	5/′3 ′reverse primer	Access number
Reference gene			
*ef1**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	AB075952
Liver metabolism			
gck	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC	XM003451020
pfklr	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC	XM003447353
pklr	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA	XM005472622
g6pca1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA	XM003448671
g6pca2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA	XM013273429
pck1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA	XM003448375
pck2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT	XM019354843
fasn	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT	XM013276809
g6pd	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT	XM013275693
asat	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC	XM003451918
alat	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG	XM005476466
gdh	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC	XM003457465
Muscle metabolism			
glut4	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG	JN900493
hk1	CGTCGCTTAGTCCCAGACTC	TGACTGTAGCGTCCTTGTGG	XM019360229
hk2	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC	XM003448615
pfkma	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG	XM019349871
pfkmb	TTTGTGCATGAGGGTTACCA	CACCTCCAATCACACACAGG	XM003441476
pkma	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA	XM005447626

*: from Yang et al. 2013.

I and II, *hk1*, and hk2; phosphofructokinase, *pfkma*, and *pfkmb*; pyruvate kinase, *pkma*). Hepatic lipogenic capacities (fatty acid synthase, *fasn*; glucose-6-phosphate dehydrogenase, *g6pd*) were examined. In addition, the enzymes involved in amino acid catabolism (glutamate dehydrogenase, *gdh*; alanine aminotransferase, *alat*; aspartate amino transferase, *asat*) were determined. A Roche Light Cycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France) was used for RT-PCR assays of each level of transcript of all metabolic genes. Assays were performed using a reaction mix of 6 µL per sample, and each of them contained 2 µL of a diluted cDNA template (1: 40), 0.24 µL of each primer (10 µM), 3 µL of Light Cycler 480 SYBR® Green I Master Mix (Roche Diagnostics) and 0.76 µL of DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany). The PCR protocol was initiated at 95°C for 10 min for the initial denaturation of the cDNA and hot-start Taq polymerase activation, followed by 45 cycles of a three-step amplification program (15 s at 95°C, 10 s at 60–64°C [according to the primer set used] and 15 s at 72°C to extend the DNA). The melting curves were systematically analyzed (temperature gradient at 1.1°C/s from 65 to 97°C, five acquisitions/1°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicates of RT and PCR amplification, respectively) and negative controls (reverse-transcriptase- and cDNA-template-free samples, respectively). For the analysis of the mRNA levels, relative quantification of target gene expression was performed using the Roche Applied Science E-Method (Pfaffl, 2001). The relative gene expression of *ef*1α was performed for the normalization of the measured mRNA in each tissue, since its relative expression was not significantly varied according to the sampling process (data not presented). In all cases, PCR efficiency was calculated from the slope of a

standard curve using serial dilutions of cDNA. In all cases, the PCR efficiency values were acceptable and ranged between 1.8 and 2.0.

6.3.6 Enzyme activity assay

The liver is the main metabolic organ, and the enzyme activity of phosphofructokinase (PFK), aspartate amino transferase (ASAT) and glutamate dehydrogenase (GDH) were detected in the liver. At weeks 36 and 41, we performed enzymatic activities for some metabolic enzymes in the liver (2 fish/replication, total = 12) (phosphofructokinase; PFK, aspartate amino transferase; ASAT, glutamate dehydrogenase; GDH) and in the muscle (2 fish/replication, total = 12) (hexokinase; HK, PFK, pyruvate kinase; PK). Tissue samples including the liver (100 mg) or the muscle (200 mg) were homogenized in seven volumes of ice-cold buffer at pH 7.4 (50 mmol L^{-1} Tris, 5 mmol L^{-1} EDTA, 2 mmol L^{-1} DTT) and a protease inhibitor cocktail (P2714; Sigma, St Louis, MO, USA). The homogenates of liver were centrifuged at 10,000 g for 20 min at 4°C, and the supernatants were used for assays of PFK (EC 2. 7. 1. 11) (Borges et al., 2014) and ASAT (EC 2.6.1.1), following the protocol of the commercial kit for enzyme assay (enzyme line, bioMe´rieux). In addition, the hepatic homogenates were sonicated for 1 min, followed by centrifugation at 10,000 g for 20 min at 4°C for the assay of GDH (EC 1.4.1.2) (Gómez-Requeni et al., 2003) .The homogenates of muscle were centrifuged at 900 g for 10 min at 4°C, and the supernatants were used for analysis of HK (EC 2.7.1.1). The hexokinase (HK) low-Km enzyme activities were assayed as previously described by Panserat et al. (2000) (Panserat et al., 2000), In addition, one more centrifugation at 10,000 g for 20 min at 4°C was conducted, and the supernatants were used for assays of PFK (EC 2. 7. 1. 11) (Borges et al., 2014) and PK (EC 2.7.1.40) (Panserat et al., 2001). Enzyme activities

were measured in duplicate at 30°C (PFK) and 37°C (HK, PK, ASAT and GDH), and the nicotinamide adenine dinucleotide phosphates were determined using spectrophotometry at 340 nm. The reactions were initiated by the addition of the specific substrate; a Power Wave X (BioTek Instrument). Deionized water was used as a blank for each assay. Enzyme activity units were defined as micromoles of a substrate converted into product per minute at the assay temperature and expressed as the value per milligram of protein. Protein concentration was measured in duplicate, according to (Bradford, 1976), using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

6.3.7 Statistical analysis

The statistical analyses of the data were performed using SPSS for Windows, version 12 (SPSS Inc., Chicago, IL, USA). An independent *t*-test analysis was conducted to evaluate effects of the early dietary stimulus (HP/LC feeding versus LP/HC-feeding fry) on the carbohydrate and its related metabolic gene expression at week 1 and week 4 (the end of stimulus). In addition, it was used to determine all parameters before the challenge (week 36), including growth performances, plasma metabolites, chemical composition in the liver and the muscle and the expression of genes related to carbohydrate metabolism. After the nutritional challenge (week 41), two-way factorial ANOVA was conducted to determine two combination factors including the effects of early dietary stimulus and the dietary carbohydrate challenge and their interactions. When the interaction of the factors was statistically significant, one-way ANOVA following Tukey's range test was used to rank the treatment combination groups. Throughout the experiment, the effects and differences were declared to be significant when $P<0.05$.

6.4 Results

6.4.1 Direct effects of the early high-carbohydrate stimulus in fish

During the period of stimulus (first-feeding with LP/HC or HP/LC diets for 4 weeks), there were no differences in mortality rates ($P>0.05$) (Table 6.3). Growth performances for the two groups of fish are presented in Table 6.3. During the stimulus period, growth response including final weight (FW), weight gain (WG), average daily gain (ADG) and specific growth rate (SGR) of fish fed the LP/HC diet was lower than that observed in fish fed the HP/LC diet ($P<0.05$). Indeed, at the end of the stimulus, the body weight of fish fed HP/LC at first-feeding was significantly higher (1.4 times) than the one observed in fish fed LP/HC at first-feeding ($P<0.05$) (Table 6.3). Note that feed intake (FI) through the end of stimulus (weeks 1 and 4) was performed at an excess amount to ensure the supply amount of diet for fry (according to commercial protocol).

The early stimulus by intake of high-carbohydrate diet exerted significant effects on glucose metabolism.

The direct effects of the intake of the high-carbohydrate diet on the expression of metabolic genes were measured at week 1 (during the stimulus period) and at week 4 (end of the stimulus period) (Table 6.4). After 1 week of feeding the high-carbohydrate diet, there were no significant differences in expression of genes involved in glycolysis in the liver, whereas the expression of several muscular glycolytic genes changed. Indeed, up-regulation of *hk2* was observed, whereas down-regulation of *pfkmb* was found ($P<0.05$). In addition, the *glut4* mRNA level was significantly decreased in LP/HC-fed fry ($P<0.05$). Moreover, significant down-regulation of genes involved in gluconeogenesis (*g6pca1*, *g6pca2*, *and pck1*) and

amino acid catabolism (*asat and alat*) was also detected in LP/HC-fed fry (P<0.05) (Table 6.4). At the end of the high-carbohydrate stimulus (week 4), the effect of hyperglucidic stimulus was observed, although it was weaker than the one observed after 1 week of stimulus. Indeed, the effects of down-regulation of genes involved in gluconeogenesis and amino acid catabolism were no more found in LP/HC-fed fry (P<0.05) at the end of stimulus (Table 6.4). By contrast, down-regulation of *glut4* and *pfkmb* and up-regulation of *hk2* mRNAs were always observed in LP/HC-fed fry (P<0.05). Additionally, up-regulation of *pkma* and *g6pd in LP*/*HC fish* was detected for the first time (P<0.05) (Table 6.4). Finally, LP/HC-fed fish had higher glycogen and triglyceride contents than that of HP/LC-fed fish (Figure 6.2A-B). Overall, intake of high-carbohydrate diet exerted significant effects on glucose metabolism, indicating that the early stimulus was effective in the present study.

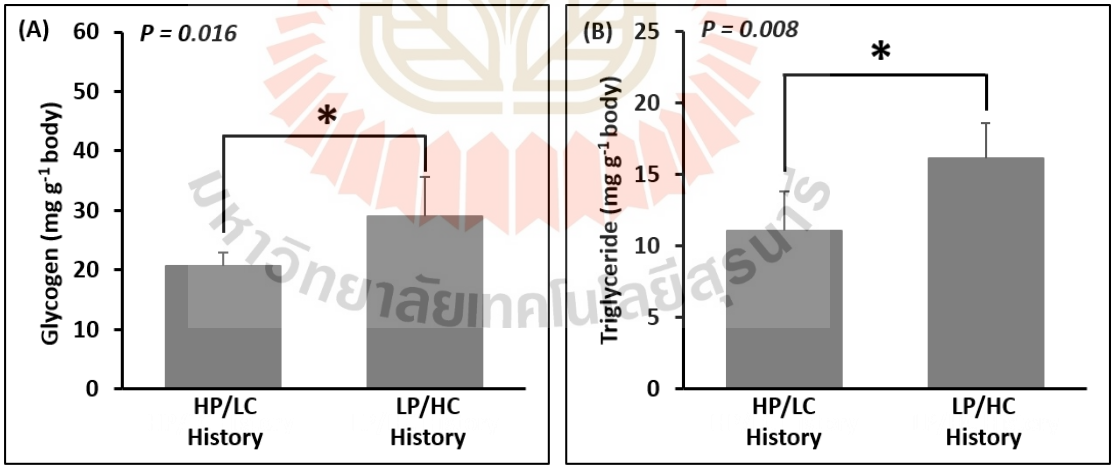

Figure 6.2 Chemical composition of fish after the early dietary stimulus. Dietary stimulus including HP/LC and LP/HC diets (History) was performed since first-feeding through 4 weeks, at the end of stimulus, the fry was sampling for determination of glycogen (A) and triglyceride (B) contents. The asterisk in the bar graph indicates a significant difference (P<0.05).

Table 6.3 Growth performances of Nile tilapia that were fed with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) (mean ± SD, $n = 6$) during the early stimulus and the feeding (4 first weeks) with a commercial diet (up to week 36).

Experimental periods	**FW**[3] **(g)**	**WG**[4] **(g)**	**ADG**[5] **(g day^{-1})**	**SGR**[6] **(% day^{-1})**	**FI**[7] **(g day^{-1})**	**FCR**[8]	**Survival rate**[9] **(%)**
1 Week (during stimulus)[1]							
HP/LC	53.7 ± 3.2 mg	44.1 ± 3.2 mg	6.3 ± 0.5 mg day^{-1}	24.5 ± 0.8	14.6 ± 0.0 mg day^{-1}	2.4 ± 0.2	No Mortality
LP/HC	52.3 ± 3.5 mg	42.7 ± 3.5 mg	6.1 ± 0.5 mg day^{-1}	24.2 ± 1.0	14.6 ± 0.0 mg day^{-1}	2.3 ± 0.2	No Mortality
P- value[2]	0.514	0.492	0.490	0.482	1.000	0.481	-
4 Weeks (end of stimulus)[1]							
HP/LC	730.7 ± 14.5 mg	721.1± 14.5 mg	25.8 ± 0.5 mg day^{-1}	15.5 ± 0.1	105.9 ± 8.3 mg day^{-1}	4.1 ± 0.2	No Mortality
LP/HC	517.1 ± 15.5 mg	507.5 ±15.6 mg	18.1 ± 0.6 mg day^{-1}	14.2 ± 0.1	88.4 ± 6.6 mg day^{-1}	4.8 ± 0.3	No Mortality
P- value[2]	<0.001	<0.001	<0.001	<0.001	0.002	0.001	-
8 Weeks (4 weeks after stimulus)							
HP/LC History	3.40 ± 0.12	3.39 ± 0.11	0.06 ± 0.00	9.94 ± 0.06	0.15 ± 0.0	1.8 ± 0.1	No Mortality
LP/HC History	2.95 ± 0.11	2.94 ± 0.12	0.05 ± 0.00	9.70 ± 0.07	0.19 ± 0.0	2.0 ± 0.1	No Mortality
P- value[2]	<0.001	<0.001	0.001	<0.001	<0.001	0.002	
24 Weeks (20 weeks after stimulus)							

Table 6.3 (Continues).

Experimental periods	FW[3] (g)	WG[4] (g)	ADG[5] (g day^{-1})	SGR[6] (% day^{-1})	FI[7] (g day^{-1})	FCR[8]	Survival rate[9] (%)
HP/LC History	42.24 ± 0.63	42.23 ± 0.63	0.25 ± 0.00	4.99 ± 0.01	0.52 ± 0.01	1.79 ± 0.05	No Mortality
LP/HC History	41.11 ± 0.95	41.10 ± 0.95	0.24 ± 0.01	4.98 ± 0.01	0.54 ± 0.00	1.81 ± 0.03	No Mortality
P- value[2]	0.036	0.032	0.001	0.036	0.001	0.393	
36 Week (32 weeks after stimulus)							
HP/LC History	219.44 ± 4.93	219.43 ± 4.93	0.87 ± 0.02	3.98 ± 0.00	1.26 ± 0.05	1.31 ± 0.05	97.7 ± 2.1
LP/HC History	216.65 ± 1.98	216.64 ± 1.98	0.86 ± 0.01	3.98 ± 0.01	1.36 ± 0.05	1.39 ± 0.04	98.2 ± 1.8
P- value[2]	0.226	0.226	0.467	0.363	0.008	0.013	0.661

[1] Note that feed intake through the end of stimulus (weeks 1 and 4) was followed according to commercial protocol. Swim-up fry were fed the experimental diet at 30% body weight during week 1, 20% body weight during week 2, and 10% body weight during weeks 3-4 [(18)]; therefore, over calculated FCR was presented.

[2] A t-test analysis was used to analyze the effects of different stimulus between high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) diets.

[3] Final body weight (FW).

[4] Weight gain (WG) = final body weight-initial body weight.

[5] Average daily gain (ADG) = (final body weight-initial body weight)/experimental days.

[6] Specific growth rate (SGR) = 100 × [(ln final body weight- ln initial body weight)/experimental days].

[7] Feed intake (FI) = dry feed fed/experimental days.

[8] Feed conversion ratio (FCR) = dry feed fed/wet weight gain.

[9] Survival rate = 100 × [(Initial number of fish- Final number of final) / Initial number of fish].

Table 6.4 mRNA levels of genes involving in carbohydrate metabolism in whole body of Nile tilapia that were fed with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) at 1 and 4 weeks post stimulus (mean ± SD, n=6).

mRNA level	1 week post stimulus			4 weeks post stimulus		
	HP/LC History	LP/HC History	*P-Value*[1]	HP/LC History	LP/HC History	*P-Value*[1]
Glycolysis						
gck	0.7 ± 0.4	1.3 ± 0.8	0.109	1.0 ± 0.4	1.3 ± 0.5	0.240
pfklr	1.2 ± 0.5	1.1 ± 0.2	0.660	1.0 ± 0.3	0.9 ± 0.2	0.422
pklr	1.6 ± 0.6	1.2 ± 0.2	0.156	1.2 ± 0.4	0.8 ± 0.1	0.610
Gluconeogenesis						
g6pca1	2.1 ± 0.8	0.3 ± 0.1	0.003	1.2 ± 0.3	1.0 ± 0.1	0.402
g6pca2	2.3 ± 0.8	0.3 ± 0.1	0.001	0.9 ± 0.3	0.7 ± 0.1	0.137
pck1	2.3 ± 1.0	0.3 ± 0.1	0.005	0.9 ± 0.6	1.0 ± 0.5	0.908
pck2	0.9 ± 0.6	1.1 ± 0.4	0.582	0.8 ± 0.2	0.8 ± 0.1	0.441
Lipogenesis						
fasn	1.6 ± 0.5	1.2 ± 0.2	0.175	1.1 ± 0.4	0.9 ± 0.1	0.191
g6pd	1.4 ± 0.5	1.1 ± 0.2	0.298	0.7 ± 0.1	0.9 ± 0.1	0.032
Amino acid catabolism						
asat	2.0 ± 0.7	0.6 ± 0.2	0.002	1.0 ± 0.3	0.8 ± 0.2	0.103
alat	1.6 ± 0.7	0.9 ± 0.2	0.042	1.0 ± 0.1	1.0 ± 0.1	0.363
gdh	1.1 ± 0.6	1.2 ± 0.2	0.628	0.5 ± 0.1	0.6 ± 0.1	0.287

Table 6.4 (Continous).

mRNA level	1 week post stimulus			4 weeks post stimulus		
	HP/LC History	**LP/HC History**	***P-Value*[1]**	**HP/LC History**	**LP/HC History**	***P-Value*[1]**
Glucose transport and glycolysis						
glut4	1.5 ± 0.5	1.0 ± 0.2	0.040	1.1 ± 0.2	0.6 ± 0.1	0.002
hk1	1.4 ± 0.5	0.9 ± 0.2	0.074	1.1 ± 0.3	0.9 ± 0.1	0.304
hk2	0.9 ± 0.3	1.4 ± 0.3	0.004	0.8 ± 0.1	1.1 ± 0.2	0.016
pfkma	1.2 ± 0.4	1.1 ± 0.1	0.712	1.0 ± 0.4	0.9 ± 0.1	0.400
pfkmb	1.7 ± 0.5	0.9 ± 0.1	0.010	1.2 ± 0.3	0.8 ± 0.2	0.030
Pkma	1.6 ± 0.7	1.3 ± 0.3	0.394	0.9 ± 0.1	1.1 ± 0.1	0.028

[1] t-test analysis was used to analyse the effects of differences stimulus between high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC).

Table 6.5 Growth performance of Nile tilapia fed with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) at before (36 wps) and after (41 wps) challenging with CHO-M and CHO-H for four weeks (mean ± SD, n=6).

Parameters	HP/LC History		LP/HC History		*P-Value*[1]		
	CHO-M	CHO-H	CHO-M	CHO-H	History	Diet	Interactions
IW (g)	211.1 ± 2.2	211.7 ± 2.0	210.1 ± 0.9	211.3 ± 2.1	0.338	0.257	0.700
FW (g)	322.3 ± 7.5	300.9 ± 6.5	331.2 ± 7.6	316.5 ± 8.1	<0.001	<0.001	0.293
WG[2] (g)	111.2 ± 7.4	89.2 ± 7.6	121.2 ± 6.9	105.3 ± 9.8	<0.001	<0.001	0.371
ADG[3] (g day^{-1})	3.4 ± 0.2	2.7 ± 0.2	3.7 ± 0.2	3.2 ± 0.3	<0.001	<0.001	0.378
SGR[4] (% day^{-1})	1.3 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	<0.001	<0.001	0.372
FI[5] (g day^{-1})	3.5 ± 0.2	3.4 ± 0.4	3.5 ± 0.3	3.3 ± 0.3	0.958	0.359	0.577
FCR[6]	1.0 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	0.034	0.013	0.214
Survival[7] (%)	91.7 ± 9.1	94.4 ± 8.6	94.4 ± 8.6	94.4 ± 8.6	0.701	0.701	0.701

[1]Two-way ANOVA was used to analyse the effects of dietary stimulus (history), challenging diet (Diet) and their interaction (History × Diet).
[2] Weight gain (WG) = final body weight- initial body weight.
[3]Average daily gain (ADG) = (final body weight-initial body weight)/experimental days.
[4]Specific growth rate (SGR) = 100 × [(ln final body weight-ln initial body weight)/experimental days].
[5]Feed intake (FI) = dry feed fed/experimental days.
[6]Feed conversion ratio (FCR) = dry feed fed/wet weight gain.
[7] Survival rate = 100 x [(Initial number of fish - Final number of final)/ Initial number of fish].

Table 6.6 Proximate composition of Nile tilapia fed with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) at before (36 wps) and after (41 wps) challenging with CHO-M and CHO-H for four weeks (mean ± SD, n=6).

Parameter	**History (before challenge)**			**HP/LC History**		**LP/HC History**		***P-Value*[1]**		
Tissue	**HP/LC**	**LP/HC**	***P- Value***	**CHO-M**	**CHO-H**	**CHO-M**	**CHO-H**	**History**	**Diet**	**Interactions**
LIVER (g kg^{-1})										
Protein	102.4 ± 2.2	109.6 ± 2.1	0.001	103.0 ± 3.2^{a}	94.2 ± 2.3^{c}	97.2 ± 3.3^{bc}	98.9 ± 2.6^{ab}	0.674	0.007	<0.001
Fat	42.7 ± 0.4	43.6 ± 0.6	0.007	30.0 ± 2.3^{d}	46.1 ± 3.2^{b}	36.7 ± 2.5^{c}	62.8 ± 2.0^{a}	<0.001	<0.001	<0.001
Ash	13.9 ± 0.1	14.0 ± 0.3	0.466	14.7 ± 0.5	14.2 ± 1.1	14.1 ± 1.0	14.7 ± 0.7	0.962	0.955	0.150
HSI[2] (%)	4.0 ± 0.1	4.0 ± 0.3	0.661	3.5 ± 0.1	4.0 ± 0.4	3.6 ± 0.3	4.4 ± 0.3	0.073	<0.001	0.168
Glycogen (mg g^{-1})	209.7 ± 4.4	236.8 ± 19.2	0.007	210.8 ± 41.8	287.0 ± 45.8	327.0 ± 31.1	342.3 ± 29.3	0.007	<0.001	0.061
Muscle (g kg^{-1})										
Protein	174.4 ± 1.8	171.8 ± 2.8	0.086	173.6 ± 4.7^{ab}	167.2 ± 4.5^{b}	178.7 ± 4.9^{a}	159.9 ± 1.9^{c}	0.530	<0.001	0.002
Fat	17.1 ± 1.5	15.5 ± 1.0	0.063	23.3 ± 3.5	41.3 ± 2.4	25.3 ± 3.7	43.5 ± 1.3	0.089	<0.001	0.899
Ash	14.1 ± 0.2	14.3 ± 0.2	0.117	15.4 ± 0.4	15.8 ± 0.9	15.9 ± 0.6	16.0 ± 0.5	0.165	0.310	0.671
Glycogen (mg g^{-1})	6.4 ± 0.3	7.5 ± 0.8	0.008	6.8 ± 2.0	8.2 ± 0.9	8.4 ± 1.2	9.2 ± 0.8	0.023	0.059	0.552

[1]Two-way ANOVA was used to analyse the effects of dietary stimulus (history), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values for four combination groups (P<0.05).

[2]Hepatosomatic index (HSI) =100 × (liver weight / body weight).

Table 6.7 Plasma metabolites of Nile tilapia fed with high-protein/low-carbohydrate (HP/LC) and low protein/high-carbohydrate (LP/HC) at before (36 wps) and after (41 wps) challenging with CHO-M and CHO-H for four weeks (mean ± SD, n=6).

Parameters	**History (before challenge)**			**HP/LC History**		**LP/HC History**		***P-Value*[1]**		
	HP/LC	**LP/HC**	***P Value***	**CHO-M**	**CHO-H**	**CHO-M**	**CHO-H**	**History**	**Diet**	**Interactions**
Glucose (mM)	3.6 ± 0.6	4.2 ± 0.9	0.185	4.3 ± 0.3	4.2 ± 0.5	4.9 ± 0.4	5.2 ± 0.3	<0.001	0.515	0.127
Triglyceride (mM)	3.0 ± 0.5	2.8 ± 0.5	0.549	1.6 ± 0.3	2.0 ± 0.3	1.6 ± 0.5	2.4 ± 0.5	0.247	0.002	0.275
BUN[2] (mM)	1.1 ± 0.1	1.1 ± 0.1	0.616	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.007	<0.001	0.294

[1]Two-way ANOVA was used to analyse the effects of dietary stimulus (history), challenging diet (Diet) and their interaction (History × Diet).

[2]BUN: blood urea nitrogen.

Therefore, the experimental fish were maintained for evaluation of the persistent effects in adulthood. No differences were observed in mortality rates between fish previously fed LP/HC and HP/LC diets, through 36 weeks of the experimental period ($p>0.05$) (Table 6.3). During the feeding period with a commercial diet, LP/HC history fish caught up their body weight with HP/LC history fish by week 36. Indeed, feed intake (FI) and feed conversion ratio (FCR) of LP/HC history fish were higher than those of HP/LC history fish ($P>0.05$) (Table 6.3).

6.4.2 Long-term effects of early high-carbohydrate stimulus in interaction with the high-dietary-carbohydrate challenge in adult fish

To the existence of nutritional programming in relation to the early high-carbohydrate stimuli history, both HP/LC history and LP/HC history fish were challenged with a high-carbohydrate diet (CHO-H) at weeks 37-41. Results of the two effects (history and challenge) and their interaction on growth performance (Table 6.5), hepatic and muscular composition (Table 6.6), plasma metabolites (Table 6.7) and mRNA levels of genes involved in intermediary metabolism (Table 6.8 and Figure 6.3-6.4) were obtained.

Regarding the growth performance, the results indicated that, independently of the two diets for the challenge, LP/HC history fish had higher final body weight, ADG and SGR and lower FCR compared to the HP/LC history fish ($P< 0.05$) (Table 6.5). As expected, irrespective of the dietary stimulus, CHO-H led to lower growth performance when compared to that in fish fed the CHO-M diet ($P<0.05$) (Table 6.5).

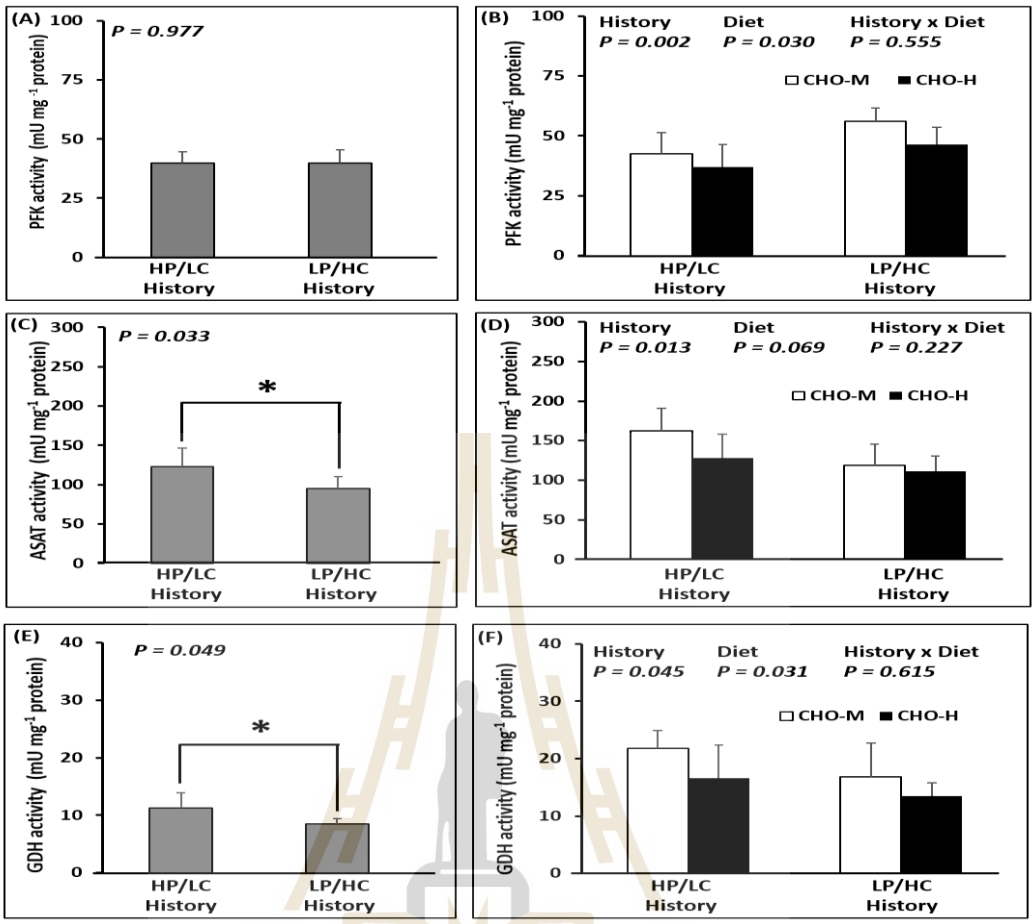

Figure 6.3 Enzyme activities (mU mg-/1 protein) in the livers of Nile tilapia that were early fed with HP/LC diet or LP/HC diet (history) and challenged in adult with CHO-M or CHO-H diets. PFK (A, B) which are involved in glycolysis, ASAT (C, D) and GDH (E, F), in amino acid catabolism were analysed in the liver samples. At week 36-41, the fish were subjected to a challenge test with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Before the challenge test, the fish were sampled to determine the effect of stimulus diet history on the PFK, ASAT and GDH activities in the liver (A, C, E). The asterisk in the bar graph indicates a significant difference ($P<0.05$). After the challenge test (week 41), the combination effects of stimulus history and challenging

diet on the PFK, ASAT and GDH activities in the liver were examined (B, D, F). Data are presented as the mean±standard deviation (SD) (n=6). Two-way ANOVA was used to analyse the effects of stimulus diets (History), challenging diets (Diet) and their interaction (History×Diet).

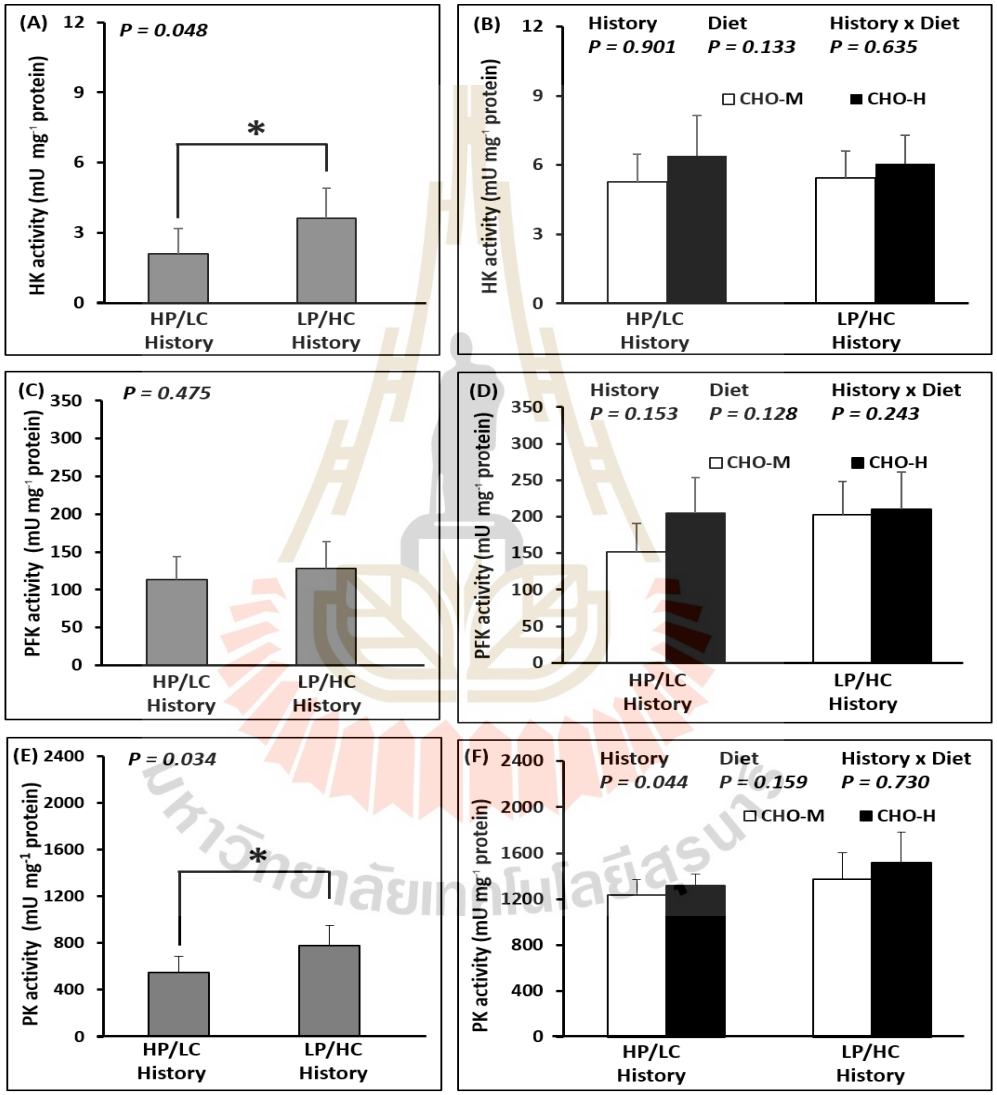

Figure 6.4 Enzyme activities (mU mg-/1 protein) in the muscles of Nile tilapia that were early fed with HP/LC diet or LP/HC diet (history) and challenged in adult with CHO-M or CHO-H diets. HK (A, B), PFK (C, D) and PK (E, F) which are involved in glycolysis were analysed in the muscle

samples. At week 36-41, the fish were submitted to a challenge test with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Before the challenge test, the fish were sampled to determine the effect of stimulus diet history on the HK, PFK and PK activities in the muscle (A, C, E). The asterisk in the bar graph indicates a significant difference ($p<0.05$). After the challenge test (week 41), the combination effects of stimulus history and challenging diet on the HK, PFK and PK activities in the muscle were examined (B, D, F). Data are presented as the mean±standard deviation (SD) (n=6). Two-way ANOVA was used to analyse the effects of stimulus diets (History), challenging diets (Diet) and their interaction (History×Diet).

The effects of the early high-carbohydrate stimulus and the high-dietary-carbohydrate final challenge on chemical composition in the liver and the muscle are presented in Table 6.6. Before the dietary challenge, early high-carbohydrate (LP/HC) history is associated with permanent effects on increased protein, lipid and glycogen in the liver ($P<0.05$). Moreover, for muscle composition (Table 6.6), early high-carbohydrate feeding history led to significantly increased glycogen in adult tilapia ($P<0.05$). Later after the dietary challenge, early high-carbohydrate history is associated with an increase of hepatic fat and glycogen in both the liver and the muscle ($P<0.05$). Moreover, dietary CHO-H led to significantly decreased hepatic protein contents ($P<0.05$) and increased fat and glycogen levels with higher his ($P<0.05$). Finally, fish fed CHO-H had lower levels of protein and higher levels of fat in the muscle ($P<0.05$). Interaction effects between nutritional

history (stimulus) and the final challenge were also found for the hepatic fat and protein in both the liver and the muscle. Indeed, LP/HC-fed fish fed the CHO-H diet had the higher fat liver and lower protein content in the muscle ($P<0.05$) (Table 6.6).

Even though just, before the final dietary challenge, early high-carbohydrate history had no significant effects on the plasma metabolites (glucose, triglyceride, and BUN) ($P>0.05$) (Table 6.7). By contrast, LP/HC history fish had higher plasma glucose and lower BUN after the final irrespective of the diets ($P< 0.05$). Finally, fish fed the CHO-H diet exhibited an increase in plasma triglyceride and a decrease in BUN ($P<0.05$) (Table 6.7).

Before the dietary challenge, fish with an early high-carbohydrate history had higher mRNA levels of hepatic *gck* and muscle *hk1* and *pkma* and higher enzymatic activities for the latter two ($P<0.05$) (Table 6.8 and Figure 6.4A, 6.4E). In addition, there was down-regulation of hepatic *asat* (mRNA and ASAT activity), *alat* (mRNA) and *gdh* (not mRNA but only glutamate dehydrogenase activity), as well as muscle *pfkmb* (only mRNA but not phosphofructokinase activity) ($P<0.05$) (Table 6.8 and Figure 6.3-6.4). The transcripts of other genes such as *pfklr*, *pklr*, *g6pca1*, *g6pca2*, *pck1*, *pck2*, *fasn*, *g6pd*, *glut4*, *hk2*, *pfkma* and hepatic PFK enzyme activity remained unchanged ($P>0.05$). When fish with HP/LC history and LP/HC history had been subjected to a final dietary challenge with CHO-M and CHO-H diets, the effects of early high-carbohydrate feeding were always detectable as reflected by the down-regulation of *asat* (mRNA and ASAT activity), *alat* (mRNA) and *pfkmb* (mRNA but not pfk activity) ($P<0.05$) (Table 6.8; Figure 6.3-6.4). However, only early high-carbohydrate history fish was associated with a decrease in hepatic gdh and an increase in muscular pk activities ($P<0.05$) (Table 6.8 and Figure 6.3-6.4). It must be

noted also that hepatic *pfklr* mRNA decreased, whereas the pfk activity increased (P< 0.05) (Table 6.8 and Figure 6.3B). There were no significant differences in expression of *gck*, *pklr*, *g6pca1*, *g6pca2*, *pck1*, *pck2*, *fasn*, *g6pd*, *glut4*, *hk1* (mRNA and its activity), *hk2* (mRNA and its activity) and *pfkma* (mRNA and its activity) (P>0.05) (Table 6.8; Figure 6.4). For the specific effects of the dietary challenge, fish fed the CHO-H diet indicated increased expression of *fasn* and *g6pd* and decreased mRNA level of *alat* (P<0.05) (Table 6.8). In addition, CHO-H resulted in reduction of PFK and GDH activities (P<0.05) (Figure 6.3B, 6.3F). The effects of the dietary challenge were no detectable for another gene expression at mRNA levels (*gck*, *pfklr*, *pklr*, *g6pca1*, *g6pca2*, *pck1*, *pck2*, *asat*, *gdh*, *glut4*, *hk1*, *hk2*, *pfkma*, *pfkmb*, *and pkma*) and enzyme activities (hepatic ASAT and muscles HK, PFK and PK) (P>0.05) (Table 6.8 and Figure 6.3). Interactions between history and the challenge were observed for mRNA levels of *pfklr*, *glut4*, *hk2*, *pfkma*, and *pkma*. In fact, HP/LC-fed fish fed on CHO-M had the highester *pfklr* mRNA level. Meanwhile, the highest *glut4* mRNA level was observed in LP/HC-fed fish fed CHO-H (P<0.05) (Table 6.8).

Table 6.8 mRNA levels of genes involved in carbohydrate metabolism in the livers and muscles of fish fed with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) at before (36 wps) and after (41 wps) challenging with CHO-M and CHO-H for four weeks (mean ± SD, n=6).

Parameter	**History (before challenge)**			**HP/LC History**		**LP/HC History**		***P Value*[1]**		
Tissue	**HP/LC**	**LP/HC**	***P Value***	**CHO-M**	**CHO-H**	**CHO-M**	**CHO-H**	**History**	**Diet**	**Interactions**
Liver glycolysis										
gck	0.5 ± 0.2	1.5 ± 0.8	0.027	0.9 ± 0.6	0.8 ± 0.3	0.7 ± 0.4	0.6 ± 0.2	0.259	0.569	0.947
pfklr	1.2 ± 0.6	1.2 ± 0.9	0.888	0.7 ± 0.2[a]	0.4 ± 0.2[b]	0.3 ± 0.1[b]	0.3 ± 0.1[b]	0.009	0.057	0.046
pklr	1.0 ± 0.3	1.5 ± 0.7	0.088	1.0 ± 0.6	0.8±0.5	1.0 ± 0.6	0.6 ± 0.2	0.418	0.122	0.503
Liver gluconeogenesis										
g6pca1	1.4 ± 0.7	0.9 ± 0.6	0.226	0.8 ± 0.5	0.6 ± 0.4	0.6 ± 0.4	0.4 ± 0.2	0.176	0.176	0.937
g6pca2	1.3 ± 1.0	0.6 ± 0.5	0.164	0.5 ± 0.3	0.4 ± 0.4	0.5 ± 0.3	0.2 ± 0.1	0.297	0.100	0.332
pck1	0.7 ± 0.4	0.6 ± 0.3	0.617	0.1 ± 0.1	0.8 ± 0.9	0.0 ± 0.1(nd)	0.1 ± 0.1(nd)	0.055	0.064	0.093
pck2	0.9 ± 0.5	0.4 ± 0.4	0.078	1.1 ± 0.7	0.5 ± 0.4	0.9 ± 0.6	0.6 ± 0.4	0.794	0.054	0.584
Liver lipogenesis										
fasn	0.7 ± 0.4	1.2 ± 0.6	0.158	0.5 ± 0.3	0.9 ± 0.8	0.4 ± 0.2	0.9 ± 0.3	0.979	0.018	0.636
g6pd	1.0 ± 0.3	1.6 ± 0.6	0.056	0.5 ± 0.3	0.8 ± 0.3	0.3 ± 0.2	1.0 ± 0.4	0.768	0.001	0.180

Table 6.8 mRNA levels of genes involved in carbohydrate metabolism in the livers and muscles of fish fed with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) at before (36 wps) and after (41 wps) challenging with CHO-M and CHO-H for four weeks (mean ± SD, n=6).

Parameter	**History (before challenge)**			**HP/LC History**		**LP/HC History**		***P Value*[1]**		
Tissue	**HP/LC**	**LP/HC**	***P Value***	**CHO-M**	**CHO-H**	**CHO-M**	**CHO-H**	**History**	**Diet**	**Interactions**
Liver amino acid catabolism										
asat	1.3 ± 0.5	0.5 ± 0.3	0.049	0.6 ± 0.3	0.8 ± 0.4	0.5 ± 0.2	0.4 ± 0.3	0.046	0.727	0.311
alat	1.3 ± 0.4	0.8 ± 0.4	0.048	1.4 ± 0.4	0.7 ± 0.4	0.7 ± 0.3	0.5 ± 0.3	0.005	0.007	0.137
gdh	0.9 ± 0.3	1.4 ± 0.5	0.053	1.1 ± 0.3	0.8 ± 0.5	0.8 ± 0.3	0.6 ± 0.3	0.158	0.096	0.684
Glucose transport and Muscle metabolism										
glut4	0.7 ± 0.2	1.1 ± 0.5	0.152	0.8 ± 0.2^{ab}	0.6 ± 0.3^{b}	0.7 ± 0.4^{ab}	1.4 ± 0.8^{a}	0.060	0.157	0.030
hk1	0.9 ± 0.2	1.5 ± 0.4	0.009	0.9 ± 0.3	1.0 ± 0.5	0.8 ± 0.3	1.4 ± 0.7	0.605	0.092	0.207
hk2	1.0 ± 0.4	1.2 ± 0.2	0.250	1.2 ± 0.4	0.9 ± 0.2	0.6 ± 0.5	1.2 ± 0.6	0.411	0.388	0.040
pfkma	0.7 ± 0.3	0.9 ± 0.4	0.369	1.0 ± 0.2	0.6 ± 0.2	0.6 ± 0.4	1.0 ± 0.5	0.865	0.873	0.019
pfkmb	1.2 ± 0.4	0.8 ± 0.1	0.044	1.2 ± 0.4	1.2 ± 0.5	0.5 ± 0.1	0.5 ± 0.2	<0.001	0.909	0.957
pkma	0.5 ± 0.2	0.7 ± 0.2	0.030	1.2 ± 0.3	1.0 ± 0.5	0.7 ± 0.2	1.1 ± 0.3	0.179	0.357	0.035

[1]Two-way ANOVA was used to analyse the effects of dietary stimulus (history), challenging diet (Diet) and their interaction (History × Diet).

Different letters indicate significant differences in the mean values for four combination groups (P<0.05).

6.5 Discussion

The concept of nutritional programming has been recently applied for better use of new feeds in aquaculture (Panserat et al., 2019, Hou and Fuiman, 2019). Because early dietary feeding is the most popular early nutritional intervention, a high-carbohydrate diet during first-feeding was applied to have original data in Nile tilapia. Indeed, several reports have described nutritional programming through early fry feeding in different, mainly carnivorous, fish species (Geurden et al., 2007, 2014; Gong et al., 2015; Liang et al., 2017; Rocha et al., 2016a,b; Fang et al., 2014). Our study, therefore, provides original data about the long-term effect of feeding high levels of carbohydrates at first-feeding on the metabolism and growth in adult Nile tilapia, an omnivorous fish species.

6.5.1 Effective effects of the early high-carbohydrate intake in tilapia fry

As expected, fish fed HP/LC-fed fry had higher weight gain (1.4 fold) than that those fed LP/HC-fed fry. It must be noted that there were no detrimental effects (decrease of survival and malformations) for the vital development of fish.

Before the test of the programming concept, we had to be sure that the tilapia "received" well the early dietary stimulus. At the end of the stimulus period, our results indicated higher whole body glycogen and triglyceride contents in fish fed LP/HC diets, which suggest much higher intake of carbohydrates in this experimental fish. Indeed, molecular data about intermediary metabolism confirm this observation at the beginning (week 1) and at the end (week 4) of the stimulus. Overall, high-carbohydrate diet stimulus suppressed muscular glucose transporter *glut4*. It also inhibited several enzymes involved in gluconeogenesis (*g6pca1, g6pca2, and pck1*) and amino acid catabolism (*asat and alat*). Although the modulatory effect was not

detectable for hepatic glycolysis, it was for muscular glycolysis (*hk2 and pfkmb*). Note that high-carbohydrate stimulus for week 4 induced lipogenetic *g6pd*. These findings suggest that fish responded to early stimuli, *i.e*., hyperglucidic diet is associated with an inhibition of gluconeogenesis, amino acid catabolism, glucose transporter and induction of lipogenesis, as it has been previously observed in other fish species (, European seabass, sturgeon, zebrafish, and gilthead seabream) with carbohydrates at first-feeding (Geurden et al., 2007,2014; Gong et al., 2015; Zambonino-Infante et al., 2019; Rocha et al., 2016b; Fang et al., 2014; HU et al., 2018). Although the results were not totally similar, our results were also in line specifically with the findings previously reported in Nile tilapia, which was acutely treated with glucose injection into yolk reserves (Kumkhong et al., 2020). Notably, but not well understood, our results indicate that at the molecular level, dietary LP/HC from the first-feeding influenced several carbohydrate-metabolism-related pathways and that the effects of stimulus at week 1 seemed to be stronger than those of detection at week 4. Combined all findings together, a hyperglucidic stimulus at early feeding with the high-carbohydrate diet are effective in changing intermediary metabolism (specially the glucose metabolism). The question was whether after the stimulus, this early dietary stimulus could be remembered by the tilapia later in life.

6.5.2 Positive long-term effects of the early high-carbohydrate stimulus on growth and metabolism in adult fish

After the stimulus, LP/HC fish exhibited higher growth performance through an increase of feed intake, which enabled them to catch up with HP/LC fish at week 36 (just before the dietary challenge), demonstrating the existence of compensatory mechanisms that have been previously observed in tilapia (Wang et al.,

2000). Our results indicated that the early high-carbohydrate diet history was associated with persistent effects on hepatic biochemical composition, including higher levels of protein, lipid, and glycogen contents and muscle glycogen in adult tilapia. Moreover, glucose metabolic pathway was also modified at molecular level. Indeed, in fish fed the early high-carbohydrate diet, there was up-regulation of glycolytic *gck, hk, and pkma* and down-regulation of amino acid catabolism (*asat and alat*). All these data were similar to what we observed previously in juvenile tilapia that were injected with glucose into the yolk (Kumkhong et al., 2020). Taken together, effects of early hyperglucidic intervention (through either glucose injection into yolk or early feeding with carbohydrates) seems to have strong impacts later in the life of tilapia, particularly by inducing glycolysis and lipogenesis and suppressing amino acid catabolism. In other words, tilapia seems to have better capacity to use glucose by sparing proteins (from amino acid catabolism).

6.5.3 Positive long-term effects of the early high-carbohydrate stimulus irrespective of the final dietary challenge on growth and metabolism of adult fish

During weeks 37-41, all fish were subjected to a dietary challenge with either a high-carbohydrate diet (CHO-H) or a medium-carbohydrate diet (CHO-M). The diets of the dietary challenge are consistent with our previous data (Boonanuntanasarn et al., 2018a,b). The growth performance is better with diet CHO-M than with CHO-H. Notably, the early high-carbohydrate diet stimulus, irrespective of the challenge diet, has a positive effect on the growth performance of adult tilapia. These observations are similar to the previous findings on the effects of the early glucose injection on the improvement of growth performance in juvenile in Nile

tilapia (Kumkhong et al., 2020). By contrast, no improvement of growth performance was observed with early hyperglucidic stimulus in other fish species such as zebrafish, gilthead seabream, rainbow trout, and European sea bass (Geurden et al., 2007, 2014; Zambonino-Infante et al., 2019; Rocha et al., 2016a,b; Fang et al., 2014; Kumkhong et al., 2020; Song et al., 2019). This effect of early feeding could be dependent on fish species. It was thus important to search for any metabolic parameters that could explain this positive programming of growth performance.

Regarding the effect of the CHO-H diets, we observed ra eduction of hepatic protein and plasmatic BUN, an elevation of hepatic fat and plasma triglyceride, and an increase in HSI. These findings were consistent with the observations reported in previous studies (Boonanuntanasarn et al., 2018b; Azaza et al., 2015; Wang et al., 2017). Notably intake of a high-carbohydrate diet did not significantly increase the post-prandial glycemia during, suggesting a high ability of Nile tilapia to regulate glucose homeostasis when fed with carbohydrates, as observed previously in omnivorous fish (Polakof et al., 2012; Kamalam et al., 2017). Indeed, high-carbohydrate intake is associated with higher lipogenic gene expressions (*fasn*, *g6pd*), lower gene expressions for amino acid catabolism (*alat*), lower enzyme activities (GDH) for amino acid catabolism and higher enzyme activities for glycolysis (PFK) in the liver. All thess expected regulations by dietary carbohydrates can explain the better use of dietary carbohydrates in tilapia (an omnivorous fish species) than in rainbow trout (a carnivorous fish species) with poor regulation of lipogenesis and amino acid catabolism in the liver (Polakof et al., 2012; Kamalam et al., 2017).

Regarding the effect of the early LP/HC diet stimulus (the programming effect), some interesting metabolic data have been observed that could be related to the better growth performance. First, lower levels of hepatic and muscle protein and higher levels of lipid and glycogen in the liver and the muscle, as well as lower plasma BUN and higher glycemia, stongly suggest that the glucose, lipid, and amino acid metabolism modifications in adult tilapia are linked to the early nutritional stimulus. (i.e., the LP/HC stimulus). These findings about plasma and metabolite compositions are similar to those in previous reports on tilapia programmed through glucose injection in yolk (Kumkhong et al., 2020) but not to those on the other fish species, for which no significance was found for these parameters in gilthead seabream, rainbow trout and sturgeon (Geurden et al., 2007, 2014; Rocha et al., 2016b; Song et al., 2019). Second, it was interesting to also analyze the metabolism at the enzymatic and molecular levels in relation to the nutritional history. Indeed, early experience of tilapia with high-carbohydrate diet is associated with lower levels of amino acid catabolism (down-regulation of *asat;* both mRNA and enzyme, *alat;* mRNA, *gdh;* enzyme) and higher levels of glycolysis (hepatic PFK and muscle PK activities even though their mRNA levels did not follow the same trends). These findings were in line with the reports described previously in juvenile tilapia stimulated with direct glucose injection (Kumkhong et al., 2020) and in omnivorous zebrafish early fed with high levels of carbohydrates (Fang et al., 2014). However, this is different to what was observed in carnivorous fish; for example, in rainbow trout fed at first-feeding with high levels of carbohydrates, no permanent effects on hepatic glucose metabolism were observed in juvenile, and only muscle glycolysis and transport were changed (Geurden et al., 2014; Song et al., 2019). By contrast, in

gilthead seabream, there were no obvious effects on glycolysis, gluconeogenesis and lipogenesis (Rocha et al., 2016a,b). In gilthead seabream, dietary glucose stimulus at larval stages. Taken together, our study proves without ambiguity that early feeding can have strong and long-term impacts on nutrient use and growth performance of Tilapia.

6.6 Conclusion

In conclusion, early nutrition can be involved in permanent changes in metabolism later in tilapia's life as it is well described in mammals (Lucas et al., 1998; Fernandez-Twinn and Ozanne, 2010). Further studies are required to better understand the mechanisms (such as epigenetics) at the origin of these observations.

6.7 Acknowledgements

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CHAPTER VII

SUMMARY

This study revealed the effect of different levels of dietary carbohydrate on growth performance, body composition, blood metabolites, hepatic size and hematic and muscular glycogen in Nile tilapia (*Oreochromis niloticus*). Also, molecular responses to different levels of dietary carbohydrates were explored. The findings could be concluded as the following:

(i) Growth response of fish depended on dietary protein contents. Fish achieved the best growth performance when they in took optimum dietary protein level. Excess of dietary protein resulting in the reduction of growth performance.

(ii) In adult fish, fish fed different levels of dietary carbohydrate (through 90 days) had similar muscular and whole body nutrient compositions.

(iii) Increase in dietary carbohydrate level led to elevation in plasma glucose but without going up to hyperglycemia)no more than 5 mM). In addition, dietary high carbohydrate level increase plasma cholesterol and triglyceride.

(iv) Increase in hepatosomatic index, hepatic glycogen and muscular glycogen were observed in fish fed high carbohydrate diet, demonstrating that, in Nile tilapia, liver and muscle responds well at a metabolic level to dietary carbohydrates.

(v) However, no down regulation of hepatic gluconeogenesis, no induction of hepatic lipogenesis and of muscle glycolysis were detected at a molecular level in tilapia fed high carbohydrate diet (even with 50% of carbohydrates) through 90 days.

More investigations of carbohydrate metabolism such as nutritional programming of carbohydrate was conducted. This study demonstrated for the first time the existence of nutritional programming in Nile tilapia, a model of omnivorous fish. Injection of glucose into yolk reserved of Nile tilapia larvae exerted the effect of nutritional programming of carbohydrate as following:

(i) An increase in the glucose level in the yolk led to an increase in the glycogen and glucose content in larvae.

(ii) In larvae at 1-week post injection (wpi), at the transcriptional level, glucose stimuli during the early alevin stage influenced carbohydrate-metabolism-related pathways including induction of glycolysis, down-regulation of gluconeogenesis and amino acid catabolism.

(iii) The early glucose stimulus persisted up to 20 weeks. Programming of glucose metabolism seems to be associated with a better use of carbohydrates, leading to a higher dietary protein-sparing effect until the fingerling. However, normal-saline-injected fish showed catch-up growth during juvenile fish (at 20 wpi).

(iv) In juvenile fish, glucose overload in the yolk sac had effect on several metabolic actors such as muscular glycogen and hepatic fat.

(v) The early glucose stimulus persisted up to 20 weeks in the carbohydrate-metabolism-related pathways including induction of glucose transport and glycolysis and down-regulation of gluconeogenesis and amino acid catabolism) even though these changes were less pronounced than those observed at 1 wpi).

(vi) The combination effects of early hyperglucidic stimuli and high-carbohydrate diet on the biochemical composition, liver size, muscle composition and plasma metabolites revealed the existence of nutritional programming.

(vi.i) High-carbohydrate diets exerted expected effects on the chemical composition of the liver and muscles: (1) reduced protein in the liver, (2) increased fat in the liver and (3) elevated glycogen in the liver and muscles.

(vi.ii) The glucose stimulus history is more pronounced when the fish are challenged with medium -and high-carbohydrate diets .Early glucose stimuli and high-carbohydrate diets synergistically reduced hepatic proteins, increased hepatic lipids and increased glycogen in the liver and muscles.

(vi.iii) Early glucose stimuli appeared to synergistically increase liver glycolysis and muscle glucose metabolism and decrease gluconeogenesis.

(vii) Although, carbohydrate diets did not exert an effect on the global DNA methylation in the liver and muscles, early nutritional stimuli could have a long-term effect on the DNA methylation of the genome of tilapia.

(viii) The long-term effects of the glucose injection were not observed on the growth performance, postprandial plasma metabolite profiles and glucose metabolism in adult Nile tilapia (Up to 33 weeks)

(ix) The long-term effects (on glucose and lipid metabolism) of early glucose injection into yolk were revealed mainly when adult tilapia were fed with a high-carbohydrate diet including decreasing proteins in liver and muscle as well as increasing hepatic size and lipid and muscle glycogen. In addition, the early glucose injection was associated with the induction of glycolytic enzymes (hepatic gck and muscle hk2, mRNA and enzymatic activities; and muscle PK activity) as well as gluconeogenesis, amino acid catabolism and lipogenesis.

This study provided original data that early feeding can have strong and long-term impacts on nutrient use and growth performance of the Nile tilapia as following:

(i) During early feeding stimulus, high-protein/low-carbohydrate (HP/LC) diet-fed fry had higher weight gain (1.4 fold) than low-protein/high-carbohydrate (LP/HC) diet-fed fry. LP/HC diet increase whole body glycogen and triglyceride contents. At week 1, high-carbohydrate diet stimulus was associated with an inhibition of gluconeogenesis, amino acid catabolism, and glucose transporter and induction of lipogenesis.

(ii) Later, HP/LC diet fed fish had higher growth performance; however, LP/HC diet-fed fish caught up with that of HP/LC diet-fed fish at week 36.

(iii) The early high-carbohydrate diet history was associated with persistent effects on hepatic biochemical composition, including higher levels of protein, lipid, and glycogen contents and muscle glycogen in adult tilapia .

(iv) The effects of early hyperglucidic intervention through early feeding with carbohydrate led to inducing glycolysis and lipogenesis and suppressing amino acid catabolism .

(v) Positive long-term effects of the early high-carbohydrate stimulus irrespective of the final dietary challenge were observed on growth performance in adult fish.

(vi) During the final dietary challenging period, high carbohydrate diet resulted in a reduction of hepatic protein and plasmatic blood urea nitrogen (BUN), an elevation of hepatic fat and plasma triglyceride, and an increase in hepatosomatic index. high-carbohydrate intake is associated with higher lipogenic gene expressions (fasn and g6pd), lower gene expressions for amino acid catabolism (alat), lower enzyme activities (GDH) for amino acid catabolism, and higher enzyme activities for glycolysis (PFK) in the liver.

(vii) During the final dietary challenging period, early LP/HC diet stimulus (the programming effect) led to improve growth performance, lower levels of hepatic and muscle protein and increase levels of lipid and glycogen in the liver and the muscle as well as lower plasma BUN.

(viii) Early experience of tilapia with high-carbohydrate diet is associated with lower levels of amino acid catabolism (downregulation of asat; both mRNA and enzyme, alat; mRNA, gdh; enzyme) and higher levels of glycolysis (hepatic PFK and muscle PK activities).

BIOGRAPHY

Suksan Kumkhong was born on 4 May 1990 in Sungnoen, Nakhon Ratchasima Thailand. In 2008 , finished high school from Rajsima Wittayalai school, Nakhon -Ratchasima. In 2013 graduated the Bachelor's degree (second honor) in school of Animal Production Technology, Suranaree University of Technology, Nakhon -Ratchasima. In 2014 began a Ph.D. studies in School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and received a scholarship from the Royal Golden Jubilee Ph.D. Program (Ph.D. 0138/2557) in Thailand and The scholarship by the French Government (Campus France file number is 931061D. He spent 1 year in the lab at National Institute for Agricultural Research INRA Nutrition metabolism Aquaculture, France for his Ph.D thesis was the effect of nutritional programming of dietary energy sources on long - term metabolic pathway in Nile tilapia (Oreochromis niloticus).