EFFECT OF SHORT PERIOD OF EARLY DIETARY CARBOHYDRATE DURING FRY STAGE ON NUTRIENT METABOLISM AND GROWTH PERFORMANCE IN JUVENILE NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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ผลของการให้อาหารคาร์โบไฮเดรตสูงในระยะปลาวัยอ่อนต่อเมตาบอลิซึมของสารอาหารและสมรรถนะการเจริญเติบโตในปลานิลวัยรุ่น (*Oreochromis niloticus*)

นางสาวณัฐนันท์ ศรีสกุลเตียว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's degree.

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ณัฐนันท์ ศรีสกุลเตียว : ผลของการให้อาหารคาร์โบไฮเดรตสูงในระยะปลาวัยอ่อนต่อเมตาบอลิซึมของสารอาหารและสมรรถนะการเจริญเติบโตในปลานิลวัยรุ่น (*Oreochromis niloticus*) EFFECT OF SHORT PERIOD OF EARLY DIETARY CARBOHYDRATE DURING FRY STAGE ON NUTRIENT METABOLISM AND GROWTH PERFORMANCE IN JUVENILE NILE TILAPIA (*OREOCHROMIS NILOTICUS*) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. สุรินทร บุญอนันธนสาร, 86 หน้า.

คำสำคัญ: ปลานิล/โภชนะเริ่มแรก/คาร์โบไฮเดรต/กลูโคสเมแทบอลิซึม

วัตถุประสงค์ของการศึกษานี้คือเพื่อศึกษาผลของ Nutritional programming ในการกระตุ้นการกินอาหารคาร์โบไฮเดรต (CHO) ในระยะเริ่มต้นของการพัฒนาการของปลานิลวัยอ่อนต่อประสิทธิภาพการเจริญเติบโตและเมแทบอลิซึมของปลานิล ในการทดลองที่ 1 มี 3 กลุ่มการทดลอง คือ 1) กลุ่มที่มีการกระตุ้นทางโภชนาการด้วยการให้อาหารที่มีคาร์โบไฮเดรตต่ำและโปรตีนสูงเป็นเวลา 3 สัปดาห์ในระยะปลาวัยอ่อน (ปลากลุ่มควบคุม) 2) กลุ่มที่มีการกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูงและโปรตีนต่ำ (HC/LP) ระยะเวลา 1 สัปดาห์ในระยะปลาวัยอ่อน (ปลากลุ่มกระตุ้นระยะสั้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูง) 3) กลุ่มที่มีการกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูงและโปรตีนต่ำ (HC/LP) ระยะเวลา 3 สัปดาห์ในระยะปลาวัยอ่อน (ปลากลุ่มกระตุ้นระยะยาวด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูง) ผลการศึกษาเมื่อเปรียบเทียบกับลูกปลากลุ่มควบคุม ปลากลุ่มกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูงระยะสั้นและ ระยะยาว มีความสัมพันธ์กับประสิทธิภาพการเจริญเติบโตที่ลดลงในลูกปลาเมื่อสิ้นสุดระยะเวลากระตุ้น ซึ่งสัมพันธ์กับการแสดงออกของยีนที่แตกต่างกันสำหรับการเผาผลาญโปรตีนและกลูโคส ตามด้วยการเติบโตแบบชดเชยในภายหลัง (Compensatory growth) ต่อจากนั้น เพื่อทดสอบ Nutritional programming ต่อเมตาบอลิซึมของคาร์โบไฮเดรต ในสัปดาห์ที่ 21–24 ทำการทดสอบ Nutritional programming ด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูง (CHO-H) ผลแสดงให้เห็นถึงการเพิ่มประสิทธิภาพการเจริญเติบโตของปลา ซึ่งแสดงให้เห็นในกลุ่มปลาที่ได้รับการกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูงและโปรตีนต่ำ (ปลากลุ่มกระตุ้นด้วย HC/LP ระยะสั้น) มีผลต่อการเพิ่มการใช้คาร์โบไฮเดรตเพื่อการเจริญเติบโตได้ดีขึ้น โดยมีผลต่อการเปลี่ยนแปลงเมตาบอลิซึมที่เชื่อมโยงกับ Nutritional programming ในระยะปลาวัยอ่อนส่งผลต่อการเพิ่มการควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับไกลโคไลซิสและการขนส่งน้ำตาลกลูโคส ปลานิลวัยอ่อนที่ได้รับการกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตในอาหารช่วงวัยอ่อน ทำให้เกิดการลดการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างกลูโคส (gluconeogenesis) และแคแทบอลิซึม (amino aci catabolism) ของกรดอะมิโน Nutritional programming การกระตุ้นด้วยทางโภชนาการด้วยการให้อาหารที่มีระดับคาร์โบไฮเดรต

สูงสามารถอธิบายการใช้ประโยชน์จากสารอาหารคาร์โบไฮเดรตได้ดีขึ้น โดยเฉพาะการเพิ่มการใช้ประโยชน์จากน้ำตาลโดยผลการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการไกลโคไลซิส (glycolysis) การทดลองที่ 2 มี 3 กลุ่มการทดลอง คือ 1) การกระตุ้นทางโภชนาการด้วยการให้อาหารที่มีคาร์โบไฮเดรตต่ำและโปรตีนสูงเป็นเวลา 3 สัปดาห์ในระยะปลาวัยอ่อน (ปลากลุ่มควบคุม) 2) กลุ่มที่มีการกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูงและโปรตีนต่ำ (HC/LP) ระยะเวลา 1 สัปดาห์ระยะปลาวัยอ่อน (ปลากลุ่มกระตุ้นระยะสั้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูง) 3) กลุ่มที่มีการกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูงและโปรตีนต่ำ (HC/LP) ระยะเวลา 3 สัปดาห์ในระยะปลาวัยอ่อน (ปลากลุ่มกระตุ้นระยะยาวด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูง) ทำการทดสอบ Nutritional programming ด้วยอาหารที่มีระดับคาร์โบไฮเดรตปานกลาง (CHO-M) ในสัปดาห์ที่ 21-24 ผลการทดลองแสดงให้เห็นถึงปลาการกลุ่มกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูงและโปรตีนต่ำระยะปลาวัยอ่อนในการกระตุ้นระยะสั้นและยาว ไม่ส่งผลต่อประสิทธิภาพการเติบโต อย่างไรก็ตาม การกระตุ้นระยะสั้นและยาว ด้วยอาหารที่มีระดับคาร์โบไฮเดรตสูง มีผลต่อการเปลี่ยนแปลงค่าเคมีในเลือดค่ากลูโคสและไตรกลีเซอไรด์เพิ่มขึ้น รวมถึงองค์ประกอบทางเคมีของไขมันในตับ ไขมันในกล้ามเนื้อและไตรกลีเซอร์ไรด์ ในตับและกล้ามเนื้อ ไกลโคเจนในกล้ามเนื้อที่เพิ่มขึ้น ในขณะที่ระดับโปรตีนในตัวปลาลดต่ำลง และ โปรตีนในเลือดลดลง มากไปกว่านั้นระดับการแสดงออกของยีนของการขนส่งกลูโคส และกระบวนการไกลโคไลซิสในกล้ามเนื้อเพิ่มสูงขึ้น และระดับของการสลายกรดอะมิโนมาใช้เป็นพลังงานลดลงในปลากลุ่มที่ถูกกระตุ้นด้วยอาหารที่มีระดับคาร์โบไฮเดรตในอาหารที่สูงในช่วงวัยอ่อน

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา ณัฐนันท์ ศรีสกุลเตียง

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา สรินทร

NATTANAN SRISAKULTIEW : EFFECT OF SHORT PERIOD OF EARLY DIETARY CARBOHYDRATE DURING FRY STAGE ON NUTRIENT METABOLISM AND GROWTH PERFORMANCE IN JUVENILE NILE TILAPIA (*OREOCHROMIS NILOTICUS*). THESIS ADVISOR : ASSOC. PROF. SURINTORN BOONANANTANASARN, Ph.D., 86 PP.

Keyword: NILE TILAPIA/NUTRITIONAL PROGRAMMING/EARLY FEEDING/ CARBOHYDRATE/GLUCOSE METABOLISM

The objective of this study was to investigate the effects of different durations of early carbohydrate (CHO) feeding stimulus on the growth performance and carbohydrate metabolism of Nile tilapia at later in life. In experiment I, the first-feeding nutritional stimulus treatments contained a low-CHO/high-protein (LC/HP) diet for 3 weeks (control group), a short duration high-CHO/low-protein (HC/LP) diet for 1 week (short-HC/LP), and a long duration HC/LP diet for 3 weeks (long-HC/LP). Compared to the control fry, the short- and long-HC/LP diets were associated with lower growth performance in the fry at the end of the stimulus period. Early stimulus was associated with differential gene expression for protein and glucose metabolism. Compensatory growth was observed later (week 20) in both short- and long-HC/LP groups. Subsequently, to test the existence of CHO metabolic programming, the experiment was divided into 2 challenge tests including high-CHO (CHO-H) or medium-CHO (CHO-M) diet challenge.

In experiment I, juvenile fish were challenged with a CHO-H diet in weeks 21–24. Our results show that early HC/LP stimulus improved the growth performance of juveniles, suggesting that a history of early HC/LP stimulus contributes to better use of dietary CHO for growth. Irrespective of the duration of early stimulus, metabolism modifications linked to early HC/LP stimulus were observed. The upregulation of the glucose gene (involved in glycolysis and transport) in juveniles that experienced early stimulus and the downregulation of genes involved in gluconeogenesis and amino acid catabolism can explain the improved use of the CHO diet in these fish. Taken together, early HC/LP feeding in tilapia fry is associated with metabolic programming. Because the long-HC/LP treatment had stronger direct negative effects on growth performance than the short-HC/LP treatment, this study suggests that a 1-week early

HC/LP feeding period could be sufficient to program juvenile tilapia for better nutrient use.

For experiment II, juvenile fish were challenged with a CHO-M diet in weeks 21–24. The results show that early HC/LP stimulus had no effects on growth performance in juveniles. However, both short and long HC/LP stimulus were associated with differences in lipid metabolism (increases in plasma triglycerides, fat contents in liver, muscle and whole body, and triglyceride content in both liver and muscle), carbohydrate metabolism (increases glycogen content in muscle and plasma glucose) and reduction in protein content in whole body. The upregulation of genes involved in muscle glycolysis and glucose transport and the downregulation of genes involved in amino acid catabolism were observed, demonstrating metabolic modulation at molecular level in juvenile fish.

In conclusion, early HC stimulus in tilapia fry is associated with metabolic programming later in life, and the effects were obvious with dietary high CHO during challenge test. Because the long-HC/LP treatment had negative effects on growth performance during stimulus period, this study suggests that a 1-week early HC/LP feeding period could be sufficient to use for nutrient stimulus.

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LIST OF ABBREVIATIONS

°C	=	Degree Celsius
µL	=	Microliter
ADG	=	Average daily weight gain
alat	=	Alanine aminotransferase
ANOVA	=	Analysis of variance
asat	=	Aspartate transaminase
BUN	=	Blood urea nitrogen
Cm	=	Centimeter
Cm^3	=	Cubic centimeter
CHO-H	=	High carbohydrate diet
CHO-M	=	Low carbohydratediet
DNA	=	deoxyribonucleic acid
ef1α	=	Eukaryotic translation elongation factor 1 alpha
fasn	=	Fatty acid synthase
FCR	=	Feed conversion ratio
FI	=	Feed intake
g	=	Gram
$g\ kg^{-1}$	=	Gram per kilogram
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
HC	=	High carbohydrate diet
HSI	=	Hepatosomatic index
hk1	=	Hexokinases 1
hk2	=	Hexokinase 2

LIST OF ABBREVIATIONS (Continued)

HP	=	High protein diet
HC/LP	=	High carbohydrate- low protein
HP/LC	=	High protein- low carbohydrate
kJ g^{-1}	=	Kilojune per gram
KOH	=	Potassium hydroxide
LP	=	Low protein diet
M	=	Meter
M	=	Molar
m^2	=	Square meters
mg	=	Miligram
mg mL^{-1}	=	Miligram per milliliters
mg g^{-1}	=	Milligrams per gram
mg l^{-1}	=	Milligram per liter
miRNAs	=	MicroRNAs
mL	=	Millilite
mM	=	Millimolar
NaCl	=	Sodium chloride
NADP	=	Nicotinamide adenine dinucleotide phosphate
NFE	=	Nitrogen-Free Extract
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
SD	=	Standard deviation
SGR	=	Specific growth rate

LIST OF ABBREVIATIONS (Continued)

WG	=	Weight gain

CHAPTER I
INTRODUCTION

1.1 Introduction

Global tilapia production has increased year by year to provide sustainable food (FAO, 2018). Research and development have been undertaken in various fields to improve tilapia production in term of productivity, meat quality and environmental impact (Nguyen, 2016). For example, study on tilapia nutrition has been carried out to develop tilapia production with least cost (Silva et al., 1989). The research on tilapia nutrition is needed to improve the productivity of tilapia production for contribution to global food security (Parata et al., 2020). Research have focus on the effects of dietary carbohydrate on growth and carbohydrate metabolism in Nile tilapia (Boonauntanasarn et al., 2018a; Boonauntanasarn et al., 2018b). Investigate of utilization and metabolism of dietary carbohydrate are important to produce low-cost feed with high quality (Geurden et al., 2013). The low-cost with high quality diet has been presented a challenge to develop in Nile tilapia for sustainable tilapia production (Pichet, 2016).

Generally, in fish, carbohydrates including starches and sugar are mainly used as energy source (Zhao et al., 2007). Dietary of carbohydrate at adequate level could improve protein sparing effects or protein retention by preventing catabolism of amino acids for energy use (Boonanuntanasarn et al., 2018a; Boonanuntanasarn et al., 2018b). In addition, optimum dietary carbohydrate could reduce nitrogen discharge in water. Since carbohydrate could be obtained from cheap feed ingredients, the efficient and maximal utilization of dietary carbohydrate would enable production of least cost feed with high quality (Shiau and Peng 1993). Recently, nutritional strategy has been proposed to be able to modulate metabolism in vertebrate. Nutritional programing describes the effects of dietary stimulus during early life period at critical developmental time on modulation of metabolic pathways during later growth phase (Panserat et al., 2017; Panserat et al., 2019). For instance, nutritional programming in Nile tilapia (*O. niloticus*) stimulus was accomplished by microinjection of 2M glucose into yolk during alevin stage and dietary

carbohydrate of first feeding during fry stage. (Kumkhong et al., 2020a; Kumkhong et al., 2020b). The method of nutritional stimulus is hypothesized to be a suitable window period for stimulus during early life development which varied according to fish species. The nutritional stimulus by first feeding is practical for tilapia farming, and adequate nutritional stimulus period is important for nutritional programming. Since long-term stimulus by feeding with high carbohydrate/low protein diet affected to retardation growth (Kumkhong et al., 2020b), which was not acceptable for tilapia farming. However, inadequate stimulus period would not be able to exert long-term effects at later in life. Therefore, the optimum period for effective nutritional stimulus is important and required to investigate for further application of nutritional programing for tilapia farming.

1.2 Research objectives

1.2.1 To study the long-term effects and optimum period of early high carbohydrate stimulus during fry stage on growth performance and metabolic responses at later juvenile stage when they were challenged with high CHO diet

1.2.2 To study the long-term effects and optimum period of early high carbohydrate stimulus during fry stage on growth performance and metabolic responses at later juvenile stage when they were challenged with low CHO diet.

1.3 hypotheses

1.3.1 There was nutritional programing of early high carbohydrate intake in Nile tilapia during fry stage, and the long-term effects depended on early nutritional stimulus period

1.3.2 The early high carbohydrate intake in Nile tilapia during fry stage had long-term effects on growth and CHO metabolism when fish were challenged with high CHO diet during juvenile stage.

1.3.3 The early high carbohydrate intake in Nile tilapia during fry stage had long-term effects on growth and CHO metabolism when fish were challenged with low CHO diet during juvenile stage.

1.4 Scope of study

In order to test the metabolic response and also nutritional programming in Nile tilapia, First, the effect of high carbohydrate diet was investigated by different early nutritional history such as control, short HC/LP (first feeding high carbohydrate-low protein diet for 1 week) and long HC/LP (first feeding high carbohydrate-low protein diet for 3 week) (1week and 3 weeks stimulus). Second, the metabolic response and nutritional programming were determined by dietary challenging, high carbohydrate diet (CHO-H) and low carbohydrate diet (CHO-M). To test the different responses, form dietary challenging CHO-H (experimental I) & CHO-M (experimental II). The metabolic response to dietary short and long HC/LP history were determined on several parameter including growth performance plasma metabolite nutrient composition in muscle and liver and glucose metabolism. A schematic view of the experiment under this thesis is provide in fig. 1.1.

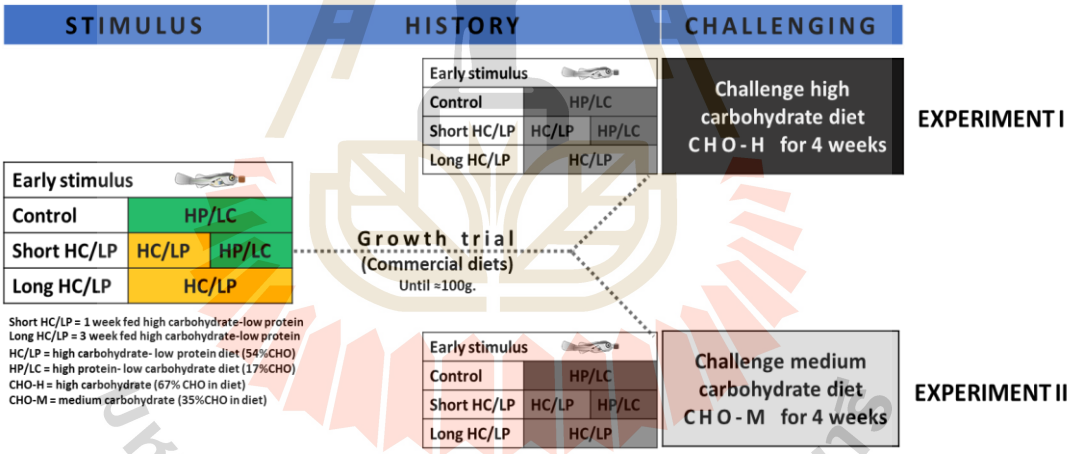

Figure 1.1 The plan of the two experiments developed in Nile tilapia focusing on early high carbohydrate response and different dietary challenging.

1.5 Expected result

This finding reveals the metabolic response form effect of early nutritional programming (short and long HC/LP). The resulting knowledge form this thesis can provide the way a better use of high carbohydrate and low carbohydratefor fish and allow incorporation of ingredient in fish diet.

1.6 References

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CHAPTER II

LITERATURE REVIEW

2.1 Nile Tilapia (*Oreochromis niloticus*)

World fish production of Nile tilapia (*O. niloticus*) showed that tilapia production has been the second most economic freshwater fish species in the world after carp species (Table 2.1). Also, in Thailand, tilapia has been the major culture freshwater fish species with th high production more than two thousand ton per year (Wang et al., 2005). The tilapia products have provided for domestic consumption and export. Among tilapia production, Nile tilapia has been dominated in farmed fish. Nile tilapia (*O. niloticus*) is an omnivorous fish species (Fig. 2.1). Its natural feed are phytoplankton, periphyton, aquatic plants, small invertebrate, benthic fauna, detritus and bacterial films associated with detritus (FAO, 2018). Tilapia is able to live in a wide range of environmental conditions, including high salinity, high temperatures, high ammonia concentrations, and low oxygen levels. This makes tilapia very suitable for aqua farming.

Table 2.1 Global aquaculture volumes of top 10 species in 2014.

ALL AQUATIC SPECIES			FINFISH AND SHELLFISH ONLY		
Species	Volume (t)	(%)	Species	Volume (t)	%
Eucheuma seaweeds	9,053,044	9	Grass carp	5,537,794	8
Japanese kelp	7,654,586	8	Silver carp	4,967,739	7
Grass carp	5,537,794	5	Cupped oysters	4,378,011	6
Silver carp	4,967,739	5	Common carp	4,159,117	6
Cupped oysters	4,378,011	4	Japanese carpet shell	4,010,703	5
Common carp	4,159,117	4	**Nile tilapia**	**3,670,260**	**5**
Japanese carpet shell	4,010,703	4	White-leg shrimp	3,668,682	5
Gracilaria seaweeds	3,751,396	4	Bighead carp	3,253,143	4
Nile tilapia	**3,670,260**	**4**	Catla	2,770,020	4
White-leg shrimp	3,668,682	4	Carassius carps	2,767,910	4
Other species	50,287,741	4	Other species	34,648,728	47
Grand total	101,139,072	50		73,832,107	100

Source: FAO FishStatJ(2016).

Figure 2.1 Nile tilapia (*Oreochomis niloticus*).

This makes tilapia very suitable for aqua farming. The water qualities that are suitable for tilapia are presented in Table 2.2. The tolerance range which was acceptable range Ammonia 0.0125 mg l^{-1}, Nitrite 0.1-0.2 mg l^{-1} and Nitrate 0-3.0mg l^{-1} respectively was reported by Agbo (2008).

Table 2.2 The optimum condition for Nile tilapia (*O. niloticus*) culture.

Rearing condition for Nile tilapia	**Optimum condition**
Air temperature ($^{\circ}$C)	30-36
Water temperature ($^{\circ}$C)	27.5-28.6
Dissolved oxygen mg l^{-1}	3.5-5.5
pH	7.0-8.0

Source; Boonauntanasarn et al., 2018a, b; Kumkhong et al., 2020a, b**.**

In tilapia production, all male tilapia culture is preferred. Sex reversal of tilapia can be change by feeding fry with dietary containing 17**α**-methyltestosterone (17**α**- MT) at 60 mg/kg feed at first feeding for 4 weeks (Rima et al., 2017). Generally, the feed is the major operational cost item, accounting 50-70% (Boonauntanasarn et

al., 2018a) of total production cost. The cost value from feed will be depending on the composition of nutrient in diet. The commercial feed for Nile tilapia varies depending on difference stage of growth showed in Table 2.3 In addition, the commercial feed (practical diets) for Nile tilapia were reported to contain protein (25-45%), lipid (5-12%), carbohydrate (20-50%), fiber (≤ 6%), total n-6 and n-3 fatty acids (0.5-1.0%), calcium (0.3%) and phosphorus (0.7%) (Pichet, 2016) (Table 2.4).

Table 2.3 The commercial diet for Nile tilapia (*O. niloticus*) on difference stage.

Growth stage	Nutrient composition					
	Moisture	Protein	Fat	Fiber	Ash	NFE*
First feeding larvae	Powdered fish meal > 40% protein, 8% crude fat					
Fry						
Fingerlings	12	40	5	4	8	31
Juvenile 35-100g	12	32	4	6	8	38
Adult 100-300g	12	32	4	8	8	38
>300g	12	25	4	8	10	41
Broodstock	12	32	3	8	8	37

* NFE: Nitrogen-free extract= 100%-(moisture+crudeprotein+crude fiber+crude lipid+ash).

Source; Aqua feed company, THAILAND.

Table 2.4 Commercial feed for Nile tilapia.

Proximate composition (% wet weight)	Commercial 40% CP	Commercial 32% CP	Commercial 30% CP	Commercial 15.5% CP
Protein	40.00	33.60	31.40	15.50
Fat	8.80	3.50	3.30	3.00
Fiber	2.90	5.40	5.70	10.00
Ash	12.90	10.40	10.90	10.20
NFE[a]	35.40	47.10	48.70	61.30
Gross energy (kJ g^{-1})	16.83	15.59	15.36	14.36

[a] NFE: Nitrogen-free extract= 100%-(moisture+crude protein+crude fiber+crude lipid+ ash).

In fish feed, three main nutrients have been classified according to their functions including body building (protein), energy giving (protein, lipid, carbohydrate), and protective and regulatory (vitamin and mineral). In Thailand, there are several carbohydrate sources especially cassava, rice, broken rice and corn. Therefore, Thailand is the source of carbohydrate for Nile tilapia. In energy giving nutrient, carbohydrate is non-protein energy source, that are available and low-cost. Normally, optimal carbohydrate levels could provide protein sparing effect (Azaza et al., 2015; Honorato et al., 2010) and reduce catabolism of other nutrients for energy (Polakof et al., 2012). Nevertheless, increase dietary carbohydrate levels could have negative impact on growth, fish health by metabolic disturbance and cause the clinical sign such as hyperglycemia (>5.5mmol) (West et al., 1994). Therefore, incorporation of optimal level of dietary carbohydrate in fish feed are required. Indeed, in fish, the ability to utilize carbohydrate varies depending on food habits (Kamalam et al., 2017). Since several energy yielding ingredient are cheap, comparing to protein ingredient, investigate of utilization and metabolism of dietary carbohydrate are important to produce low-cost feed with high quality. The low-cost with high quality diet has been presented a challenge to develop in Nile tilapia for sustainable tilapia production.

2.2 Carbohydrates

Carbohydrates are organic substances which have the general chemical formula as $(CH_2O)_n$ (n is the number of carbon in the molecule). In plant sources, there are various forms of carbohydrates such as starches, cellulose and sugar (Langley-Evans et al., 2009). Generally, in fish, carbohydrates including starches and sugar are energy source. Indeed, fish do not have a dietary requirement for carbohydrates because fish can efficiently produce glucose from noncarbohydrate (gluconeogenesis) precursor such as lactate, pyruvate and amino acids (NRC, 2011). Dietary inclusion of carbohydrate could improve protein sparing effects or protein retention by preventing catabolism of amino acids for energy use (Hardy et al., 2010). In addition, optimum dietary carbohydrate could reduce nitrogen discharge in water (Hillestad et al., 2001). Since carbohydrate could be obtained from cheap feed

ingredients, the efficient and maximal utilization of dietary carbohydrate would enable production of least cost feed with high quality. Moreover, the carbohydrate ingredients particularly starch generally provides as binders for feed preparation facilitating pellet binding, stability, and floatability. Like other vertebrates, in fish, carbohydrates are classified as non-essential nutrients which provide an energy source for fish (Boonanuntanasarn et al., 2018b). The carbohydrates are incorporated in practical diets as much as possible for minimal feed cost. Indeed, fish exhibits diversity in feeding habits including carnivores, omnivores, and herbivores (Harpaz and Uni, 1999). The carnivorous fish generally are less efficient to utilize carbohydrate as energy source whereas the omnivorous and herbivorous fish are better efficient to utilize carbohydrate as energy source (Panserat et al., 2002; Wang et al., 2005; Figueiredo-Silva et al., 2013; Azaza et al., 2015). Therefore, carbohydrates could provide more efficient protein-sparing effect for growth for the omnivorous and herbivorous comparing with the carnivorous fish. The digestion and metabolism of carbohydrate thus vary among fish habits and species which are required to investigate to contribute the efficient and precision use of dietary carbohydrate.

2.3 Utilization of carbohydrate in fish

Various factors influence carbohydrate utilization in fish (Table 2.5). Sources of carbohydrate including gelatinized potato starch, wheat starch, Maize starch and soybean meal are important factor affecting carbohydrate utilization (Li et al., 2016; Song et al., 2019), In addition, different fish species based on feeding habits could use carbohydrate as energy source differently. In carnivorous fish, rainbow trout (*Oncorhynchus mykiss*) could use dietary carbohydrate rage 18-27 percent (Suárez et al., 2002; Yamamoto et al., 2001). Comparing with the carnivorous fish, the omnivorous (Nile tilapia) fish could use higher carbohydrate level 22-45 percent (Ali and Al-asgah, 2001; Wang et al., 2005). In herbivorous, Grass carp (*Ctenopharyngodon idellus)* could utilization source of carbohydrate range 20-47 percent (Li et al., 2013; Tian et al., 2012). Altogether, fish exhibit diversity in utilization of carbohydrate as energy source; therefore, the digestion and metabolism of carbohydrate would also vary according to fish species.

Table 2.5. The ability utilization of carbohydrate of fish.

Feeding behavior	Fish species	Carbohydrate source	Optimum level (%)	Reference
Carnivorous	Rainbow trout *Oncorhynchus mykiss*	Gelatinized potato starch	18-27	Yamamoto et al., 2001
		Gelatinized maize starch	20	Suárez et al., 2002
	Gilthead sea bream *Sparus aurata*	Gelatinized maize starch	18	Fernández et al., 2007
	European sea bass *Dicentrarchus labrax*	Precooked starch and wheat shorts	25	Perez et al., 1997
Herbivorous	Grass carp *Ctenopharyngodon idellus*	Maize starch	38	Li et al., 2014a
		Wheat starch	33	Tian et al., 2012
	Indian major carp - Rohu *Labeo rohita*	Dextrin and starch	45	Mohapatra et al., 2003
		Dextrin	36	Jafri, 1998
	Blunt snout bream *Megalobrama amblycephala*	Cassava starch	30	Li et al., 2013
Omnivorous	Gibel carp *Carassius auratus gibelio*	Maize starch	30	Li et al., 2014b
		Maize starch and α-starch	28	Tan et al., 2009
	Nile tilapia *Oreochromis niloticus*	Maize grain and wheat bran	48	Ali and Al-asgah, 2001
	Hybrid tilapia *Oreochromis niloticus x O. aureus*	Maize starch	22-46	Wang et al., 2005
	Silver barb *Puntius gonionotus*	Dextrin	26	Mohanta et al., 2009
	Yellow fin sea bream *Sparus latus*	Raw maize starch	20	Wu et al., 2007a

Source; apply from Kamalam et al., 2017.

2.4 Carbohydrate metabolism

When animal consumed the starch, alpha-amylase (α-amylase) hydrolyzes the starch components including amylose (α-1,4) and amylopectin (α-1,6) to yield maltose or short chain dextrin. Subsequently, sucrase-isomerases further hydrolyze maltose or short chain dextrin into glucose. Then glucose is transported across the villi of gut lumen (Fig. 2.2). (Krogdahl et al., 2005; NRC, 2011). Glucose is transported through sodium dependent glucose symporter (SGLT1) and glucose transporter (GLUT2) from intestinal lumen into blood stream. When fish intake dietary carbohydrates, glucose will be absorbed for use as energy substrate. Four main metabolic pathways are involved in utilization of carbohydrate as energy source including glycolysis (liver), glycogenesis (liver), gluconeogenesis (liver) and glucose transport (liver and muscle). Since there are interactions of metabolism of nutrients among carbohydrate, protein and lipid, two main pathways are also involved in the metabolism of carbohydrate such as amino acid catabolism and lipogenesis. The main organ of glycolysis pathway is liver, and the series of pathways occur in cytoplasm. Excess glucose is stored as glycogen via glycogenesis in liver and muscle for reservation of energy (glycogen and fat). In addition, glucose is a substrate to produce lipid via lipogenesis in adipose tissue. Glycogenolysis is the process of the breakdown of glycogen into glucose when the body lacks energy.

Glycolysis is a catabolized process “breakdown of sugar". Hexose particularly glucose is converted into pyruvate (three-carbon keto-acids). First, glucose receives a phosphate from ATP and converts glucose-6-phosphate (G6P) and subsequently fructose-6-phosphate (F6P). The F6P converts fructose-1,6-bisphosphate (F1,6BP), and ATP is also used in this reaction. Two 3-carbon intermediates including glyceraldehyde-3-phosphate (G3P) are converted to 3-phosphoglyceroyl phosphate (1,3 BPG). The series of glycolysis process use 2 ATP molecules but provides 4 ATP molecules. Therefore, 2 ATP molecules and pyruvate are obtained from the glycolysis processes (Fig. 2.3). There are a number of enzymes involved in the series of glycolysis processes. Among them, three enzymes in liver including Glucokinase(*gck*), Phosphofructokinase, liver (*pfklr*) and Pyruvate kinase liver (*pklr)* and enzyme in muscle consisting of Hexokinase (*hk2*), Phosphofructokinase muscle (*pfkm*) and Pyruvate kinase (*pkma*) (McDonald et al., 2011.

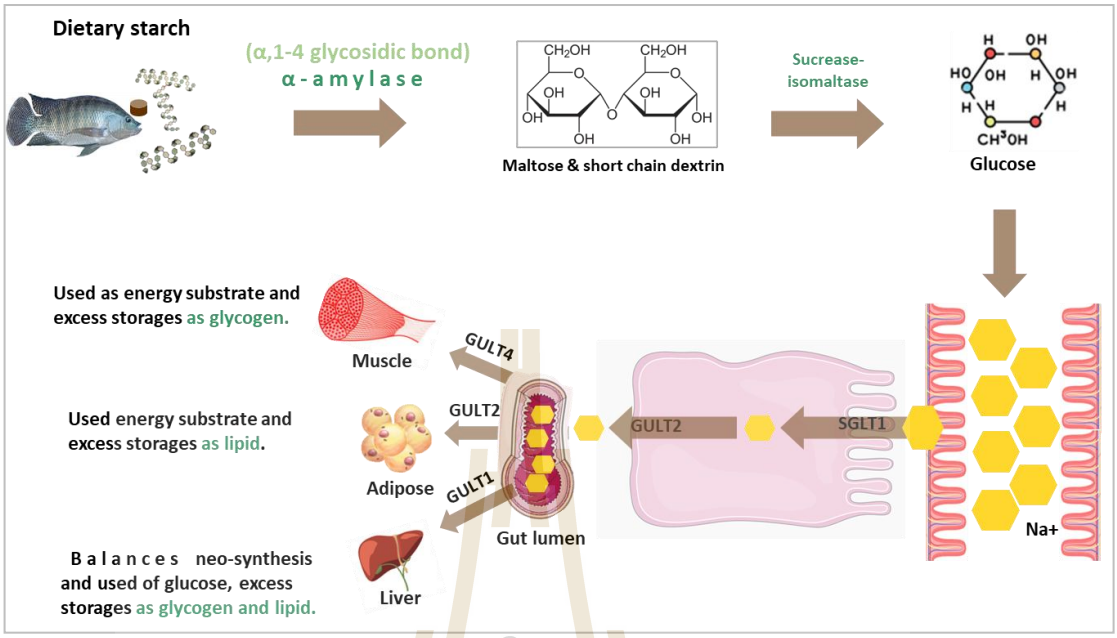

Figure 2.2 Digestion and absorption of carbohydrate: apply from Kamalam et al., 2017.

Subsequently, pyruvate is converted to acetyl Co A enters the Krebs cycle (citric acid cycle). To acetyl Co A enters the Krebs cycle (citric acid cycle). Citric acid cycle or tricarboxylic acid cycle involves in a cyclic process to oxidize pyruvate to convert to Nicotinamide Adenine (NADH), Flavin Adenine Dinucleotide (FADH) and Guanosine triphosphate (GTP) (Fig. 2.3).

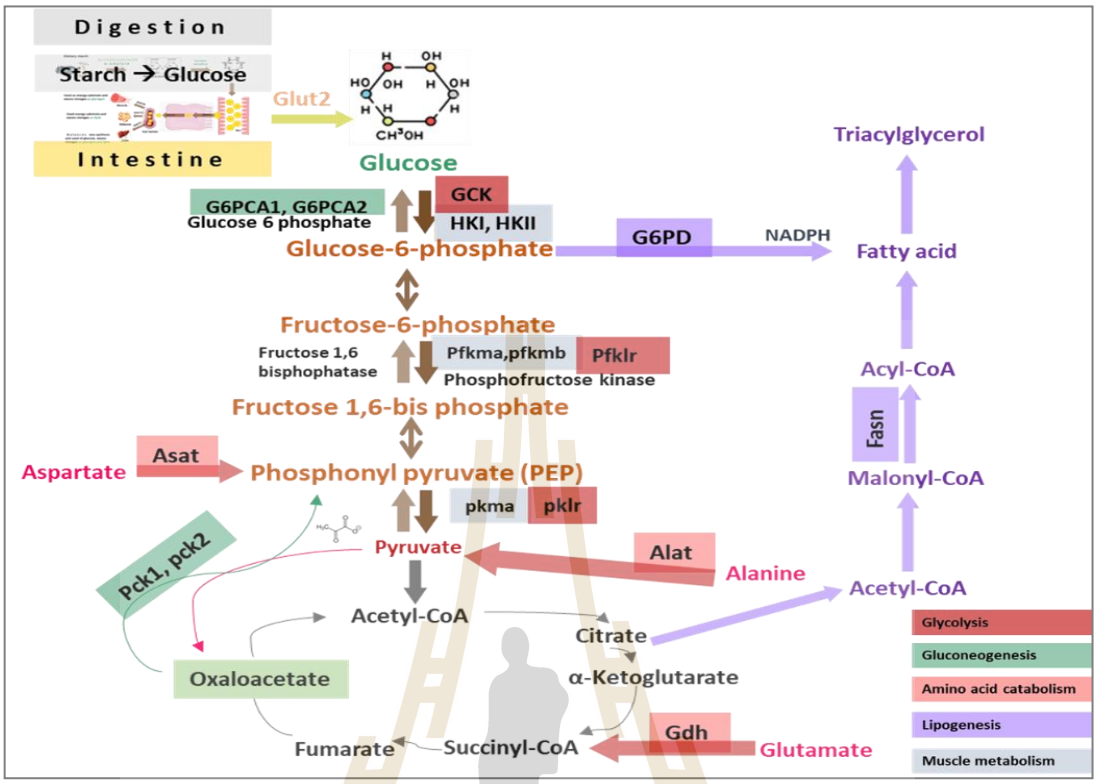

Figure 2.3 The metabolic gene pathway involved in carbohydrate metabolism Kamalam et al., 2017.

Gluconeogenesis is opposite pathway of glycolysis which produce glucose to sustain glucose homeostasis. Gluconeogenesis is series process to synthesize glucose from organic compounds such as amino acids after deamination. First, two pyruvate molecules form oxaloacetate. Then, the oxaloacetate converts to phosphoenolpyruvate. Phosphoenolpyruvate is changed to fructose-1,6-biphosphate, and then to fructose-6- phosphate. Fructose-6-phosphate is converted glucose-6-phosphate by the enzyme phosphohexose isomerase. Glucose is formed from glucose-6-phosphate in the cell's endoplasmic reticulum via the enzyme glucose-6-phosphatase (Fig. 2.3). To form glucose, a phosphate group is removed, and glucose-6-phosphate and ATP becomes glucose and ADP. Also, ATP is used during this process. Indeed, a number of enzymes involved in gluconeogenesis. Three major enzymes consisting of Glucose-6-phosphatase, fructose-1,6-bisphosphatase and glucose-6-phosphatase were evaluated the gluconeogenic pathways (McDonald et al., 2011).

Amino acid catabolism is series of pathways to catabolize amino acids and convert them to be sugars. First, the amino group is removed from amino acids, the amino group converts to be ammonium, and ammonium enters the urea cycle and change to be urea. After removing of amino groups. The remaining portion of amino acid (organic acids) are oxidized to be alpha-keto acid. The alpha-keto acid enters TCA cycle, to produce energy. For example, glutamate can be converted by a transamination reaction to alpha-ketoglutarate, which can be oxidized in the citric acid cycle. Oxaloacetate is produced from aspartate and asparagine. Succinyl-CoA is produced from isoleucine, valine, and methionine. Alpha-ketoglutarate is produced from arginine, glutamate, glutamine, histidine and proline.

The acid can also enter glycolysis, where it will be eventually converted into pyruvate. The pyruvate is then converted into acetyl-CoA so that it can enter the TCA cycle and convert the original pyruvate molecules into ATP, or usable energy for the organism (McGaha et al., 2012).

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Lipogenesis is series of pathways in which sugars are converted into fatty acids. The fatty acids are used to produce triglycerides and other lipid molecules (Song et al., 2019). When energy is excessive in the body, most of the newly synthesized fatty acids are esterified to convert triglycerides for storage. The lipogenic pathways are

involved in conversion of glucose to fatty acids. First, in mitochondria, glucose enters glycolysis and tricarboxylic acid (TCA) cycle to produce citrate. The citrate is transported to cytosol and then produces acetyl-CoA by ATP-citrate lyase (ACLY). Acetyl-CoA carboxylases 1 (ACC1) converts acetyl-CoA to malonyl-CoA. Fatty acid synthase (FASN) converts malonyl-CoA into palmitate which is the key rate-limiting enzyme in lipogenesis mechanism. Palmitate is the first fatty acid product which can later enter desaturation and/or elongation to produce other complex fatty acids (Song et al., 2019).

2.5 Nutritional programming

Nutritional programming describes the process of exposure to environmental stimuli during critical period of development that exerts permanent effects on physiology or metabolism of the organism (Langley-Evans et al., 2009; Hou and Fuiman 2020). In mammals, the process of quality or quantity of nutrients nutrient consumption by mother during pregnancy brings to permanent change in physiology or metabolism at later in life. In fish, the effects of environmental stimuli during early development (critical period) leads to modulate physiology or metabolism at later in life. (Rocha et al., 2015; Rocha et al., 2016a,b and Turkmen et al., 2017) Consequently, the stimulus (by environment and/or nutrition) from the first experience affects permanent changes to the physiology or metabolism of the organism. Dietary n-3 polyunsaturated fatty acids (PUFA) in pregnant mothers might have effects on growth of their children. For example, lactation Danish mother consumed fish oil for 4 months comparing with olive oil. The result demonstrated that fish oil intake during lactation increased docosahexaenoic acid (DHA: 22:6n-3) of red blood cell of infant at 2.5-fold, comparing with that of olive oil. At 2.5 year, children in the fish oil group had larger head circumference and body mass index (Laurizen et al., 2005)

In vertebrates, there is a great developmental plasticity in a period of life which has ability to permanently change their physiologic and metabolic pathways. For example, it was studied in avian model. This study was to investigate the long-term effect of prenatal protein to albumen removal (albumen-deprived group and

replacement with saline) during early embryonic development on growth and metabolism. Compared to non-manipulated or sham-manipulated hens, albumen-deprived hens had significantly lower body weight. Moreover, albumen-deprived hens had lower number of eggs and egg weight, suggesting that albumen removal diminished the reproductive capacity. Plasma triiodothyronine level were increase in albumen-deprived hens. At 10 weeks, decrease glucose tolerance was observed in albumin-deprived hens. The effects of albumen removal were transmitted into their offspring. The offspring of albumen-deprived hens had low body weight and relative residual yolk weight at hatching. Therefore, prenatal protein undernutrition (albumen removal) in avian had long-term effects on the body weight and reproductive performance (Willems et al., 2015)

Nutritional programming effects was studied in rainbow trout (*Oncorhynchus mykiss*) (Marandel et al., 2016; Liu et al., 2017), Seabass (*Dicentrarchus labrax*), Seabream (*Sparus aurata*) (Turkmen et al., 2017; Izquierdo et al., 2015), Atlantic salmon (*Salmo salar L.*) (Vera et al., 2017; Clarkson et al., 2017), and zebrafish (*Danio rerio*) (Fig. 2.4) (Fang et al., 2014; Rocha et al., 2015a,b). Generally, rainbow trout, which is carnivorous fish, has poor ability to use dietary carbohydrate as energy source. Two main pathways involve in carbohydrate metabolism pathways: glycolysis and gluconeogenesis. Therefore, study on these important carbohydrate metabolisms during ontogenesis in rainbow trout would be useful for modulation of carbohydrate metabolism to improve the efficient use of carbohydrate as energy source in rainbow trout. It was showed that, at stage 23 of trout fry, the metabolic of gene *pfkl* which is the gene encoding phosphofructokinase of glycolysis pathway; *pfkl* was expressed. It was showed that, at stage 31 of trout fry, *g6pc, fbp and pck* which is the gene encoding glucose-6-phosphatase, fructose-1,6 bisphosphatase and phosphoenolypyruvate kinase, respectively, of gluconeogenesis pathways were expressed. Therefore, these two stages of development were ontogenic expression of several enzymes involving in carbohydrate metabolism which were suggested to be suitable stage for studying of nutritional programming effects pf carbohydrate in rainbow trout (Marandel et al., 2016).

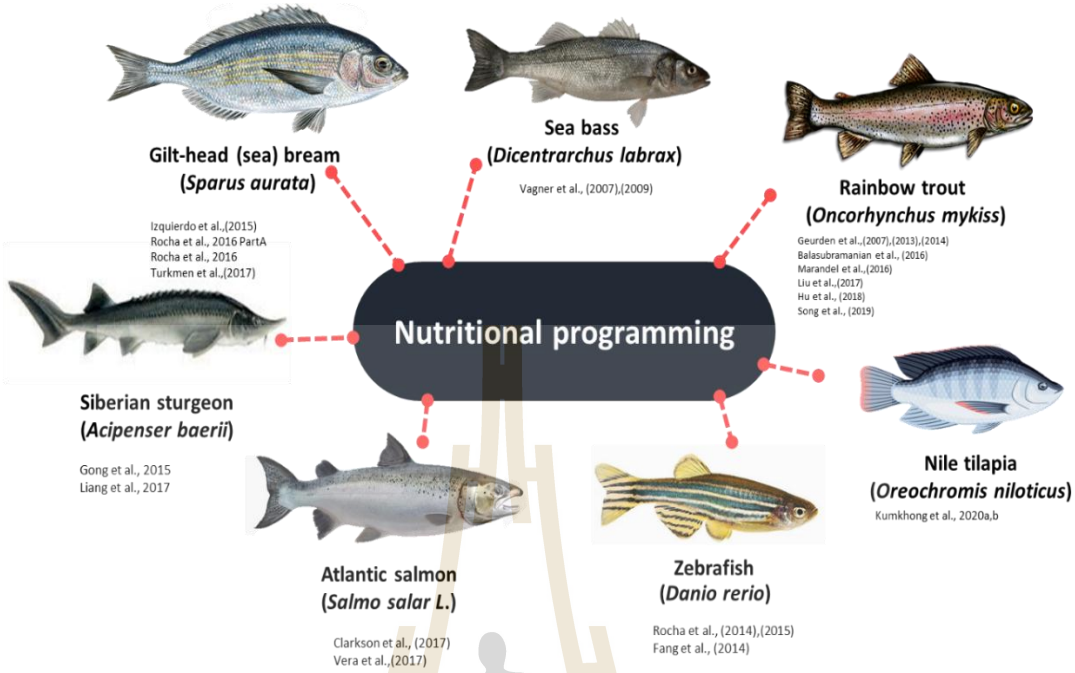

Figure 2.4 Nutritional programming in fish to improve use of new feed in aquaculture.

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Nutritional programming effects was studied in rainbow trout (*Oncorhynchus mykiss*) (Marandel et al., 2016; Liu et al., 2017), Seabass (*Dicentrarchus labrax*), Seabream (*Sparus aurata*) (Turkmen et al., 2017; Izquierdo et al., 2015), Atlantic salmon (*Salmo salar L.*) (Vera et al., 2017; Clarkson et al., 2017), and zebrafish (*Danio rerio*) (Fig. 2.4) (Fang et al., 2014; Rocha et al., 2015a,b). Generally, rainbow trout, which is carnivorous fish, has poor ability to use dietary carbohydrate as energy source. Two main pathways involve in carbohydrate metabolism pathways: glycolysis and gluconeogenesis. Therefore, study on these important carbohydrate metabolisms during ontogenesis in rainbow trout would be useful for modulation of carbohydrate metabolism to improve the efficient use of carbohydrate as energy source in rainbow trout. It was showed that, at stage 23 of trout fry, the metabolic of gene *pfkl* which is the gene encoding phosphofructokinase of glycolysis pathway; *pfkl* was expressed. It was showed that, at stage 31 of trout fry, *g6pc, fbp and pck* which is the gene encoding glucose-6-phosphatase, fructose-1,6 bisphosphatase and phosphoenolypyruvate kinase, respectively, of gluconeogenesis pathways were expressed. Therefore, these two stages of development were ontogenic expression of several enzymes involving in carbohydrate metabolism which were suggested to be suitable stage for studying of nutritional programming effects pf carbohydrate in rainbow trout (Marandel et al., 2016).

Moreover, nutritional programming in Nile tilapia (*O. niloticus*) stimulus (Fig.2.5) was accomplished by microinjection of either 2M glucose or 0.85% Nacl (saline) into yolk during alevin stage. The results showed that at 1-week post injection, up-regulation of genes involved in glycolysis pathway (*phlr, hk1*, and *pkma*), glucose transport pathway (*glut4*) and down-regulation of genes related to gluconeogenesis (*g6pca1*, *g6pcal2* and *pck1*) and amino acid catabolism pathway (*asat, alat*) ($P<0.05$) were observed. The permanent effects of glucose stimulus during alevin stage were observed in juvenile fish. During 20 weeks after early glucose stimulus, the effects of glucose stimuli was detected to modulate several physiologic and metabolic pathways. As a result, up-regulation of genes involved in glycolysis pathway (*gck, hk1*and *hk2*) and glucose transport pathway (*glut4*) and down-regulation of genes related to gluconeogenesis (*g6pca1*, *g6pcal2* and *pck1*) and amino acid catabolism pathway (*asat, alat*) ($P<0.05$) were observed in glucose stimulus fish. In addition,

glucose stimulus increased the pyruvate kinase (*pk*) activity. Moreover, after challenging the glucose stimulus fish with high carbohydrate (67% CHO; CHO-H) or low carbohydrate (37% CHO; CHO-M) during week 20-24, the CHO-H diet induced expression of glycolysis and lipogenesis (*gck, pklr, hk1, hk2, fpkma, fasn* and *g6pd*) and inhibited the expression of gluconeogenesis and amino acid catabolism (*g6pca1, pck1, pck2, asat, alat* and *gdh*). Therefore, the glucose stimulus into yolk was found to be effective to induce nutritional programing in Nile tilapia which could exert permanent metabolism effect latter life (Kumkhong et al., 2020a).

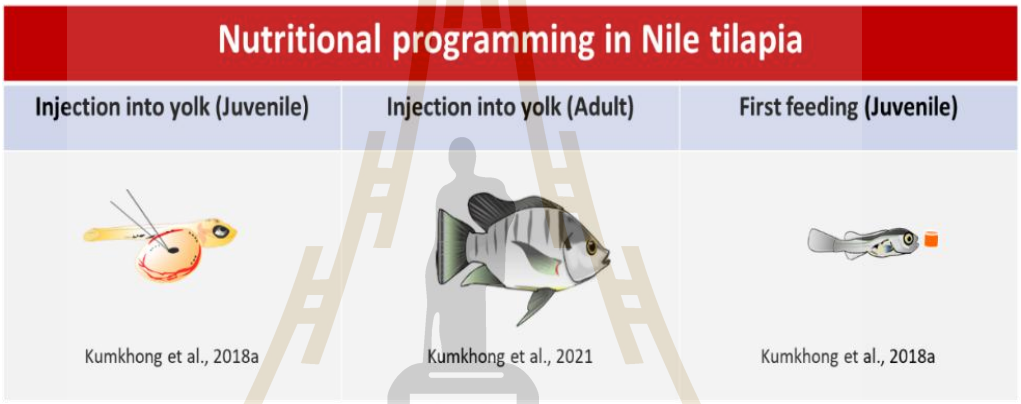

Figure 2.5 Nutritional programming in Nile tilapia (*Oreochromis niloticus*).

Early glucose injection on long-term zoo parameter (nutrient composition, plasma metabolite and carbohydrate metabolism were revealed similarly in Kumkhong et al., 2020a) However, the effect form juvenile fish are presented strong effect than adult stage. Taken together the impacts of nutritional programming between juvenile and adult phases after glucose injection might be due to difference life stage associated changes in carbohydrate metabolism for energy use in tilapia (FAO, 2019). It might also be due to that the programming effect may be erased after a long-time stage of development in life because the epigenetic marks at the origin of the programming could be reversible (Kumkhong et al., 2021).

The hyperglucidic stimulus was also performed using first feeding of Nile tilapia fry. Early intervention stimulus was achieved by feeding fry with high-protein/ low-carbohydrate (HP/LC) diet or high-carbohydrate/ low-protein (HC/LP) diet since first

feeding for 4 weeks in Nile tilapia (*O. niloticus*). There was several permanent effect of LP/HC diet-fed including 1) modulate hepatic composition, 2) increase glycogen in muscle, 3) lower enzyme involved in amino acid catabolism pathway, 4) higher enzyme involved in glycolysis. Subsequently, after challenging with difference dietary carbohydrate level, early high carbohydrate stimulus group has effect on adult tilapia including 1) increase utilization of glucose, which had protein-sparing effect for growth, 2) induce lipogenesis pathway, 2) decrease amino acid catabolism pathway. Stimulus low-protein/high-carbohydrate (LP/HC) diet at early fry stage for 4 weeks affected to retardation growth (Kumkhong et al., 2020b). Stimulus period is important for nutritional programming, because long-term affected to retardation growth.

2.6 Nutritional stimulus for nutritional programing effects

In order to develop nutritional programming in fish, the suitable nutritional stimulus is an important factor. For nutritional stimulus, the stage of metabolic plasticity is hypothesized to be a suitable window period for stimulus which varied according to fish species (Table 2.6). For example, microinjection of glucose into yolk reserves during embryogenesis or early larvae development were demonstrated to effective for nutritional stimulus (Rocha et al., 2014; Rocha et al., 2015). Maternal nutrient transfer by broodstock feeding was also used for nutritional stimulus (Fernandez-Palacious et al., 2015). Nutritonal stimulus at the onset of exogenous feeding were also demonstrated to be a period for nutritional stimulus (Geurden et al., 2007; Fang et al 2014; Geurden et al., 2014; Marandel et al., 2016). For example, in Trout (*O. mykiss*), stimulus by first feeding with 60% carbohydrate for 3 days showed the permanent effect on digestive enzyme (amylase and maltase), indicating a positive long-term physiological change (Geurden et al., 2007; Geurden et al., 2014; Marandel et al., 2016).

Moreover, in zebrafish, the effect of glucose injection was observed on long lasting change in the expression of gene (Table 2.7) in larvae involving glucose transport and glucose metabolism. The programming concept was evaluated in juveniles and had little effect on the long-term modulation of carbohydrate metabolic genes in Zebrafish (Fang et al., 2014; Rocha et al., 2014; Rocha et al., 2015).

Moreover, for nutritional programming in Nile tilapia (*O. niloticus*) (Table 2.8), carbohydrate stimulus was accomplished by microinjection of 2M glucose into yolk during alevin stage and dietary carbohydrate of first feeding during fry stage. The concept to fish nutrition provides numerous possibilities to improve carbohydrate utilization in other species (Kumkhong et al., 2020a, Kumkhong et al., 2020b).

Table 2.6 Summary enzyme activity through nutritional programming concept difference species.

Enzyme	Marandel et al.,2016 (Rainbow trout)				Geurden et al., 2014 (Rainbow trout)			Fang et al., 2014 (Zebrafish)					Song et al., 2019 (Rainbow trout)	
	Measure enzyme activity at different stage of development				Challenge diet for 65 days (days 105 – 170) with challenge feed (27% CHO 35% CP 16% CF)			Stimulus with 60 % Maltose dextrin at different stage for 3 days. Challenge with high carbohydrate (35 % CHO; 48% CP; 10 % CF) for 7 days.					Stimulus with 30% starch at first feeding for 4 weeks. Challenge with LP diet for 11 weeks.	
	Stage 6	Stage 15	Stage 22	Stage 23	Stage 31	HP	VLP	Control	FF-3	FF-5	YE- 3	YE- 5	Stimulus period	Challenge period
gck	nd	nd	nd	nd	nd	NS	NS	D	BC	C	B	A	XX	NS
hkl						NS	NS						NS	NS
pfklr	-	-	-	X	XX								XX	NS
pklr	-	-	-	-	X			B	B	A	A	A	X	NS
g6pca	-	-	-	-	X	NS	NS	AB	AB	C	A	A	NS	NS
fbpase	nd	nd	nd	nd	X								NS	NS
pepck	nd	-	-	X	XX			A	AB	B	AB	B		
pepck-C	-	-	-	-	X									
gdh						NS	NS							
hkm						NS	NS						NS	
pfkm														
pfkma													NS	NS
pfkmb													X	NS
pkma													X	XX
glut4													NS	XX
Amylase						NS	NS	B	A	B	B	ND		

*X; Significant at P<0.05 ,XX Significant at P<0.01,NS = Non-significant **Capital A,B,C,D shown significant at p <0.001; (A>B>C>D) Geurden et al., 2014 in juvenile rainbow trout (50 g) were induced by the early 5-day feeding of diets with low (VLP) and high (HP) protein/ carbohydrate ratio on gene expression in rainbow trout juveniles fed the challenge diet for 65 days (days 105–170) with challenge feed (27% CHO 35% CP 16% CF) Temperature was maintained at 18°C (spring water) VLP (65% CHO, 20% CP, 10 %CF) HP (<1% CHO, 60% CP, 11 %CF)

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CHAPTER III

EFFECT OF SHORT PERIOD OF EARLY HIGH CARBOHYDRATE AND ITS RELATION TO THE CARBOHYDRATE CHALLENGING IN JUVENILE TILAPIA FED WITH HIGH CARBOHYDRATE (CHO-H)

3.1 Abstract

The objective of this study was to investigate the effects of different durations of early carbohydrate (CHO) feeding stimulus on the growth performance and CHO metabolism of Nile tilapia later in life. The first-feeding nutritional stimulus treatments were a low-CHO/high-protein diet for 3 weeks (control group), a short duration high-CHO/low-protein (HC/LP) diet for 1 week (short-HC/LP group), and a long duration HC/LP diet for 3 weeks (long-HC/LP group). As expected, compared to the control fry, the short- and long-HC/LP diets were associated with lower growth performance in the fry at the end of the stimulus period, which was associated with differential gene expression for protein and glucose metabolism, followed by compensatory growth later. Subsequently, to test the existence of CHO metabolic programming, juvenile fish were challenged with a high-CHO diet in weeks 21–24. Our results show that early HC/LP stimulus improved the growth performance of juveniles, suggesting that a history of early HC/LP stimulus contributes to better use of dietary CHO for growth. Interestingly, metabolism modifications linked to early HC/LP stimulus were observed in many of the parameters, irrespective of the duration of early stimulus. The upregulation of the glucose gene (involved in glycolysis and transport) in juveniles that experienced early stimulus and the downregulation of genes involved in gluconeogenesis and amino acid catabolism can explain the improved use of the CHO diet in these fish. Taken together, early HC/LP feeding in tilapia fry is associated with highly significant metabolic programming later in life. Moreover, because the long-HC/LP treatment had stronger direct negative effects on growth performance than the short-HC/LP treatment, this study suggests

that a 1-week early HC/LP feeding period could be sufficient to program juvenile tilapia for better nutrient use.

Keywords: Nile tilapia; nutritional programming; early feeding; carbohydrate; glucose metabolism

3.2 Introduction

The most important challenge to overcome in fish nutrition is developing a cost-effective diet with maximal growth performance that promotes both human and animal welfare, and sustainable and environmentally friendly aquaculture. Several studies have sought alternative and novel ingredients in aquafeeds (Hua et al., 2019; Cottrell et al., 2020). Alternatively, new nutritional strategies must be developed to assist in the efficient use of alternative ingredients and/or specific nutrients. Recently, a new concept called nutritional programming has attracted increased attention; nutritional programming alters the ability of fish to better utilise specific nutrients. Nutritional programming or metabolic programming describes the effects of nutrient intervention on quality and/or quantity during early life, and how these effects persist and influence the modulation of metabolic and physiological pathways later in life. This concept is related to the nutritional status of mammals and fish during early life and the subsequent metabolic consequences at later life stages (Lucas, 1998; reviewed in Panserat et al., 2019; Hou and Fuiman, 2020). Nutritional programming has been successfully used to improve the utilisation of plant-based ingredients (Geurden et al., 2013; Izquierdo et al., 2015; Clarkson et al., 2017).

Metabolic programming using dietary carbohydrate (CHO) has been extensively studied in mammals in association with diseases such as diabetes and metabolic syndrome (Symonds, 2009; Vaiserman, 2017). CHO programming has also been investigated in carnivorous and omnivorous fish (Geurden et al., 2007, 2014; Fang et al., 2014; Gong et al., 2015; Rocha et al., 2016a, 2016b; Liang et al., 2017; Zambonino-Infante et al., 2019; Song et al., 2019; Kumkhong et al., 2020a, 2020b, 2021). The effects of nutritional programming vary according to fish species and/or feeding habits. For example, in rainbow trout (Oncorhynchus mykiss), early feeding with a high-CHO (HC) diet was later associated with the upregulation of enzymes

related to muscle glycolysis and glucose transport (Geurden et al., 2014; Song et al., 2019). In contrast, although an early HC diet increased the expression of the glucokinase enzyme and suppressed the expression of the glucose-6-phosphatase gene in European sea bass (Dicentrarchus labrax), these effects on CHO metabolism did not persist to the juvenile stage (Zambonino-Infante et al., 2019). The nutritional programming effects of early hyperglucidic stimulus are particularly strong in omnivorous fish (Fang et al., 2014; Rocha et al., 2014; Kumkhong et al., 2020a, 2020b, 2021).

Nile tilapia (*Oreochromis niloticus*), which are omnivorous fish, can effectively use CHO as an energy source (Kamalam et al., 2017; Shiau and Peng, 1993; Boonanuntanasarn et al., 2018a). The Nile tilapia is an economically important freshwater fish worldwide, and its production is estimated to increase annually (FAO, 2018). Moreover, the Nile tilapia is a model omnivorous aquaculture-related experimental fish, particularly for CHO metabolism. Indeed, Nile tilapia have high glucose homeostasis abilities and can adapt to CHO intake by inducing glycolysis and lipogenesis and inhibiting gluconeogenesis (Boonanuntanasarn et al., 2018a, 2018b). The first experiment on CHO nutritional programming was conducted by injecting glucose into yolk reserves during the larval stage. Its effects on CHO metabolism were later detectable in the juvenile and adult stages (Kumkhong et al., 2020a, 2021). Although glucose injection into the yolk reserve can be applied for metabolic programming, this technique is not feasible on a large scale in tilapia farming. The results of a second experiment on nutritional programming, which involved early feeding using an HC diet for 4 weeks during the fry stage, were also positive (Kumkhong et al., 2020b). However, the 4 weeks of early HC feeding directly decreased the growth performance of the fish fry, although compensatory growth occurred later (Kumkhong et al., 2020b).

To further the application of nutritional programming in tilapia farming and improve the early feeding stimulus protocol, this study investigated the optimal method for early HC feeding stimulus by comparing short (1 week) and long (3 weeks) duration HC feeding treatments. Our data show that the two different durations of early HC feeding had similar effects on CHO metabolism at the molecular level. Significant effects of early HC feeding stimulus were found in

several intermediary metabolic actors during the juvenile stage. In particular, when juvenile fish were challenged with an HC diet, we detected positive effects on growth performance and CHO use, leading us to conclude that the optimal period for early HC feeding stimulus in tilapia.

3.3 Materials and methods

3.3.1 Experimental design, diet, and fish rearing

All experimental procedures involving fish were approved by the Ethics Committee of the Suranaree University of Technology Animal Care and Use Committee (approval no. A-18/2562). The Nile tilapia broodstock (800–1000 g) was maintained in an earthen pond (800 m2) at the University Farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The tilapia broodstock was fed with a commercial diet of 30% crude protein (CP) and 4% crude fat (CF) at 3% body weight daily.

The experimental design was completely randomised, with three treatment stimuli, each of which was replicated six times (using six brood females). Figure 3.1 demonstrates the experimental plan, including the stimulus and challenge periods. Nile tilapia larvae were collected from six brood females (replicates). During the stimulus period, the control Nile tilapia fry (50 fish/replicate) were fed a low-CHO (LC)/high-protein (HP) diet for 3 weeks (control). For the two dietary stimulus treatments, the Nile tilapia fry (50 fish/replicate) were either fed an HC/low-protein (LP) diet for 1 week followed by 2 weeks of the control diet (short-HC/LP treatment), or the HC/LP diet for 3 weeks (long-HC/LP treatment). All experimental fish were then fed a commercial diet (32% CP+4% CF) up to week 20. During weeks 21–24, all the experimental fish were challenged with an HC diet. Six fish from each replicate were randomly selected, cultured (at a density of 1 fish/100 L), and fed an HC diet for 4 weeks. The compositions of the experimental difference stimulus diets Table 3.1 and challenge diet (Table 3.2) their proximate compositions, including moisture, crude protein (CP), crude fat (CF), crude fibre, and ash, which were analysed according to the standard methods of the Association of Official Analytical Chemists (AOAC, 1990). Dead fish were recorded to determine the survival rate, and growth performance was monitored. Throughout the experimental period, the air and water

temperatures were recorded daily. They ranged from 30.0–35.0℃ and 26.0–29.0℃, respectively. The dissolved oxygen was determined weekly using a dissolved oxygen meter; it was maintained at 5.0±0.7 mg/L (average±SD). A pH meter was used to determine the pH, which ranged from 7.5–8.5. The dissolved oxygen and pH were both within acceptable ranges for tilapia growth.

Table 3.1 Ingredients of the two stimulus diets (diets at first feeding).

Ingredients (%)	Stimulus diets	
	HP/LC	HC/LP
Fish meal	86	36
Rice flour	10	56
Soybean oil	2	6
Fish premix [a]	2	2
Proximate composition (% dry weight)		
Dry matter	95.78	95.66
Protein	49.26	24.71
Fat	9.69	9.92
Fiber	0.58	0.48
Ash	23.81	10.81
NFE [b]	16.67	54.09
Gross energy (kJ g^{-1})	17.80	18.60

[a] Vitamin and trace mineral mix provided the following (IU/kg or g/kg 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, 5000 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, 1000 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.

Table 3.2 Ingredients of the challenge diets for two experiments.

Ingredients (%)	Challenging diet
	High carbohydrate (CHO-H)
Fish meal	14
Soybean meal	6
Rice flour	70
Rice bran	3
Soybean oil	4
Fish premix [a]	1
Dicalcium phosphate	2
Proximate composition (% dry weight)	
Dry matter	95.73
Protein	15.49
Fat	6.48
Fiber	0.86
Ash	6.03
NFE [b]	66.88
Gross energy)kJ g^{-1} (	17.20

[a] Vitamin and trace mineral mix provided the following (IU/kg or g/kg 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, 5000 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, 1000 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.

3.3.2 Fish sampling, blood collection, and proximate nutritive analysis

For fry sampling, 4 h after feeding, fish were euthanised by exposing them to ice water. During the stimulus period, in week 1, three pools of three fish from each replicate (n = 6 replicates) were sampled and stored at −80°C for metabolic gene expression and whole-body triglyceride and glycogen content analyses. In addition, in week 3, these analyses were conducted using samples of one fish per replicate (n = 6 replicates). After week 20 (before the challenge) and

week 24 (after the challenge), three fish per replicate (n = 6 replicates) were randomly sampled. The fish were anaesthetised with clove oil (40 mg L−1) 5 h after feeding. To analyse blood metabolites, blood samples (from two fish) were collected from the caudal vein using a hypodermic syringe and mixed with 1.0% (v/v) of 15% ethylenediaminetetraacetic acid. Plasma was collected by centrifuging the samples at 3000 ×g for 10 min at 4°C and was stored at −80°C until analysis. After bleeding, portions of liver and muscle tissue were collected and stored at −80°C for the analysis of metabolic gene expression. Subsequently, other liver and muscle tissue samples were collected to determine their proximate nutritive composition and their triglyceride and glycogen content. In addition, one fish per replicate (n = 6 replicates) was sampled to analyse the proximate nutritive composition of the whole body

3.3.3 Proximate chemical composition and glycogen and triglyceride content

Proximate chemical analyses, including CP, CF, and ash analyses, were performed according to the Association of Official Analytical Chemists (1990). The glycogen content in 1-week-old fry (three fish) and 3-week old fry (one fish), and the liver (100 mg) and muscular (200 mg) glycogen compositions of the juvenile fish were determined according to the hydrolysis technique described by Good et al. (1933), with modifications (Kumkhong et al., 2020a). To analyse triglycerides, the whole bodies of the 1-week old fry (three fish) and 3-week old fry (one fish), and the liver and muscle samples, were homogenised in liquid nitrogen, and 100 mg of each sample was homogenised again in 1 mL of 5% IGEPAL in a deionised water solution. The samples were heated to 90°C for 5 min and then cooled down to room temperature. The heating and cooling steps were repeated. Subsequently, to remove any insoluble material, the supernatants were collected after centrifuging the reaction mixture at 10,000 ×g and 4°C for 10 min. The supernatants were diluted with deionised water, and triglycerides were measured using the glycerol-3 phosphate oxidase-sodium N-ethyl-N-(3-sulfopropyl)-m-anisidine method (Bucolo and David, 1973).

3.3.4 Blood chemical analysis

The plasma metabolites, including glucose, triglycerides, cholesterol, and blood urea nitrogen (BUN), were measured using two fish per replicate. The plasma glucose levels were quantitatively analysed using Trinder's method (Trinder, 1969). The plasma triglyceride levels were evaluated using 3-sulfopropyl-m-anisidine (Bucolo and David, 1973). Cholesterol was quantitatively determined using the cholesterol oxidase phenol–aminophenazone method (Flegg, 1973). Plasma protein levels were evaluated using the Biuret method (Gornall et al., 1949). The plasma BUN levels were analysed using a modified indophenol colorimetric method (Weatherburn, 1967).

3.3.5 Total RNA extraction, cDNA synthesis, and real-time RT-PCR for metabolic gene expression

Quantitative real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) was performed to measure the mRNA levels of CHO metabolism-related genes in the whole bodies of the 1-week old fry and 3-week old fry and the liver and muscle of juvenile fish. The expression of genes involved in hepatic glycolysis, including glucokinase (gck), phosphofructokinase (pfklr), and pyruvate kinase (pklr), and gluconeogenic enzymes, including glucose-6-phosphatase (g6pca1 and g6pca2), phosphoenolpyruvate carboxykinase cytosolic (pck1), and phosphoenolpyruvate carboxykinase (pck2) was assessed. The analysis of lipogenic capacity included fatty acid synthase (fasn) and glucose-6-phosphate dehydrogenase (g6pd) determination. The examination of the expression of enzymes related to amino acid catabolism included glutamate dehydrogenase (gdh), alanine aminotransferase (alat), and aspartate amino transferase (asat). In the muscle samples, genes involved in glucose utilisation, including glucose transporter (glut4), and enzymes related to muscular glycolysis (hexokinase I/II, hk1, and hk2; phosphofructokinase, pfkma, and pfkmb; and PK and pkma) were analysed.

Total RNA was extracted from 1-week-old fry (three fish) and 3-week-old fry (one fish) and from the liver (50 mg) and muscle (100 mg) samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quantity and quality of total RNA were measured using a NanoDrop system (Thermo Fisher, Madison, WI, USA) and 1% agarose gel electrophoresis, respectively. One microgram of total RNA was used

for cDNA synthesis (duplicated for each sample) using a SuperScript III RNAseH-Reverse transcriptase kit (Invitrogen) (Table 3.3), with random primers (Promega, Madison, WI, USA), following the manufacturer's protocol. Reverse transcription was performed in duplicate for each sample. Each PCR assay included replicate samples, and duplicate reverse transcription and real-time PCR amplification assays were performed to analyse the mRNA levels. Table 3.5 shows the primer sequences used for the real-time RT-PCR. Reverse transcriptase- and cDNA-template-free samples were used as negative controls. Relative quantification of experimental gene expression was conducted using the Roche Applied Science E-Method (Table 3.4), according to Pfaffl (2001). As the relative gene expression of ef1α did not differ significantly among the experimental groups (data not shown), the relative mRNA level of ef1α was used to normalise the measured mRNA levels. In all cases, the PCR efficiency was determined from the slope of the standard curve using serial dilutions of cDNA. Throughout the analysis, the PCR efficiency values ranged between 1.8 and 2.0.

Table 3.3 Master mix used for cDNA synthesis.

Solution	**Volume (µl)**
Reaction I	
Total RNA 1µg	10
Random primer	1
RNA luciferase (2 µg/ µl)	0.5
PCR NUC (dNTP)	1
Reaction II	
DI	1.5
TP 5X buffer	4
dTT (0.1M)	1
Rnase out	1
Ss III RT	0.5
Total	20.5

Table 3.4 Master mix used for real-time quantitative polymerase chain reaction.

Solution	Volume (µl)
2X Light Cycle 480 SYBR® Green	3
1 µg cDNA	2
Forward primer 10 mM	0.12
Reward primer 10 mM	0.12
DNAse/RNAse free water	0.76
Total	6

Table 3.5 Primers used for mRNA quantification by qRT-PCR in carbohydrate metabolism.

Gene	Name	5'/3' Sense primer	5'/3' Antisense primer
Gck	Glucokinase	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC
Pkma	Pyruvate kinase muscle	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA
Pklr	Pyruvate kinase liver	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA
glut4	Glucose transporter type 4 muscle	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG
Hk2	Hexokinase 2 muscle	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC
Hk1	Hexokinase 1	CGTCGCTTAGTCCCAGACTC	TGACTGTAGCGTCCTTGTGG
Pfkma	phosphofructokinase, muscle a	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG
Pfkmb	phosphofructokinase, muscle b	TTTGTGCATGAGGGTTACCA	CACCTCCAATCACACACAGG
Pfkla	Phosphofructokinase, liver	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC
FASN	Fatty Acid Synthase	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT
G6PD	glucose-6-phosphate dehydrogenase	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT
G6pca.1	Glucose-6-phosphatase 1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA
G6pca.2	Glucose-6-phosphatase 2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA
Pck1	Phosphoenolpyruvate carboxykinase 1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA
Pck2 F2	Phosphoenolpyruvate carboxykinase 2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT
ASAT	Aspartate amino transferase	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC
ALAT	Alanine amino transferase	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG
GDH	Glutamate dehydrogenase	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC
EF-1a	Eukaryotic translation elongation factor 1 alpha	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC

3.3.6 Statistical analysis

All data were analysed using SPSS for Windows version 12) SPSS Inc., Chicago, IL, USA .(One-way analysis of variance was performed and, where significant differences were observed, the Tukey's range test was used to rank the treatment groups .The effects and differences were considered significant at $P<0.05$.

3.4 Result

The results of this study are presented as follows: the direct effects of the early stimulus treatments (weeks 1–3), Section 3.2 corresponds to the intermediary phase (weeks 4–20), and Section 3.3 concerns the final challenge (weeks 20–24).

3.4.1 Direct effects of early HC/LP feeding on growth and CHO metabolism in fry

During the stimulus period, fish fry were fed an HC/LP diet for 1 week (short-HC/LP fish) or 3 weeks (long-HC/LP fish). There were no significant differences in the mortality or malformation rates of the fish fry during the early stimulus feeding periods (Table 3.6). Compared with the LC/HP diet-fed fry (control fish), the short- and long-HC/LP fry showed lower growth performance including final weight (FW), weight gain (WG), average daily gain (ADG), and specific growth rate (SGR) and a higher feed conversion ratio (FCR) (Table 3.6).

Table 3.6 Growth performances of the Nile tilapia that were fed the HC/LP diet for 1 week (Short HC/LP) and 3 weeks (Long HC/LP) and control LC/HP during the stimulus (mean±SD, n=6).

Experimental periods	FW3 (g)	WG4 (g)	ADG5 (g day-1)	SGR6 (% day-1)	FCR[7]	Survival rate[8]
Week 1 (during stimulus) 1						
Control	33.72±1.27[a]	26.32±1.16[a]	3.76±0.17[a]	21.68±0.68[a]	0.59±0.04[b]	
Short HC/LP	31.83±1.00[b]	24.28±0.96[b]	3.47±0.14[b]	20.57±0.72[b]	0.65±0.04[a]	No
Long HC/LP	31.88 ± 0.30[b]	24.05±0.20[b]	3.44±0.03[b]	20.05±0.12[b]	0.68±0.01[a]	mortality
P -value[2]	0.005	0.001	0.001	0.001	0.001	
Week 2 (during stimulus) 1						
Control	45.03±1.29[a]	37.63±1.11[a]	2.69±0.08[a]	12.91±0.29[a]	2.09±0.03[b]	
Short HC/LP	39.01±1.34[b]	31.46±0.99[b]	2.25±0.07[b]	11.74±0.14[b]	2.24±0.02[a]	No
Long HC/LP	39.98±0.97[b]	32.15±0.94[b]	2.30±0.07[b]	11.64±0.17[b]	2.25±0.02[a]	mortality
P -value[2]	<0.001	<0.001	<0.001	<0.001	<0.001	
Week 3 (during stimulus) 1						
Control	125.92±3.71[a]	118.52±3.95[a]	5.64±0.19[a]	13.50±0.34[a]	1.54±0.05	
Short HC/LP	117.16±2.51[b]	109.61±2.72[b]	5.22±0.13[b]	13.06±0.30[b]	1.54±0.05	No
Long HC/LP	118.23±0.77[b]	110.40±0.83[b]	5.26±0.04[b]	12.93±0.09[b]	1.55±0.02	mortality
P -value[2]	<0.001	<0.001	<0.001	0.005	0.718	
Week 4 (After stimulus)[1]						
Control	378.98±22.77[a]	371.59±22.80[a]	13.27±0.81[a]	14.06±0.29[a]	1.21±0.04	
Short HC/LP	359.67±13.37[ab]	352.12±13.48[ab]	12.58±0.48[ab]	13.80±0.25[ab]	1.19±0.02	No
Long HC/LP	339.27±26.45[b]	331.44±26.44[b]	11.84±0.94[b]	13.45±0.28[b]	1.24±0.04	mortality
P -value[2]	0.021	0.019	0.019	0.006	0.106	

[1]Note FW; mg, WG; mg, ADG; mg/day, SGR;mg day-1.

[2]One-way ANOVA analysis of Variance was used to analyze the effects of different stimulus between Control, Short HC/LP and Long HC/LP stimulus with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) diets.

[3]Final body weight (FW) = initial body weight - final body weight

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

[8]Survival rate = 100 × [(Initial number of fish – Final number of final)/Initial number of fish]

The effects of early HC/LP stimuli on the expression of genes encoding enzymes involved in hepatic glycolysis (gck, pfklr, and fklr), gluconeogenesis (g6pca1, g6pca2, pck1, and pck2), lipogenesis (fasn and g6pd), amino acid catabolism (asat, alat, and gdh), glucose transport (glut4), and muscular glycolysis (hk1, hk2, pfkma, pfkmb, and pkma) were examined (Table 3.7). After week 1 of the stimulus period, the HC/LP diet had upregulated the expression of hepatic gck and muscle pkma ($P< 0.05$). In addition, this diet downregulated the expression of g6pca1, asat, alat, and glut4 ($P<0.05$). However, there were no significant changes in the expression of pfklr, pklr, g6pca2, pck1, pck2, fasn, g6pd, gdh, hk1, hk2, pfkma, or pfkmb ($P>0.05$). After week 3 of the stimulus period, feeding with the HC/LP diet increased the mRNA levels of g6pd, hk2, pfkmb, and pkma in the long-HC/LP fry and pfkma in both the short- and long-HC/LP fry ($P<0.05$). Moreover, the HC/LP stimuli diet was always associated with downregulated expression of asat, alat, and glut4 ($P<0.05$). In contrast, no reduction in the expression of g6pca1 or elevation in that of gck were detected ($P>0.05$). The expressions of pfklr, pklr, g6pca2, pck1, pck2, fasn, gdh, and hk1 also remained unchanged ($P>0.05$).

Regarding the metabolites, after week 1 of the HC/LP stimulus period, the glycogen and triglyceride content of the whole body was elevation) Fig .3A-B .(After week 3 of the HC/LP stimulus period, the increases in the glycogen and triglyceride content continued in the long-HC/LP fry .However, only a small elevation in triglycerides and no further effects on glycogen were observed in the short-HC/LP fry (Fig .3C-D).

Table 3.7 mRNA levels of genes involved in carbohydrate metabolism in the whole body of the Nile tilapia that were fed the HC/LP diet for 1 week (Short HC/LP) and 3 weeks (Long HC/LP) and control LC/HP during the stimulus (mean±SD, n=6).

	Week 1 (During stimulus)				Week 3 (During stimulus)			
	Control	Short HC/LP	Long HC/LP	P value	Control	Short HC/LP	Long HC/LP	P value
Glycolysis								
gck	0.31±0.10^{b}	0.60±0.20^{a}	0.64±0.24^{a}	**0.017**	0.77±0.15	0.89±0.13	0.95±0.17	**0.138**
pfklr	1.12±0.26	1.28±0.35	1.21±0.24	**0.652**	0.44±0.20	0.59±0.16	0.58±0.19	**0.307**
pklr	1.51±0.84	1.27±0.39	0.97±0.31	**0.281**	0.48±0.25	0.32±0.10	0.28±0.21	**0.217**
Gluconeogenesis								
g6pca1	1.41±0.19^{a}	1.01±0.20^{b}	1.01±0.32^{b}	**0.017**	2.51±0.30	2.20±0.25	2.08±.52	**0.148**
g6pca2	1.08±0.72	1.28±0.46	1.23±0.32	**0.804**	0.50±0.16	0.52±0.22	0.44±0.10	**0.728**
pck1	1.14±0.72	1.60±0.84	1.04±0.38	**0.342**	0.56±0.19	0.43±0.11	0.45±0.12	**0.234**
pck2	0.76±0.45	0.98±0.80	0.90±0.76	**0.850**	0.97±0.28	0.86±0.10	0.77±0.08	**0.164**
Lipogenesis								
fasn	0.85±0.32	1.22±0.16	1.06±0.29	**0.092**	0.76±0.13	0.97±0.19	0.86±0.12	**0.072**
g6pd	1.06±0.38	1.27±0.20	1.33±0.41	**0.360**	0.67±0.16^{b}	0.85±0.17^{ab}	0.95±0.18^{a}	**0.017**
Amino acid catabolism								
asat	1.47±0.50^{a}	0.91±0.32^{b}	0.87±0.30^{b}	**0.028**	1.02±0.12^{a}	0.88±0.24^{ab}	0.65±0.21^{b}	**0.014**
alat	1.56±0.37^{a}	1.17±0.26^{b}	1.16±0.13^{b}	**0.032**	0.98±0.17^{a}	0.69±0.12^{b}	0.75±0.08^{b}	**0.004**
gdh	1.01±0.22	1.18 0.10	1.11±0.20	**0.270**	0.92±0.17	0.98±0.23	0.93±0.15	**0.806**
Glucose transport and glycolysis								
glut4	1.56±0.24^{a}	1.00±0.24^{b}	0.95±0.42^{b}	**0.007**	1.11±0.31^{a}	0.81±0.15^{b}	0.79±0.09^{b}	**0.033**
hk1	1.18±0.36	0.93±0.58	0.95±0.32	**0.545**	0.89±0.17	0.92±0.06	0.93±0.05	**0.810**
hk2	1.12±0.37	1.43±0.40	1.47±0.54	**0.370**	0.78±0.20^{b}	1.00±0.14^{ab}	1.19±0.22^{a}	**0.006**
pfkma	1.11±0.58	1.54±0.42	1.25±0.28	**0.280**	0.60±0.15^{b}	0.91±0.14^{a}	0.94±0.19^{a}	**0.004**
pfkmb	1.19±0.47	1.24±0.22	1.18±0.25	**0.945**	0.87±0.16^{b}	0.79±0.09^{b}	1.08±0.18^{a}	**0.012**
pkma	0.82±0.26^{b}	1.52±0.43^{a}	1.46±0.50^{a}	**0.019**	0.81±0.08^{b}	0.92±0.16^{b}	1.08±0.09^{a}	**0.005**

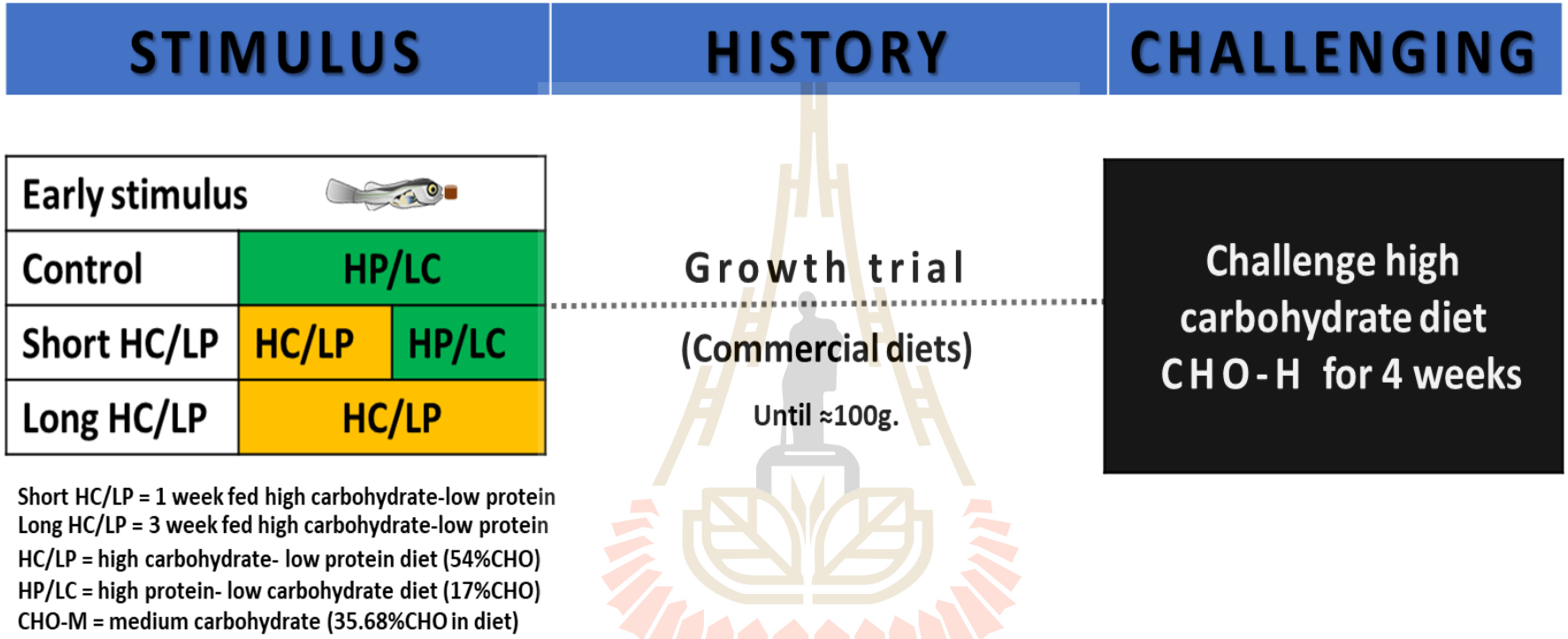

Figure 3.1 Experimental plan and sampling regimes during the challenging with high carbohydrate diet (CHO-H).

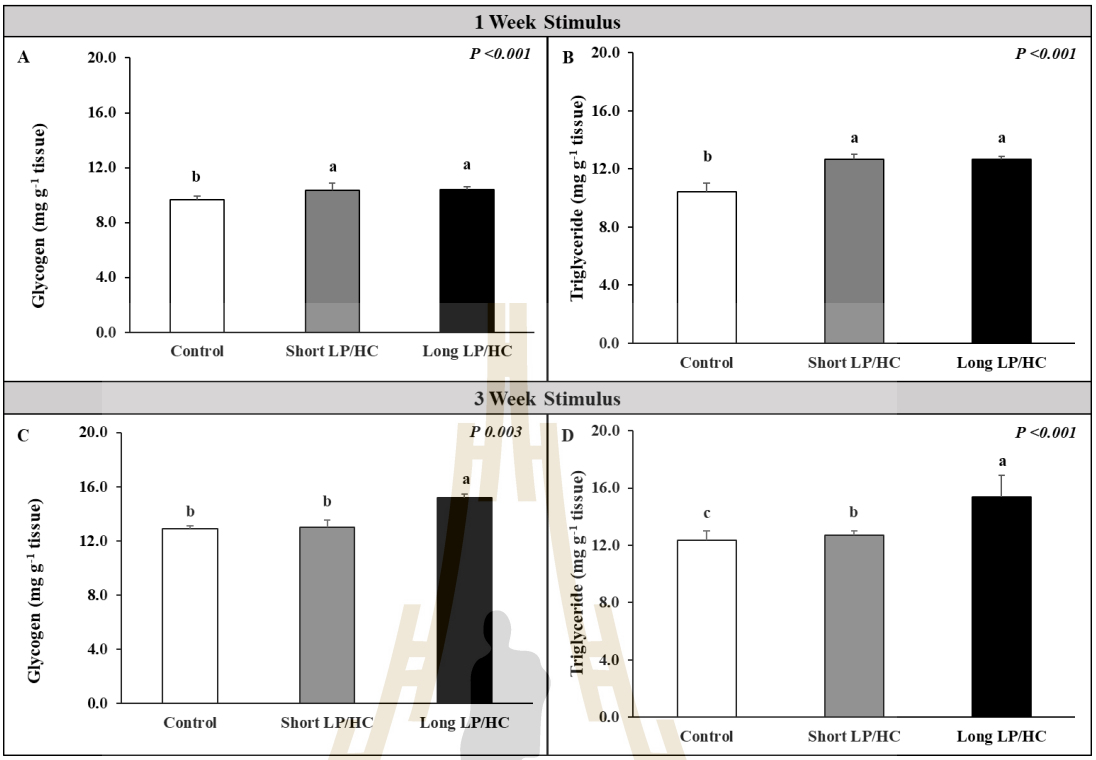

Fig .3.2 Glycogen and Triglyceride contents in whole larvae of fish after the early dietary stimulus. (fig.3A; glycogen content in whole body at stimulus 1 week, fig 3B; triglyceride content in whole body at stimulus 1 week, fig 3C glycogen content in whole body at stimulus 3-week, fig 3D; triglyceride content in whole body at stimulus 3 week respectively)

3.4.2 Long-term effects of early HC/LP stimuli on the growth performance and CHO metabolism of juvenile fish fed a commercial diet (up to week 20)

Table 3.8 shows that the growth performance (FW, WG, ADG, SGR, FCR) of the short- and long-HC/LP fish caught up to that of the control fish by week 20 (juvenile fish) ($P>0.05$). The effects of an HC/LP stimulus history on the nutritive composition of the liver, muscle, and whole body of juvenile fish are shown in Table 3.9. There were no significant differences in the protein or ash content of the liver, muscle, and whole body among the experimental fish ($P>0.05$). While the fat content in the muscle and whole body was similar among all the experimental fish ($P>0.05$), the long-HC/LP fish had a higher hepatic fat content than the control fish ($P<0.05$).

The hepatic and muscular glycogen content was similar among the experimental groups (P>0.05). There were also no significant differences in the muscular triglyceride content, whereas both the short- and long-HC/LP fish had a higher hepatic triglyceride content than the control fish (P<0.05). The hepatosomatic index was similar in all experimental groups (P>0.05). Finally, there were no significant differences in the plasma metabolites (including glucose, triglyceride, BUN, cholesterol, and total protein) of the juvenile fish (P>0.05) (Table 3.10).

Table 3.8 Long-term effects of early HC/LP stimulus on the growth performance of juvenile fish fed a commercial diet (up to week 20) (mean±SD, n=6).

Experimental periods	FW3 (g)	WG4 (g)	ADG5 (g day−1)	SGR6 (% day−1)	FCR[7]	Survival rate[8]
Week 8						
Control	2.95±0.15^{a}	2.93±0.15^{a}	0.0517±0.004^{ab}	9.41±0.09^{a}	0.95±0.04^{b}	
Short HC/LP	2.95±0.16^{a}	2.93±0.16^{a}	0.0533±0.005^{a}	9.40±0.10^{a}	0.90±0.03^{b}	No mortality
Long HC/LP	2.48±0.18^{b}	2.47 ± 0.18^{b}	0.0450±0.005^{b}	9.09±0.13^{b}	1.07±0.07^{a}	
P -value [2]	**<0.001**	**<0.001**	**0.025**	**<0.001**	**<0.001**	
Week 12						
Control	14.97±0.73^{a}	14.95±0.73^{a}	0.18±0.01^{a}	8.21±0.06^{a}	1.50±0.06^{a}	
Short HC/LP	14.37±0.57^{a}	14.35±0.57^{a}	0.17±0.01^{a}	8.15±0.04^{a}	1.46±0.04^{ab}	No mortality
Long HC/LP	12.83±0.81^{b}	12.82±0.81^{b}	0.15±0.01^{b}	8.02±0.07^{b}	1.42±0.04^{b}	
P -value [2]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.035**	
Week 16						
Control	53.55±5.40^{a}	53.53±5.40^{a}	0.48±0.05^{a}	7.29±0.09^{a}	1.56±0.27	
Short HC/LP	47.55±3.17^{ab}	47.53±3.17^{ab}	0.42±0.03^{ab}	7.18±0.06^{a}	1.58±0.12	No mortality
Long HC/LP	41.38±5.47^{b}	41.37±5.47^{b}	0.37±0.05^{b}	7.06±0.12^{b}	1.84±0.45	
P -value [2]	**0.002**	**0.002**	**0.002**	**0.002**	**0.259**	
Week 20						
Control	93.20±0.91	93.18±0.91	0.67±0.01	6.22±0.01	1.67±0.12	99.17±1.39
Short HC/LP	91.98±0.84	91.97 0.84	0.66±0.01	6.22±0.01	1.61±0.06	100.00±0.00
Long HC/LP	92.03±1.02	92.02±1.02	0.66±0.01	6.18±0.06	1.63±0.11	99.72±0.68
P -value [2]	**0.063**	**0.063**	**0.063**	**0.111**	**0.591**	**0.290**

[1]Note FW;mg,WG;mg,ADG;mg/day,SGR;mg day-1

[2]One-way ANOVA analysis of Variance was used to analyze the effects of different stimulus between Control, Short HC/LP and Long HC/LP stimulus with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) diets.

[3]Final body weight (FW) = initial body weight - final body weight

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

[8]Survival rate = 100 × [(Initial number of fish – Final number of final)/Initial number of fish]

Table 3.9 Proximate composition in liver, muscle and whole body of the Nile tilapia that were fed the HC/LP diet for 1 week (Short HC/LP) and 3 weeks (Long HC/LP) and control LC/HP during before challenge (week 20) (mean±SD, n=6).

	Week 20 (Before challenge)			
	Control	Short HC/LP	Long HC/LP	P value
Liver(%)				
Protein	9.43±0.60	9.49±0.38	9.74±0.73	**0.404**
Fat	4.47±0.25[b]	4.65±0.20[ab]	4.76±0.21[a]	**0.011**
Ash	1.23±0.24	1.31±0.22	1.34±0.39	**0.661**
HSI (%)	1.50±0.13	1.48±0.13	1.51±0.07	**0.922**
Glycogen (mg g-1 tissue)	46.8±0.67	47.01±1.27	47.35±1.67	**0.753**
Triglyceride) mg g^{-1} tissue(	14.24±0.44[c]	25.30±1.26[b]	29.04±2.12[a]	**<0.001**
Muscle (%)				
Protein	13.92±0.72	13.54±0.49	13.72±0.42	**0.263**
Fat	1.58±0.09	1.61±0.26	1.55±0.23	**0.808**
Ash	1.32±0.38	1.30±0.21	1.32±0.20	**0.975**
Glycogen (mg g-1 tissue)	4.37±0.09	4.44±0.33	4.58±0.29	**0.364**
Triglyceride (mg g-1 tissue)	4.72±0.24	4.76±0.15	4.99±0.19	**0.068**
Whole body (%)				
Protein	11.3±0.75	11.48±0.90	12.17±1.13	**0.071**
Fat	3.41±0.19	3.25±0.16	3.31±0.17	**0.092**
Ash	3.81±0.22	3.78±0.17	3.82±0.37	**0.953**

Table 3.10 Plasma metabolites of the Nile tilapia that were fed the HC/LP diet for 1 week (Short HC/LP) and 3 weeks (Long HC/LP) and control LC/HP during before challenge (week 20) (mean±SD, n=6).

	Week 20 (Before challenge)			
	Control	**Short HC/LP**	**Long HC/LP**	**P value**
Glucose)mM(	2.50±0.18	2.59±0.22	2.59±0.19	**0.693**
Triglyceride)mM(	1.45±0.12	1.49±0.12	1.51±0.05	**0.553**
BUN)mM(	0.65±0.35	0.44±0.25	0.40±0.26	**0.300**
Cholesterol (mM)	2.25±0.14	2.25±0.02	2.28±0.04	**0.818**
Total protein (g/L)	28.59±1.76	27.43±0.90	27.19±0.57	**0.126**

The effects of an HC/LP stimulus history on the gene expression of enzymes related to CHO metabolism are shown in Table 6. After week 20, an HC/LP diet stimulus history led to the upregulation of pfklr and fasn in both the short- and long-HC/LP fish, whereas it elevated the expression of pklr, hk2 and pkma in only the long-HC/LP fish ($P<0.05$). In contrast, the expression of pck2 and asat ($P<0.05$) was downregulated in both the short- and long-HC/LP fish, and a decrease in the expression of g6pca1, alat, and glut4 was observed in the long-HC/LP fish ($P<0.05$). There were no significant alterations observed in the expression of gck, g6pca2, pck1, g6pd, gdh, hk1, pfkma, or pfkmb ($P>0.05$) (Table 3.11).

Table 3.11 mRNA levels of genes involved in carbohydrate metabolism in the liver and muscle of the Nile tilapia during before challenge (mean±SD, n=6).

	Week 20 (Before challenge)			
	Control	Short HC/LP	Long HC/LP	P value
Glycolysis				
gck	1.71±0.16	1.90±0.16	1.74±0.17	**0.136**
pfklr	1.03±0.09^{b}	1.20±0.05^{a}	1.25±0.10^{a}	**0.001**
pklr	0.55±0.20^{b}	0.76±0.18^{ab}	0.99±0.38^{a}	**0.037**
Gluconeogenesis				
g6pca1	1.99±0.12^{a}	1.90±0.09^{ab}	1.73±0.17^{b}	**0.011**
g6pca2	0.56±0.21	0.70±0.32	0.62±0.19	**0.626**
pck1	0.62±0.17	0.55±0.11	0.54±0.12	**0.560**
pck2	2.17±0.29^{a}	1.41±0.16^{b}	1.26±0.28^{b}	**<0.001**
Lipogenesis				
fasn	0.31±0.28^{b}	0.94±0.22^{a}	0.98±0.32^{a}	**0.001**
g6pd	1.35±0.14	1.68±0.26	1.50±0.28	**0.089**
Amino acid catabolism				
asat	2.06±0.13^{a}	1.49±0.14^{b}	1.42±0.16^{b}	**<0.001**
alat	1.03±0.19^{a}	1.06±0.17^{a}	0.56±0.29^{b}	**0.002**
gdh	0.87±0.11	0.92±0.14	0.93±0.11	**0.685**
Glucose transport and glycolysis				
glut4	1.13±0.15^{a}	1.27±0.23^{a}	0.61±0.36^{b}	**0.001**
hk1	0.23±0.03	0.24±0.05	0.24±0.05	**0.852**
hk2	0.68±0.12^{b}	0.76±0.04^{ab}	0.84±0.09^{a}	**0.024**
pfkma	0.82±0.06	0.84 ±0.17	0.72±0.22	**0.399**
pfkmb	0.99±0.13	0.94±0.12	1.09±0.15	**0.154**
pkma	0.54±0.20^{b}	0.81±0.28^{b}	1.30±0.25^{a}	**<0.001**

3.4.3 Early dietary HC stimuli exerted positive effects on growth and CHO metabolism in juvenile fish challenged with an HC diet for four weeks

After week 20 (juvenile fish), all experimental fish, including the LC/HP diet-fed control fish, short-HC/LP diet-fed fish, and long-HC/LP diet-fed fish, were challenged with an HC diet for 4 weeks. Table 3.12 shows the growth performance and survival rates of the fish during the challenge period. Our results show that an early HC-LP stimulus history affected growth performance. Both the short- and long-HC/LP stimuli improved growth performance, including FW, WG, ADG, and SGR, when compared to the LC/HP control diet ($P<0.05$). In addition, a significantly lower FCR was observed in the short- and long-HC/LP fish than in the control fish ($P<0.05$). There were no significant differences in survival rate during the challenge period (Table 3.12).

Table 3.12 Growth performances of the Nile tilapia during challenging with high carbohydrate (CHO-H) for 4 weeks (mean±SD, n=6).

Experimental periods	FW3 (g)	WG4 (g	ADG5 (g day−1)	SGR6 (% day−1)	FCR[7]	Survival rate[8]
Week 24 (during challenging with CHO-H)						
Control	125.50±9.09[b]	125.48±9.09[b]	0.75±0.06[b]	5.37±0.04[b]	2.20±0.17[a]	97.22±6.81
Short HC/LP	143.50±8.24[a]	143.48±8.24[a]	0.86±0.05[a]	5.45±0.03[a]	1.91±0.11[b]	97.22±6.81
Long HC/LP	138.33±8.02[a]	138.31±8.02[a]	0.83±0.05[a]	5.43±0.04[a]	1.98±0.11[b]	100.00±0.00
P -value [2]	**0.006**	**0.006**	**0.006**	**0.006**	**0.006**	**0.616**

[1] Note FW;mg,WG;mg,ADG;mg/day,SGR;mg day-1

[2] One-way ANOVA analysis of Variance was used to analyze the effects of different stimulus between Control, Short HC/LP and Long HC/LP stimulus with high-protein/ low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) diets.

[3] Final body weight (FW) = initial body weight - final body weight

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

[8] Survival rate = 100 × [(Initial number of fish – Final number of final) / Initial number of fish]

The long-term effects of early HC/LP stimulation, in combination with an HC diet challenge, on the nutrient compositions of the liver, muscle, whole body, and plasma metabolites were investigated. Table 4 shows the nutrient compositions of the liver, muscles, and whole bodies of the experimental fish. While an increase in hepatic glycogen was detected only in the long-HC/LP fish, muscular glycogen was elevated in both the short- and long-HC/LP fish, compared with the control fish ($P<0.05$). In addition, while early HC/LP stimulation led to an increase in the muscular fat content of only the long-HC/LP fish, it increased the muscular triglyceride and whole-body fat content of both the short- and long-HC/LP fish ($P<0.05$). Moreover, both the short- and long-HC/LP fish had lower protein levels than the control fish ($P<0.05$). There were no significant differences in the ash content of the liver, muscle, or whole-body samples ($P>0.05$) (Table 3.13). Table 3.14 shows the plasma metabolites in the experimental fish. Early HC/LP stimulation was associated with an increase in plasma glucose and a decrease in total protein in both the short- and long-HC/LP fish ($P<0.05$). In addition, compared with the control fish, a significant increase in triglycerides was observed in the long-HC/LP fish ($P<0.05$). There were no significant differences in the plasma BUN or cholesterol levels among the experimental groups ($P>0.05$) (Table 3.14).

Table 3.13 Proximate composition in liver, muscle, and whole body of the Nile tilapia during challenge with CHO-H (mean±SD, n=6).

	Week 24 (Challenging with CHO-H)			
	Control	Short HC/LP	Long HC/LP	P value
Liver(%)				
Protein	12.21±1.05	11.81±1.60	11.35±0.28	**0.430**
Fat	3.92±0.37	4.25±0.30	4.38±0.54	**0.168**
Ash	1.22±0.11	1.15±0.10	1.23±0.17	**0.549**
HSI (%)	2.57±0.44	2.16±0.24	2.25±0.42	**0.172**
Glycogen (mg g-1 tissue)	63.35±3.67^{b}	69.19±5.78^{ab}	72.71±2.89^{a}	**0.006**
Triglyceride (mg g-1 tissue)	27.19±1.64	28.57±1.86	29.43±1.14	**0.076**
Muscle (%)				
Protein	14.16±1.01	13.38±0.60	13.08±0.49	**0.643**
Fat	2.13±0.15^{b}	2.13±0.36^{b}	2.78±0.51^{a}	**0.011**
Ash	1.83±0.36	1.94±0.08	1.94±0.13	**0.055**
Glycogen (mg g-1 tissue)	7.45±1.21^{b}	10.46±0.94^{a}	10.62±0.59^{a}	**<0.001**
Triglyceride (mg g-1 tissue)	5.32±0.27^{b}	5.90±0.39^{a}	6.14±0.62^{a}	**0.005**
Whole body (%)				
Protein	14.65±0.38^{a}	13.26±0.37^{b}	13.16±0.59^{b}	**<0.001**
Fat	3.84±0.41^{b}	5.23±0.70^{a}	5.47±0.52^{a}	**<0.001**
Ash	3.76±0.17	3.80±0.21	3.86±0.21	**0.686**

Table 3.14 Plasma metabolites of the Nile tilapia during challenge with CHO-H.

	Week 24)Challenging with CHO-H(			
	Control	Short HC/LP	Long HC/LP	P value
Glucose (mM)	3.59±0.02^{b}	4.01±0.05^{a}	4.03±0.05^{a}	**<0.001**
Triglyceride (mM)	1.70±0.01^{b}	2.09±0.54^{ab}	2.27±0.08^{a}	**0.018**
BUN (mM)	1.31±0.77	0.97±0.56	0.72±0.43	**0.259**
Cholesterol (mM)	2.54±0.07	2.54±0.03	2.57±0.05	**0.555**
Total protein (g/L)	40.86±0.45^{a}	39.25±0.45^{b}	39.11±0.27^{b}	**<0.001**

Table 3.13 shows the long-term effects of early HC/LP stimulation, in combination with an HC diet, on the expression of genes encoding enzymes involved in CHO metabolism. The upregulation of gck, pfklr, pklr, glut4, pfkma, and pfkmb was observed in both the short- and long-HC/LP fish (P<0.05). Compared with the control

diet, the long-HC/LP stimuli induced the expression of fasn and hk2 ($P<0.05$) and upregulated pkma more strongly than the short-HC/LP stimuli ($P<0.05$). The suppression of pck2, alat, and hk1 was observed in both the short- and long-HC/LP fish ($P<0.05$). No significant changes in g6pca1, g6pca2, pck1, g6pd, asat, or gdh were related to an early HC/LP stimulus history ($P>0.05$).

Table 3.15 mRNA levels of genes involved in carbohydrate metabolism in the liver and muscle of the Nile tilapia challenge with CHO-H (mean±SD, n=6).

	Week 24 (After Challenge)			
	Control	Short HC/LP	Long HC/LP	P value
Glycolysis				
gck	1.59±0.23^{b}	2.54±0.31^{a}	2.78±0.39^{a}	**<0.001**
pfklr	1.79±0.09^{b}	2.85±0.32^{a}	2.94±0.61^{a}	**<0.001**
pklr	1.94±0.10^{b}	2.58±0.19^{a}	2.64±0.52^{a}	**<0.001**
Gluconeogenesis				
g6pca1	1.85±0.13	1.82±0.14	1.89±0.30	**0.752**
g6pca2	1.97±0.18	1.99±0.13	2.01±0.34	**0.792**
pck1	1.72±0.18	1.86±0.16	2.09±0.42	**0.107**
pck2	1.42±0.20^{a}	0.64±0.18^{b}	0.80±0.21^{b}	**<0.001**
Lipogenesis				
fasn	0.74±0.07^{b}	0.87±0.09^{b}	1.17±0.22^{a}	**<0.001**
g6pd	1.97±0.30	2.37±0.18	2.34±0.40	**0.074**
Amino acid catabolism				
asat	1.62±0.32	1.63±0.31	2.32±0.86	**0.077**
alat	2.44±0.19^{a}	1.27±0.08^{b}	1.38±0.27^{b}	**<0.001**
gdh	2.34±0.88	1.79±0.38	1.77±0.63	**0.272**
Glucose transport and glycolysis				
glut4	0.70±0.13^{b}	1.28±0.20^{a}	1.23±0.19^{a}	**<0.001**
hk1	0.85±0.10^{a}	0.52±0.23^{b}	0.59±0.11^{b}	**0.007**
hk2	0.55±0.21^{b}	0.82±0.29^{ab}	1.15±0.35^{a}	**0.009**
pfkma	1.94±0.07^{b}	2.34±0.12^{a}	2.47±0.12^{a}	**<0.001**
pfkmb	1.37±0.11^{b}	2.13±0.07^{a}	2.18±0.08^{a}	**<0.001**
pkma	0.51±0.06^{c}	0.79±0.19^{b}	1.46±0.35^{a}	**<0.001**

3.5 Discussion

The positive effects of CHO programming in Nile tilapia were demonstrated for the first time in our recent studies (Kumkhong et al., 2020a, 2020b, 2021). However, to apply this nutritional programming technology in large-scale fish farming, more practical stimulus methods had to be developed. The use of a nutritional stimulus in fry could be the easiest method to apply in aquaculture, but it must be optimised. Indeed, our first study (Kumkhong et al., 2020b) was extremely promising for CHO programming, but the serious decrease in growth during the long stimulus period (4 weeks) before transfer to aquaculture was not satisfactory. Therefore, this study investigated the effects of different durations of HC (CHO>50%) dietary stimulus (either 1 or 3 weeks) during the fry stage on the growth performance and CHO metabolism of the fish. The objective was to suggest the optimal duration of HC/LP stimulus to improve the use of an CHO-H diet (CHO>60%) by juvenile tilapia.

3.5.1 Early intake of an HC diet by fry for 1 or 3 weeks influenced CHO metabolism similarly, but growth performance was less affected by the 1-week treatment

Our results show that Nile tilapia fry fed an HC/LP diet had lower growth performance than the control fish, as expected owing to the lower protein content of their diet (Azaza et al., 2015; Boonanuntanasarn et al., 2018a,b). This decrease in growth performance was compensated for after the stimulus period. Compensatory growth in fish is well documented (Wang et al., 2000), and was observed in our previous study using a 4-week stimulus period (Kumkhong et al., 2020b). However, as expected, the short-HC/LP fish recovered to the same weight as the control fish sooner than the long-HC/LP fish (at weeks 4 and 20, respectively). Early stimulation with an HC/LP diet influenced CHO metabolism similarly, regardless of stimulus duration. HC/LP stimulation was associated with an increase in glycolytic and lipogenic gene expression and glycogen content, and a decrease in gluconeogenic gene expression, as previously shown in Nile tilapia (Boonanuntanasarn et al., 2018a, 2018b); however, most of these effects (including glycolysis and gluconeogenesis) were no longer visible in the short-HC/LP fish during the 2 weeks following the stimulus period, when they were fed the control diet. Importantly, the downregulation of amino acid catabolic gene expression also occurred in the two

treatment groups, owing to the lower protein content of the HC/LP diets. Similar effects of 4 weeks of HC dietary stimulus have been previously demonstrated in Nile tilapia fry (Kumkhong et al., 2020b). Effects of larval feeding with an HC diet on the expression of glycolytic and glucogenic genes have also been observed in European sea bass (Zambonino-Infante et al., 2019).

3.5.2 Early HC intake had long-term effects on the lipogenesis and CHO metabolism of juveniles fed a commercial diet (up to week 20)

Before the final challenge (at week 20), we analysed the juvenile fish, which were being fed a commercial diet. Overall, we found that lipid and glucose metabolism were modified in response to early HC stimulus. Indeed, the long-HC/LP stimulus was associated with differences in lipid metabolism (increases in plasma triglycerides, fat content, and hepatic lipogenic gene expression), as well as CHO metabolism (increases in glycolysis in the muscles and liver and in glycogen content and decreases in hepatic gluconeogenic gene expression) and amino acid catabolism (decreases in amino acid catabolic gene expression). In the short-HC/LP treatment, fewer parameters were modified, but they were changed in the same direction as those in the longer stimulus treatment, as illustrated by the increase in lipogenesis (fasn) and decrease in gluconeogenesis (pck2) and amino acid catabolism (asat) observed in the juvenile fish at week 20. Interestingly, in our previous study (Kumkhong et al., 2020b), the effects of early HC/LP stimulation for 4 weeks gave the same results as shown here, including the induction of glycolysis in the liver and muscles and the suppression of amino acid catabolism. Although the juvenile fish were fed a commercial diet (not rich in CHO), we noticed the results of metabolic programming in the fish owing to their first feeding, confirming the existence of metabolic programming in tilapia (Khumkhong et al., 2020b). However, these long-term effects were less intense when the early stimulus period was shorter. The question then, was to determine whether the effects of this metabolic programming would become more intense when the fish were fed the challenge CHO-H diet.

3.5.3 Early dietary HC stimuli exerted positive effects on growth performance and CHO metabolism in juvenile fish challenged with an CHO-H diet

To test whether early feeding with high levels of CHO could be useful for improving CHO use later in life, we fed the experimental fish an HC diet (CHO > 60%) in weeks 20–24. Impressively, the 1-week and 3-week HC/LP stimulus treatments were both associated with better growth performance and FCR than the control (no early HC stimulus), demonstrating that early HC stimulus has strong effects later in life. These data confirm the findings of previous studies, in which tilapia were exposed to early feeding with an HC diet for 4 weeks (Kumkhong et al., 2020b) or glucose injection into their yolk reserves (Kumkhong et al., 2020a). In both studies, CHO stimulus was linked to improved growth performance. It must be noted that these positive effects could be species-dependent, as no effect on growth performance was found in several other fish species, including rainbow trout (Geurden et al., 2007, 2014; Song et al., 2019), European sea bass (Zambonino-Infante et al., 2019), gilthead seabream (Rocha et al., 2016a, 2016b), and zebrafish (Fang et al., 2014).

It was important to determine whether the improved growth performance in the “programmed” fish could be associated with metabolic programming. Indeed, early HC/LP stimulus was associated with high glycolysis and glucose transporter-associated gene expression, as well as low gluconeogenesis-associated gene expression in the long-HC/LP fish. However, many of these actors were also modified in the short-HC/LP fish, suggesting that a short stimulus duration can also durably modify CHO metabolism. Similar changes were observed for lipid metabolism (i.e., higher fat content, plasma triglyceride levels, and lipogenic gene expression) in the long-HC/LP fish, but only fasn expression was modified in the short-HC/LP fish. Finally, protein metabolism could also have been modified as lower plasma BUN/protein levels and a decrease in amino acid catabolic genes were found. Overall, these results (higher glycolysis and lipogenesis, and lower gluconeogenesis and amino acid catabolism) can explain the improved growth performance of early stimulated fish fed an HC diet. These results agree with previous findings in tilapia after early feeding with HC/LP for 4 weeks or glucose injection into their yolk

(Kumkhong et al., 2020a, 2021). In European sea bass, larval feeding with an HC diet influenced CHO metabolism, increasing hepatic glycogen and decreasing gluconeogenesis in the juvenile stage, although no effect on glucose gene expression were detected (Zambonino-Infante et al., 2019). In rainbow trout, first feeding with high dietary CHO also had long-term effects on muscle glycolysis and glucose transport (Geurden et al., 2014; Song et al., 2019), whereas in gilthead seabream, an early high glucose diet had no significant effects on glycolysis, gluconeogenesis, or lipogenesis (Rocha et al., 2016a, 2016b). However, only Nile tilapia showed better growth performance among juveniles after early HC stimulus. The relationship between CHO programming and fish trophic levels requires further study.

3.5.4 Application of metabolic programming in tilapia farming

The first evidence of CHO nutritional programming in Nile tilapia was achieved via glucose injection into yolk reserves (Kumkhong et al., 2020a, 2021). However, this microinjection method is not feasible for use in tilapia farming. In contrast, changes in first feeding could easily be used in aquaculture, and our first study, which used early HC stimulus for 4 weeks, was extremely promising for metabolic programming, although the direct decrease in growth could be problematic (Kumkhong et al., 2020a). This study provides the valuable information that HC feeding stimulus for just 1 (short) or 3 (long) weeks during early development is sufficient to produce positive CHO programming. The short-HC/LP treatment was as effective in improving growth performance as the long-HC/LP treatment, and its negative effects on growth during the stimulus period were comparably weaker. Therefore, this study suggests that a 1-week HC/LP feeding stimulus during the fry stage could be a powerful method of nutritional programming to improve the utilisation of dietary CHO in later life in Nile tilapia.

The concept of nutritional programming by dietary carbohydrate has been recently applied for sustainable better use of new ingredient in aquaculture. According for the early dietary feeding is one of the potential critical periods of metabolic plasticity and, therefore, has become the popular early nutritional interference, a high carbohydrate-low protein diet (HC/LP) during first feeding was applied to Nile tilapia to obtain primary data. Nowadays, there are several reports have explained nutritional programming through early fry feeding in different fish

species, mainly carnivorous species (Geurden et al., 2007; Geurden et al., 2014; Gong et al., 2015; Ling et al., 2017; Rocha et al., 2016a; Rocha et al., 2016b; Fang et al., 2014) and other species (kumkong et al., 2020a; kumkhong et al., 2020b). therefore, in this study provides primary data on the effect of short period of early dietary carbohydrate during fry stage on nutrient metabolism and growth performance in juvenile Nile tilapia (Oreochromis niloticus), an omnivorous fish species.

In conclusion, early dietary high carbohydrate stimulus at first feeding short and long HC/LP stimulus shown permanent effect later in life. Nutritional programming effect could modulate intermediate metabolism, form short and long HC/LP early stimulus. The early stimulus could improve better growth performance. Moreover, the strong effect at early stimulus HC diet for 3 weeks (long HC/LP history) was evaluate permanent effect in juvenile Nile tilapia.

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CHAPTER IV

EFFECT OF SHORT PERIOD OF EARLY HIGH CARBOHYDRATE AND ITS RELATION TO THE CARBOHYDRATE CHALLENGING IN JUVENILE TILAPIA FED WITH MEDIUM CARBOHYDRATE DIET (CHO-M)

4.1 Abstract

Carbohydrate (CHO) is a cheap source of energy, and investigation of the efficient use of CHO has been intensively carried out in fish. Nutritional programming of CHO would have effects on the modulation of CHO metabolism in long term. Therefore, the present study aimed to investigate the effects of different durations of early high carbohydrate (HC) feeding stimulus on the growth performance and CHO metabolism of Nile tilapia at later in juvenile stage. The first-feeding nutritional stimulus treatments included a low-CHO/high-protein diet for 3 weeks (control group), a short duration high-CHO/low-protein (HC/LP) diet for 1 week (short-HC/LP group), and a long duration HC/LP diet for 3 weeks (long-HC/LP group). Subsequently, after CHO stimulus during fry stage, fish were fed commercial feeds until week 20. To test the existence of CHO metabolic programming, during week 20 –24 weeks, juvenile fish were challenged with medium carbohydrate diet (CHO-M), which contained flour as CHO source and similar nutrient composition with commercial feed, for 4 weeks (weeks 21–24). Experimental juvenile fish were evaluated their growth performance, proximate composition, blood metabolite chemistry and expression of genes that are related to CHO metabolism. Our results show that early HC/LP stimulus had no effects on growth performance in juveniles. Interestingly, metabolism modifications linked to early HC/LP stimulus were observed in several parameters. The results indicated that both short and long HC/LP stimulus were associated with differences in lipid metabolism (increases in plasma triglycerides, fat contents in liver, muscle and whole body, and triglyceride content in both liver and muscle), as well as carbohydrate metabolism (increases glycogen content in muscle and plasma glucose). Similar reduction in protein content in whole body in this study

was observed. The upregulation of genes involved in muscle glycolysis and glucose transport in juveniles and the downregulation of genes involved in amino acid catabolism were observed, demonstrating metabolic modulation at molecular level. Taken together, although early HC/LP feeding in tilapia fry had no effects growth performance, this stimulus is associated with metabolic modulation, suggesting an effective for positive nutritional programming of metabolism in Nile tilapia.

Keywords: Nile tilapia; nutritional programming; early feeding; carbohydrate; glucose metabolism

4.2 Introduction

Carbohydrate (CHO) has been used as energy source in fish particularly for omnivorous fish. Efficient utilization of dietary CHO has been reported to improve protein sparing effects which contribute least cost feed with high quality. The most important challenge to overcome in fish nutrition is developing a cost-effective diet with maximal growth performance that promotes both human and animal welfare, and sustainable and environmentally friendly aquaculture. Several studies have sought alternative and novel ingredients in aquafeeds (Hua et al., 2019; Cottrell et al., 2020). Utilization of dietary CHO and its metabolic responses have been demonstrated which vary according to fish species and/or feeding habits. The glycogen in liver and muscle and plasma metabolites (glucose, triglycerides and cholesterol) was linked to the increased of high-CHO diet. The best growth performance was found in fish fed CHO at modulate level. The dietary balance of proteins and carbohydrates show the best growth performance. Strong effect regulation of expression and activity for genes involved in glucose use in liver and muscle were detected, confirming the metabolic adaptation of tilapia to CHO diet (Boonanuntanasarn et al., 2018a,b).

In order to improve the efficient use of CHO, new strategy to modulate the CHO metabolism has been considered such as nutritional programming. Nutritional programming or metabolic programming describes the effects of nutrient intervention on quality and/or quantity during early life, and how these effects persist and influence the modulation of metabolic and physiological pathways later in life. This

concept is related to the nutritional status of mammals and fish during early life and the subsequent metabolic consequences at later life stages (Lucas, 1998; reviewed in Panserat et al., 2019; Hou and Fuiman, 2020). Recently, a new concept called nutritional programming has attracted increased attention; nutritional programming alters the ability of fish to better utilise specific nutrients. Nutritional programming has been successfully used to improve the utilisation of plant-based ingredients (Geurden et al., 2013; Izquierdo et al., 2015; Clarkson et al., 2017). Hyperglucidic stimulus by glucose injection during alevin stage had long-term effects in juvenile Nile tilapia fish led to improved growth performance, increased glycolysis in liver and muscle and decreased gluconeogenesis (Kumkhong er al., 2020a). The positive effects of glucose injection stimulus on muscular glycolysis and glycogen content were proved to existed through adult fish. However, it had no effect on growth performance in adult fish (Kumkhong et al., 2021). The effects of early feeding with high carbohydrate stimulus in tilapia fry to promote of CHO utilization linked to carbohydrate metabolism pathway increase of glycolysis and lipogenesis decreased of glucose transport and amino acid catabolism (Kumkhong et al., 2020b). In contrast, for carnivorous fish, in rainbow trout (*Oncorhynchus mykiss*), early feeding with a high-CHO (HC) diet was later associated with increase enzymes related to muscle glycolysis and glucose transport (Geurden et al., 2014; Song et al., 2019). Although an early HC diet increased glucokinase enzyme and suppressed the expression of the glucose-6-phosphatase gene in European sea bass (*Dicentrarchus labrax*), these effects on CHO metabolism did not persist to the juvenile stage (Zambonino-Infante et al., 2019).

Nile tilapia (*O. niloticus*), which are omnivorous fish, can effectively use CHO as an energy source (Kamalam et al., 2017; Shiau and Peng, 1993; Boonanuntanasarn et al., 2018a). The Nile tilapia is an economically important freshwater fish worldwide, and its production is estimated to increase annually (FAO, 2018). Moreover, the Nile tilapia is a model omnivorous aquaculture-related experimental fish, particularly for CHO metabolism. Indeed, Nile tilapia have high glucose homeostasis abilities and can adapt to CHO intake by inducing glycolysis and lipogenesis and inhibiting gluconeogenesis (Boonanuntanasarn et al., 2018a, 2018b). To further the application of nutritional programming in tilapia farming and improve the early feeding stimulus protocol, this

study investigated the optimal method for early HC feeding stimulus by comparing short (1 week) and long (3 weeks) during juvenile stage with carbohydrate diet.

4.3 Material and methods

4.3.1 Experimental fish and diet, experimental design and fish culture

Nile tilapia (*O. niloticus*) used in the present study was obtained from a broodstrock that was culturedat the Suranaree University of Technology farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Broodstock (0.8-1.5 kg) was cultured fed with a commercial feed (30% CP, 4%CF at 30% body weight) at 9:00 and 16.30 daily. Table 3.1 shows the details the feed formulation of two diet for the early feeding, high-protein/low-carbohydrate (HP/LC) and high-carbohydrate/low-protein (HC/LP) diets, and diet for the final challenge, the low carbohydrate diet (CHO-M; 36 % of carbohydrate in diet). All male fish were used in the present study to prevent the confounding effect associate with sex-dimorphism. Consequently, HP/LC and HC/LP diets were supplemented with 17α-methyltestosterone (17MT) at 60mg/kg. The male tilapia, using global consumption, and several reports have revealed no accumulation of 17MT in fish at harvesting size. The ingredients of the two stimulus diets (Table 3.1) and challenge diet (Table 4.1). The experimental plan (Fig4.1), investigates the effect of the early high carbohydrate (stimulus period), the first-feeding HC/LP diet during fry. Each experimental treatment contains 6 replications (120 larvae/cages). In total fry, (≈10mg) were randomly distributed into cages (40*40*60 cm^3). To exclude the possible effects of the environment during the stimulus phase, cages were in one cement pond (2*2*8 m^3) (six replications; 100 fry/replication), and short HC/LP fed high carbohydrate for 1-week, long HC/LP were fed high carbohydrate for 3 weeks, after stimulus phase both short and long HC/LP fish were fed commercial supplemented 17MT in diet in table 3.1. In contrast, fish control group was fed HP/LC or commercial diet supplemented 17MT for 4 weeks daily at 09:00, 11:00, 15:00, 17:00 respectively. Subsequently, experimental fish were continually cultured in cement pond and fish fed with a commercial diet *ad libitum* twice daily (at 09:00 and 16:00) up to week 20 or before challenge, and growth response every month was determined. During weeks 20-24, for the dietary challenging period, the early dietary stimulus (control history, short and long HC/LP

history) challenging with low carbohydrate diet (CHO-M) containing 36% CHO in the diet, was employed in a completely randomized design using six replicates (n=6/cage; 2x2x0.8m^3) Further, growth performance was determined. The experimental period, dead fish were record daily. Water and air temperature were determined daily, which has in range of 25.5-28.5°C and 29.2-35.7°C, respectively. Dissolved oxygen (DO) and pH were record weekly using a DO meter and pH meter, DO levels of 4.1±0.7mg/l (average±SD) and pH of 7.3±0.5 (average±SD), which were acceptable ranges, were observed.

Table 4.1 Ingredients of the challenge diets for experiments.

Ingredients (%)	Challenging diet
	Medium carbohydrate (CHO-M)
Fish meal	35
Soybean meal	30
Rice flour	15
Rice bran	18
Soybean oil	-
Fish premix [a]	-
Dicalcium phosphate	2
Proximate composition (% dry weight)	
Dry matter	95.77
Protein	35.67
Fat	6.90
Fiber	2.89
Ash	12.92
NFE	37.40
Gross energy (kJ g^{-1})	17.60

a Vitamin and trace mineral mix provided the following (IU/kg or g/kg 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, 5000 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, 1000 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.

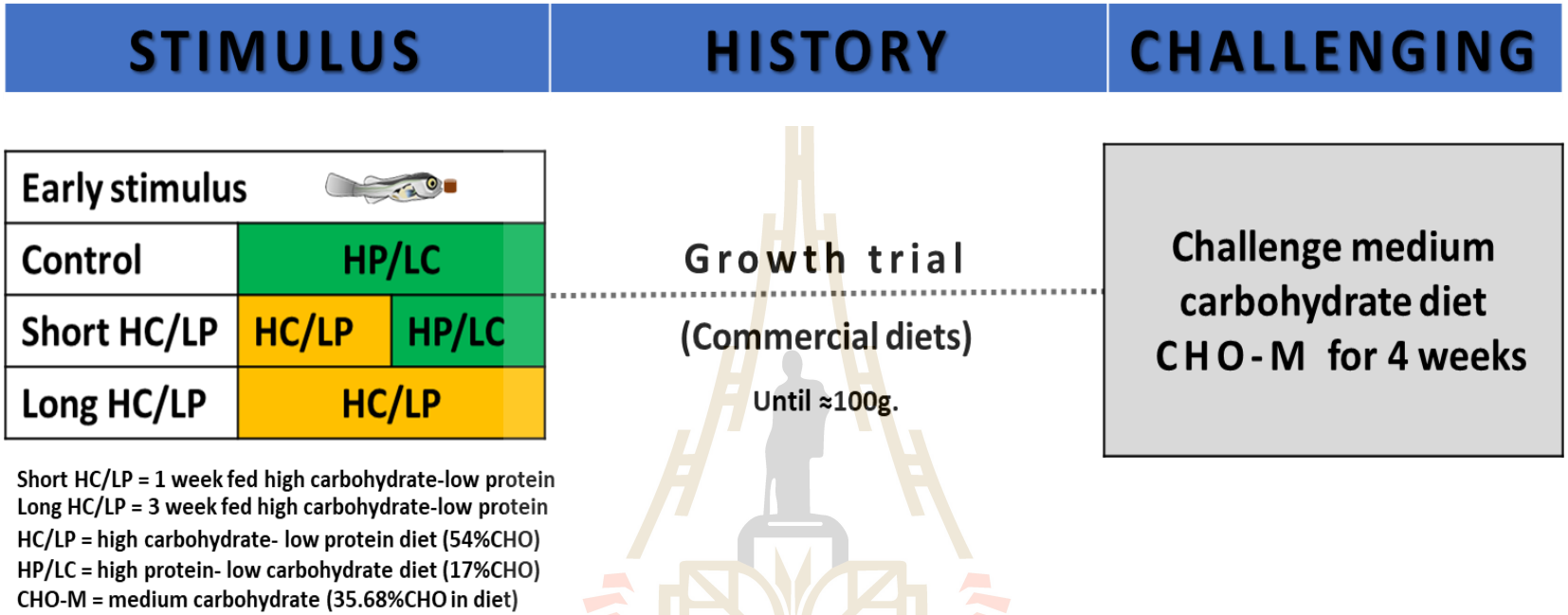

Figure 4.1 Experimental plan and sampling regimes during the challenging with medium carbohydrate (CHO-M).

4.3.2 Fish sampling and blood collections

At week 1 after stimulus (short HP/LC and short HC/LP), Total six replicates (3 fish/ replication) whole bodies of fry were sampling for analysis of metabolic response (mRNA) and levels of glycogen and triglyceride content (TAG). At week 3 or the end of stimulus, whole bodies of fry (1fish/replicated; total= 6 replicated) were collected to analysis as well as at week 1. For sampling at 5 hours after last meal (corresponding to the peak of postprandial glycaemia in tilapia), fish were snap freezing (the process by which temperature below -70°C using liquid nitrogen). At week 20, (before challenge), two fish per pound (total=12 fish per treatment) were sampling for analysis of blood metabolites and metabolic response of gene expression (mRNA) as well as chemical composition in the liver, muscle and whole body. At 5 hours after the last meal, fish were anaesthetized with 10% clove oil. Consequently, blood sampling was collected from the caudal vein suing hypodermic syringe and mixed with K_2EDTA (at 1.5mg/ml). Plasma was collected after centrifugation at 3000×g for 10 min at 4°C and stored at −80°C for plasma metabolite determination. Then, liver, muscle and whole bodies tissue samples were obtained and frozen in liquid nitrogen and kept at −80°C until analysis. At week 24, (challenging period), to investigate the early dietary stimulus and the dietary challenging (CHO-M), were collected for analysis of metabolic response (gene expression). Note all result before challenging since early feeding to 20 weeks of age. The fish for determine growth performance as similar to experiment I.

4.3.3 Determination Growth performance and feed utilization

Growth performance during week 1-4 (10 fish per cage, dry weight), at grow-out stage (week 8-20) ten fish were sampling to determine the growth performance including weight gain, average daily gain, specific growth rate, feed conversion ratio. In addition, the challenging with difference carbohydrate challenging and protein challenging, six fish were random to test effect form history (6 fish=CHO-H per cage: 6 fish=CHO-M per cage). Moreover, survival rate was determined. The growth performance, hepatosomatic index and survival rate parameters are showed as following; 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

4.3.4 Chemical composition and glycogen and triglyceride analysis

Chemical composition (two fish per replicate, total 12 fish per treatment) including ash (Burned in furnace at 550°C for 3-4 hour), moisture (Place the dish with sample in the oven at 105°C for 3 hour), crude protein, crude fat. In addition, liver and whole body was determined the chemical composition such as crude protein, crude fat, ash, and moisture according to AOAC, 2000. The hepatic and muscular glycogen contents were determined. The glycogen content was measured using a modified hydrolysis method (Good and Somogyi, 1933). The sample was ground in 1 molar 37%HCL in total 1,000 µl. An aliquot 200 µl was obtained, neutralised 50 µl (5molar KOH), and subsequently centrifuged at 12,000 rpm at 4°C for 10 min to measure free glucose content. Free glucose was measured using commercial glucose kit (Earba), according to protocol. The total glycogen form aliquot was boiled at 105°C for 30 min (5time) and then neutralised using 600 µl (total glycogen) 5 molar KOH 150 µl. After centrifuge at 12,000 rpm at 4°C 10 min, the total glucose was analysed using the glucose kit. The glycogen content was calculated from the glucose amount after subtracting the total glucose with free glucose levels according to Good and Somogyi, 1933. To analyse triglyceride, 100 mg of the sample was homogenised in 1 ml of 5 % Igepal in a deionised water solution using a Dounce homogenizer. Samples were heated at 90°C in a water bath for 5 min for 3 time and subsequently cooled down to room temperature. Then, centrifugation was performed at 12,000 rpm for 15 min at 4°C to remove any insoluble material, and the supernatants were collected and diluted with deionised water. Triglyceride was measured using a commercial triglyceride kit (Earbar), following the manufacturer's instructions.

4.3.5 Blood chemical analysis

Plasma metabolite analysis (two fish per replicate, total 12 fish per treatment) including glucose, triglyceride, cholesterol, blood urea nitrogen (BUN) and total protein was evaluated. Plasma glucose was quantitatively analyzed using Trinder's method (Barham and Trinder, 1972). Triglyceride levels was determined using glycerol-3-phosphate oxidase-sodium N-ethyl-N-(3-sulfopropyl) m-anisidine

(GPO-ESPAS) method described by Bucolo and David, 1973. Cholesterol was estimated using cholesterol oxidase phenol+aminophenazone (CHOD-PAP) technique described by Flegg, 1973. Blood urea nitrogen (BUN) content was measured in duplicate using a modified indophenol colorimetric method Weatherburn, 1967. Total protein was analyzed the Biuret method Gornall et al., 1949.

4.3.6 Gene expression analysis

Analysis of the expression of gene that are involved in carbohydrate metabolism. Fish muscular and hepatic were sampling form two fish per replicated (100 mg in muscle; 50mg in liver). To evaluated the metabolic gene involved in i.e., glycolysis (*gck*, glucokinase; *hk1*, hexokinase 1; *pfklr*, phosphofructokinase-6; pklr, *pkm*, pyruvate kinase, both liver and muscle isoforms), gluconeogenesis (*pck1* and *pck2*, phosphoenolpyruvate carboxykinase, both cytosolic and mitochondrial isoforms; FBP, fructose-1,6- bisphosphatase; *g6pca1 and g6pca2*, glucose-6-phosphatase), lipogenesis (*fasn*, fatty acid synthase; g6pd, glucose-6-phosphate dehydrogenase enzyme), amino acid catabolism (*asat*, aspartate transaminase; *alat*, alanine aminotransferase; *gdh*, glutamate dehydrogenase) glucose transporter in muscle (*glut4*). Gene expression levels was determined by quantitative real-time RT-PCR.

4.3.7 Total RNA extraction

Twelve fish in total form each treatment was sampling for total RNA extraction using TRIzol® reagent (Invitrogen Corp., Carlsbad, CA). Prepare tissue into the tube 50 mg (liver) and 100 mg (muscle) were homogenized with 1:20 and 1:10 of TRIzol® reagent respectively. The homogenate was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Then, 200 µl of chloroform (1:5 TRIzol®) was added, investing by hand for 15 seconds and incubated for 5 minutes at room temperature. Then, centrifugation of the mixture at 12,500 rpm for 5 minutes at 4°C. Collect upper phase (RNA only) and transfer into new tube, add an equal volume of isopropanol (1:1) for precipitate the total RNA at room temperature for 30 minutes. Centrifugation at 12,500 rpm for 30 minutes at 4°C, total RNA pellet was collected and washed with 80% ethanol. The washed RNA pellet was obtained by centrifugation at 12,500 rpm for 15-30 minutes at 4°C. The RNA pellet was air-dried, then dissolved in RNase-free water. The quantity of total RNA was evaluated by nano drop (Thermo scientific) accept the nucleic acid is used

to assess the purity of total RNA. A ratio was accepted at 1.8-2.0 according to protocol (accept pure). Finally, the quality of total RNA was determined by agarose gel electrophoresis based on the absence on 28S and 18S ribosomal RNA bands.

4.3.8 First strand cDNA synthesis

The total RNA concentration was measured. One microgram (µg) of total RNA form liver and muscle were mixed in nuclease-free water. To synthesize cDNA, 100 units of SuperScript III enzyme, 40 units of an RNase OUT enzyme, Reverse-Transcriptase (200unit/µl) Kit (Invitrogen) with random primers (500µg/ml) (Promega, Charbonnières, France) was used with a sample of 1 µg of total RNA (duplicate for each sample, total 12 for each treatment group), following the manufacturer's protocol (Table 3.3). The reaction mixture was incubated at 70°C for 5 minutes and then transfer to incubate on ice immediately for 5 minutes. Subsequently, the mixture for cDNA synthesis. added, mixed gently, and incubated the initial reaction at 25°C for 10 minutes (annealing). Then, the reaction mixture was incubated at 42°C for 90 minutes (synthesis of cDNA). The reaction was stopped by incubating at 70°C for 15 min. cDNA was store at –20°C until used for polymerase chain reaction (PCR).

4.3.9 Real-time RT PCR

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 the whole bodies of fry (week 1, pool of three fish/replication, n=18 per treatment; week 3, two fish/replication, n= 12 per treatment) as well as the liver (50 mg) and muscle (100 mg) (weeks 20 and 24, two fish/replication, n=12 per treatment). The PCR reaction was prepared in a final volume of 6 µl of Light Cycler® 480 SYBR Green I Master mix (Roche) described in Table 3.4. The sequence of primers of each gene determined are showed in Table 3.5 Each quantitative PCR was performed in duplicates for individual samples. The reference gene expression in this study uses elongation factor-1 (EF1α). The PCR protocol was denatured at 95°C for 10 min for and hot-start Taq polymerase activation was followed by 45 cycles of a three-step amplification program (15 s at 95°C, 10 s at 60°C and 15 s at 72°C to extend the DNA). The melting curves was systematically analyse at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR was assay

include replicate samples and negative controls. For the analysis expression of mRNA levels, relative quantification of target gene expression was perform using the Roche Applied Science E-Method following Pfaffl, 2002. The relative gene expression of reference gene was analyzed for the normalization of the measured mRNA in each sample.

4.3.10 Statistical Analysis

All data (growth performance, gene expression levels, proximate chemical composition and glycogen and triglyceride content, plasma metabolite) were analyzed using one-way analysis of variance (ANOVA) using SPSS for Windows (Release 14) (SPSS Inc., Chicago, IL, USA). When significant differences were found among the groups (control, short HC/LP and long HC/LP), Tukey's procedure was used to rank the groups. Differences between experimental groups were deemed to be significant at $p<0.05$,

4.4 Result

In this study, CHO-M diet resembled commercial diet (commercial diet containing CHO approximately 38-40%). After week 20 (juvenile fish), all experimental fish including the LC/HP diet-fed control fish, short-HC/LP diet-fed fish, and long-HC/LP diet-fed fish, were tested with the CHO-M for 4 weeks. Table 4.2 shows the growth performance and survival rate of the fish during challenge period. Our results show that both short and long HC/LP stimuli had no effect on growth performance, including FW, WG, ADG, SGR and FCR, when compared to the control fish ($P>0.05$). In addition, there were no significant differences in survival rate during the challenge period (Table 4.2).

Table 4.2 Growth performances of the Nile tilapia during challenging with medium carbohydrate (CHO-M) for 4 weeks (mean±SD, n=6).

Experimental periods	FW[3] (g)	WG[4] (g)	ADG[5] (g day^{-1})	SGR[6] (% day^{-1})	FCR[7]	Survival rate[8]
Control	133.00±12.41	132.98 12.41	0.79±0.07	5.40±0.06	2.07±0.20	97.22±6.81
Short HC/LP	139.17±6.21	139.14±6.21	0.83±0.04	5.43±0.03	1.97±0.10	97.22±6.81
Long HC/LP	140.17±9.09	140.15±9.09	0.84±0.05	5.42±0.04	1.96±0.12	97.22±6.81
P- value [2]	0.396	0.396	0.405	0.479	0.346	1.000

[1] Note FW;mg,WG;mg,ADG;mg/day,SGR;mg day^{-1}

[2] One-way ANOVA analysis of Variance was used to analyze the effects of different stimulus between Control, Short HC/LP and Long HC/LP stimulus with high-protein/low-carbohydrate (HP/LC) and low-protein/high-carbohydrate (LP/HC) diets.

[3] Final body weight (FW) = initial body weight - final body weight

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

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

The long-term effects of early HC/LP stimulation, in combination with an CHO-M challenge, on the nutrient compositions of the liver, muscle, whole body, and plasma metabolites were investigated. Table 4.3 shows the nutrient composition of the liver, muscle, and whole bodies of the of the experimental fish. Both short and long HC/LP fish had increased fat content and triglyceride content in liver, compared with the control fish ($P<0.05$). In addition, while early HC/LP stimulation led to an increase in the muscular fat content of only the long-HC/LP fish, it increased the muscular triglyceride and whole-body fat content of both the short- and long-HC/LP fish ($P<0.05$). Moreover, both the short- and long-HC/LP fish had lower whole body protein levels than the control fish ($P<0.05$). There were no significant differences in the ash content of the liver, muscle, or whole-body samples ($P>0.05$) (Table 4.3). Table 4.4 shows the plasma metabolites in the experimental fish. Early HC/LP

stimulation was associated with an increase in plasma glucose and a decrease in total protein in both the short- and long-HC/LP fish (P<0.05). In addition, compared with the control fish, a significant increase in triglycerides was observed in both short and long-HC/LP fish (P<0.05). There were no significant differences in the plasma BUN or cholesterol levels among the experimental groups (P>0.05) (Table 4.4)

Table 4.3 Proximate composition in liver, muscle and whole body of the Nile tilapia during challenge with medium carbohydrate (CHO-M) for 4 weeks (mean±SD, n=6).

	Week 24 (Challenging with CHO-M)			
	Control	Short HC/LP	Long HC/LP	P value
Liver (%)				
Protein	13.12±0.42	12.56±0.36	12.58±0.57	**0.084**
Fat	3.79±0.21 ^{b}	3.54±0.27^{b}	4.33±0.35^{a}	**0.001**
Ash	1.11±0.07	1.17±0.08	1.12±0.10	**0.400**
HSI (%)	2.12±0.33	1.92±0.62	1.93±0.67	**0.789**
Glycogen (mg g^{-1} tissue)	60.40±3.07	63.46±5.55	63.02±5.78	**0.527**
Triglyceride (mg g^{-1} tissue)	22.60±0.20^{b}	26.25±2.47^{ab}	28.32±4.16^{a}	**0.010**
Muscle (%)				
Protein	13.78±0.64	13.07±0.64	13.19±0.96	**0.254**
Fat	1.73±0.25^{b}	2.00±0.31^{ab}	2.22±0.12^{a}	**0.010**
Ash	1.86±0.16	1.97±0.09	1.91±0.13	**0.337**
Glycogen (mg g^{-1} tissue)	5.01±0.90^{b}	8.62±0.37^{a}	9.50±0.71^{a}	**<0.001**
Triglyceride (mg g^{-1} tissue)	4.02±0.42^{b}	5.41±0.76^{a}	5.78±0.90^{a}	**0.002**
Whole body (%)				
Protein	15. 26±0.60^{a}	13.87±0.49^{b}	13.92±0.71^{b}	**0.002**
Fat	3.37±0.57^{b}	4.49±0.57^{a}	4.44±0.49^{a}	**0.004**
Ash	3.89±0.14	3.85±0.18	3.95±0.29	**0.688**

Table 4.4 Plasma metabolites of the Nile tilapia during challenge with CHO-M for 4 weeks (mean±SD, n=6).

	Week 24 (Challenging with CHO-M)			
	Control	**Short HC/LP**	**Long HC/LP**	**P value**
Glucose (mM)	3.31±0.01[b]	3.75±0.04[a]	3.97±0.11[a]	**<0.001**
Triglyceride (mM)	1.54±0.01[b]	2.04±0.03[a]	2.16±0.08[a]	**<0.001**
BUN (mM)	1.57±0.50	0.97±0.56	0.89±0.50	**0.078**
Cholesterol (mM)	2.53±0.06	2.53±0.07	2.56±0.02	**0.593**
Total protein (g/L)	42.04±0.62[a]	39.71±0.50[b]	39.57±0.64[b]	**<0.001**

Table 4.5 shows the long-term effects of early HC/LP stimulation, in combination with CHO-M diet, on the expression of genes encoding enzymes involved in CHO metabolism. The upregulation of *hk1, hk2, pfkma, pfkmb, pkma* was observed in both the short- and long HC/LP fish (P<0.05). Compared with the control diet, the long-HC/LP stimuli induced the expression of *glut4* (P<0.05). The suppression of *alat* was observed in both the short- and long-HC/LP fish (P<0.05). No significant changes in *gck, pfklr, pklr, g6pca1, g6pca2, pck1, fasn, g6pd, asat,*or *gdh* were related to an early HC/LP stimulus history (P>0.05).

Table 4.5 mRNA levels of genes involved in carbohydrate metabolism in the liver and muscle of the Nile tilapia during challenge with CHO-M for 4 weeks (mean±SD, n=6).

	Week 24 (Challenging with CHO-M)			
	Control	Short HC/LP	Long HC/LP	P value
Glycolysis				
gck	1.53±0.56	2.41±0.77	2.19±0.45	**0.060**
pfklr	2.32±0.39	2.82±0.60	2.79±0.52	**0.181**
pklr	2.10±0.26	2.61±0.41	2.66±0.54	**0.062**
Gluconeogenesis				
g6pca1	1.89±0.25	1.70±0.37	1.87±0.31	**0.528**
g6pca2	1.95±0.24	2.00±0.31	2.01±0.38	**0.935**
pck1	1.88±0.24	1.88±0.27	1.84±0.35	**0.959**
pck2	1.07±0.29	0.83±0.15	0.81±0.16	**0.099**
Lipogenesis				
fasn	0.93±0.10	1.06±0.23	0.87±0.21	**0.259**
g6pd	1.69±0.31	2.16±0.35	2.09±0.40	**0.074**
Amino acid catabolism				
asat	2.00±0.27	1.91±0.96	1.88±0.50	**0.946**
alat	2.64 ± 0.39^{a}	1.74 ± 0.28^{b}	1.72 ± 0.32^{b}	**<0.001**
gdh	2.56±0.60	1.71±0.80	2.29±0.78	**0.157**
Glucose transport and glycolysis				
glut4	0.94 ± 0.04^{b}	0.70 ± 0.09^{b}	1.53 ± 0.43^{a}	**<0.001**
hk1	0.30 ± 0.12^{b}	0.47 ± 0.07^{b}	1.55 ± 0.69^{a}	**<0.001**
hk2	0.43 ± 0.16^{b}	1.02 ± 0.15^{a}	0.78 ± 0.24^{a}	**<0.001**
pfkma	1.94 ± 0.10^{b}	2.16 ± 0.12^{a}	2.11 ± 0.17^{ab}	**0.022**
pfkmb	1.32 ± 0.05^{b}	1.91 ± 0.13^{a}	1.91 ± 0.13^{a}	**<0.001**
pkma	0.37 ± 0.13^{b}	0.63 ± 0.06^{a}	0.63 ± 0.10^{a}	**<0.001**

4.5 Discussion

The concept of nutritional programming by dietary carbohydrate has been applied in aquaculture. Several study stimuli since early dietary feeding is one of the effective periods of metabolic plasticity. Therefore, it has become the popular early nutritional interference, a high carbohydrate during first feeding was applied to Nile tilapia to obtain primary data (Boonanuntanasarn et al., 2018a, Boonanuntanasarn et al., 2018b, Kumkhong et al., 2020a, Kumkhong et al., 2020b). Nowadays, there are several reports especially in carnivorous species (Geurden et al., 2007; Geurden et al., 2014; Gong et al., 2015; Ling et al., 2017; Rocha et al., 2016a; Rocha et al., 2016b; Fang et al., 2014). In this study provides the first primary data on the effect of short period of early high dietary carbohydrate during fry stage combination with medium carbohydrate containing in diet (CHO-M challenging) on nutrient metabolism and growth performance and plasma metabolite and chemical composition in juvenile Nile tilapia (*Oreochromis niloticus*), an omnivorous fish species.

To test whether early feeding with high levels of CHO could be useful for improving CHO use later in life, we fed the experimental fish CHO-M diet (35% CHO in diet) in weeks 20-24. Both short and long HC/LP stimulus treatments did not have any effects on growth performance during the challenge test. Similarly, the finding of previous studies, there were no long-term effects of nutritional programming on growth performance in zebrafish and rainbow trout. For example, zebrafish which were exposed to early feeding with high carbohydrate (35% CHO in diet challenge test) for 1 week had no long-term effects on growth performance (Fang et al., 2014). Early feeding of carnivorous rainbow trout which received a high carbohydrate diet (25% dextrin in diet challenge test) for 1 week had no effects on growth performance at later in life (Geurden et al., 2007). However, growth performance of tilapia which was exposed to early feeding with high carbohydrate diet for 4 weeks (Kumkhong et al., 2020b) or glucose injection into their yolk reserves (Kumkhong et al., 2020a) or first feeding with HC/LP diet for 1 or 3 weeks (Srisakultiew et al., 2022) improved when fish were challenged with high carbohydrate during juvenile and/or adult stages. Taken together, whether the nutritional programming exhibited effect on growth performance in long-term depended on fish species and/or level of dietary

CHO challenge at later in life. It is required to study whether the long-term effects of early HC stimulus would exert modulation effects on other parameters.

The long-term effects of metabolic programming of short or long HC stimulus on plasma metabolite and nutritive composition were investigated in juvenile fish. Our results indicated that both short and long HC/LP stimulus were associated with differences in lipid metabolism (increases in plasma triglycerides, fat contents in liver, muscle and whole body, and triglyceride content in both liver and muscle), as well as CHO metabolism (increases glycogen content in muscle and plasma glucose). Interestingly, in previous study (Kumkhong et al., 2020b), the effects of early high carbohydrates diet stimulus for 4 weeks resulted in the same results, including lipid and glycogen contents and muscle glycogen in adult tilapia, demonstrating the induction of lipid metabolism. In addition, similar findings were observed in juvenile tilapia which were fed CHO-M diet (Kumkhong et al., 2020b). Similar reduction in protein content in whole body in this study was also found in adult tilapia that was fed with HC diet during fry stage (Kumkhong et al., 2020b). Taken together, these results of modulation of proximate composition in whole body, muscle and liver as well as plasma metabolites in the fish owing to their first feeding, confirming the existence of metabolic programming in tilapia.

Early HC/LP stimulus was associated with high muscle glycolysis and glucose transporter-associated gene expression (induction of *hk2, pfkma, pfkmb* and *pkma*), as well as up-regulation of expression of *glut4* and *hk1* in the long-HC/LP fish. Similar changes were observed for protein metabolism could also have been modified a decrease in amino acid catabolic gene (*alat*) in both short and long-HC/LP fish. There were no changes in expression of any genes that are related to lipid metabolism. The results suggesting that both short and long stimulus duration can also durably modify CHO and its related metabolism such as glycolysis, glucose transport, and amino acid catabolism. These results agreed with previous findings in tilapia after first feeding with HC diet for 4 weeks in fry stage or glucose injection into their yolk (Kumkhong et al., 2020a, 2020b). Larval feeding with high carbohydrate diet influenced CHO metabolism, decreasing gluconeogenesis in the juvenile stage, although no effect on glucose gene expression were detected in European seabass (Zambonino-Infante et al., 2019). Similar induction in muscle glycolysis and glucose transport in rainbow

trout during fry stage was observed when they were fed with high carbohydrate diet (Geurden et al., 2014; Song et al., 2019). However, in gilthead seabream, an early high glucose diet had no significant effects on glycolysis, gluconeogenesis, or lipogenesis (Rocha et al., 2016a, 2016b). Taken together, our study proves that early feeding can have effect and long-term impacts on metabolic modulation in the Nile tilapia.

In conclusion, although there were effects on growth performances, early stimulus of HC diet had long-term effects on several parameters of liver, muscle and body composition in juvenile fish as well as in plasma metabolites. These finding demonstrated the persist effects of nutritional programming of HC in at metabolism level in Nile tilapia.

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CHAPTER V
CONCLUSION

This study revealed the effect of initial early feeding of dietary carbohydrate on growth performance, chemical composition, plasma metabolites, and gene involved in carbohydrate metabolism in Nile tilapia (*Oreochromis niloticus*). The findings could be concluded as the following:

(i) Nutritional programming was achieved by either short or long HC/LP.

(ii) Nutritional programming of both short and long CHO stimulus persisted and has long-term effects include induction of glycolysis and lipogenesis and reduction of gluconeogenesis and amino acid catabolism

(iii) At week 20, the compensatory growth in which low growth rate according to stimulus with HC/LP diet could caught up control fish.

(iv) At week 20-24, when fish were challenged with high CHO diet, the effects of nutritional programming of both short and long HC/LP stimulus diet appeared to be stronger.

(v) Combination of early high carbohydrate diet stimulus (history) and high CHO challenging appeared to improve growth performance at later in life.

(vi) During challenge with CHO-M diet for 4 weeks, the early feeding can have effect and long-term impacts on metabolic modulation in the Nile tilapia.

(vii) The persist effects of nutritional programming of HC in at metabolism level in Nile tilapia.

BIOGRAPHY

Nattanan Srisakultiew was born on 16 January 1997 in Muang, Nakhon Ratchasima Thailand. In 2015, finished high school from Suranaree Wittaya school (89 th), Nakhon -Ratchasima. In 2018 graduated the Bachelor's degree in school of Animal Production Technology, Suranaree University of Technology, Nakhon -Ratchasima. In 2019 began a Master degree in School of Animal Technology and Innovation, Program Biotechnology for Aquaculture, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and received a scholarship from Suranaree University of Technology. I spent 3 year in the lab at Suranaree University of Technology for my Master thesis was the Effect of short period of early dietary carbohydrate during fry stage on nutrient metabolism and growth performance in juvenile Nile tilapia (*Oreochromis Niloticus*).