EFFECTS OF HIGH CARBOHYDRATE DIET ON CARBOHYDRATE METABOLISM AND EPIGENETIC MODIFICATIONS IN NILE TILAPIA (*Oreochromis niloticus*)

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ผลของอาหารคาร์โบไฮเดรตสูงต่อเมตาบอลิซึมของคาร์โบไฮเดรต และการเปลี่ยนแปลงอีพิเจเนติกส์ในปลานิล (*Oreochromis niloticus*)

นางสาวสิริจรรยา ธงชัยไตรวัฒน์

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สิริจรรยา ธงชัยไตรวัฒน์ : ผลของอาหารคาร์โบไฮเดรตสูงต่อเมตาบอลิซึมของคาร์โบไฮเดรตและการเปลี่ยนแปลงอีพิเจเนติกส์ในปลานิล (*Oreochromis niloticus*) EFFECTS OF HIGH CARBOHYDRATE DIET ON CARBOHYDRATE METABOLISM AND EPIGENETIC MODIFICATIONS IN NILE TILAPIA (*Oreochromis niloticus*) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.สุรินทร บุญอนันธนสาร, 149 หน้า.

คำสำคัญ: ปลานิล/เมตาบอลิซึมของคาร์โบไฮเดรต/การให้อาหารซ้ำ/โภชนาการของพ่อแม่พันธุ์/อีพิเจเนติกส์

ปลานิล (*Oreochromis niloticus*) เป็นปลาที่มีความสามารถในการใช้อาหารคาร์โบไฮเดรตได้ดี ในการเลี้ยงปลานิลปลามักประสบกับสภาวะอดอาหาร โดยเกษตรกรพยายามที่จะให้ปลานิลกลับมากินอาหารหลังจากได้อดอาหาร (refeeding) เพื่อชดเชยการเจริญเติบโตที่สูญเสียไปในระหว่างการอดอาหาร จึงเป็นที่น่าสนใจว่าปลานิลจะมีการตอบสนองต่ออาหารที่มีระดับคาร์โบไฮเดรตที่แตกต่างกันอย่างไร และมีผลต่อการเปลี่ยนแปลงอีพิเจเนติกส์อย่างไร (epigenetics) เมื่อกลับมากินอาหารหลังจากอดอาหาร ดังนั้นในการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษา ผลของการอดอาหารและการกลับมากินอาหารคาร์โบไฮเดรตสูง/โปรตีนต่ำ (HC/LP) และอาหารคาร์โบไฮเดรตต่ำ/โปรตีนสูง (LC/HP) ในปลานิลวัยรุ่นและปลานิลโตเต็มวัย ต่อเมแทบอลิซึมของคาร์โบไฮเดรต (การทดลองที่ 1) และผลของอาหารดังกล่าวต่อการเปลี่ยนแปลงอีพิเจเนติกส์ (การทดลองที่ 2) รวมทั้งผลของอาหารที่มีคาร์โบไฮเดรตสูงต่อต่อเมแทบอลิซึมของคาร์โบไฮเดรตและการเปลี่ยนแปลงอีพิเจเนติกส์ในปลานิลพ่อแม่พันธุ์ (การทดลองที่ 3)

การทดลองที่ 1 ปลาได้ถูกอดอาหารเป็นระยะเวลา 4 วัน และกลับมาให้อาหารอีกครั้งเป็นระยะเวลา 4 วัน ด้วยอาหาร HC/LP หรือ LC/HP ผลการศึกษาพบว่า หลังจากอดอาหาร เมื่อปลากลับมากินอาหารอีกครั้ง การกินอาหาร HC/LP และ LC/HP สามารถชดเชยน้ำหนักตัวที่สูญเสียไปจากการอดอาหาร เพิ่มระดับไตรกลีเซอไรด์ในพลาสมา เพิ่มการสะสมของไกลโคเจน และไตรกลีเซอไรด์ในตับ เพิ่มระดับไตรกลีเซอไรด์ในกล้ามเนื้อในปลาระยะวัยรุ่นและโตเต็มวัย พบการเพิ่มโปรตีนในพลาสมาและการเพิ่มลิพิดในตับของปลานิลระยะวัยรุ่น และพบการเพิ่มระดับคอเลสเตอรอลในพลาสมาของปลานิลระยะโตเต็มวัย เมื่อเปรียบเทียบระหว่าง HC/LP และ LC/HP ผลการศึกษาพบว่าอาหาร HC/LP ส่งผลต่อการเพิ่มระดับกลูโคสในพลาสมา เพิ่มการสะสมของไกลโคเจน ไตรกลีเซอไรด์ และลิพิดในเนื้อเยื่อ รวมทั้งไตรกลีเซอไรด์ในกล้ามเนื้อ นอกจากนี้อาหาร HC/LP สามารถกระตุ้นการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการไกลโคไลซิสในตับและกล้ามเนื้อ รวมไปถึงการกระตุ้นการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างไขมันและการขนส่งกลูโคสในกล้ามเนื้อ อาหาร HC/LP ลดการแสดงออกของยีนที่เกี่ยวข้องต่อกระบวนการกลูโคนีโอเจเนซิสและการสลายกรดอะมิโนในตับและกล้ามเนื้อ

ผลการศึกษาครั้งนี้สรุปได้ว่าปลานิลมีกระบวนการเมแทบอลิซึมที่ตอบสนองต่อการ refeeding ระยะสั้นด้วยอาหารคาร์โบไฮเดรตสูงคล้ายคลึงกับการได้กินอาหารคาร์โบไฮเดรตสูงในระยะยาว

การทดลองที่ 2 พบว่าการ refeeding ด้วยอาหาร HC/LP และ LC/HP ส่งผลต่อการเปลี่ยนแปลงอีพิเจเนติกส์ การ refeeding ด้วยอาหาร HC/LP และ LC/HP ส่งผลต่อการลดปริมาณ 5-hmdC ในตับของปลาระยะวัยรุ่น และปริมาณ 5-hmdC และ 5-cadC ของปลาระยะโตเต็มวัย อีกทั้งยังเหนี่ยวนำให้เกิดสภาวะไฮเปอร์เมทิลเลชัน (hypermethylation) ไฮเปอร์อะเซทิลเลชัน (hyperacetylation) ของฮิสโตนในตับและกล้ามเนื้อ และพบว่าการ refeeding ด้วยอาหารทั้งสองสูตรกระตุ้นการแสดงออกของยีนที่เกี่ยวข้องกับเอนไซม์ที่ควบคุมกระบวนการดีเอ็นเอเมทิลเลชัน และโปรตีนฮิสโตน นอกจากนี้การ refeeding ด้วยอาหาร HC/LP ทำให้เกิดการเปลี่ยนแปลงทางอีพิเจเนติกส์ที่มีความจำเพาะต่อเนื้อเยื่อ ได้แก่ ไฮโปเมทิลเลชันของ H3K36 และไฮโปอะเซทิลเลชันของ H3K9 ในตับของปลานิลระยะวัยรุ่น และพบว่าการ refeeding ด้วยอาหาร HC/LP ส่งผลต่อการเกิดไฮโปเมทิลเลชันของดีเอ็นเอ และไฮโปอะเซทิลเลชันของ H3K9 ในกล้ามเนื้อของปลานิลระยะโตเต็มวัย

การทดลองที่ 3 พบว่าอาหาร HC/LP กระตุ้นการแสดงออกของยีน *glut4* ในกล้ามเนื้อ และส่งผลให้แม่พันธุ์ปลามีอัตราความดกไข่เพิ่มขึ้น แต่น้ำหนักไข่และดัชนีสืบพันธุ์ลดลง สำหรับพ่อแม่พันธุ์ปลานิลพบว่า อาหาร HC/LP ส่งผลให้ระดับกลูโคสและไตรกลีเซอไรด์ในพลาสมาเพิ่มขึ้น ระดับโปรตีนในพลาสมาลดลง และองค์ประกอบทางเคมีของตับและกล้ามเนื้อเปลี่ยนแปลง และพบว่าอาหาร HC/LP กระตุ้นการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการไกลโคไลซิส (ตับและกล้ามเนื้อ) และการสร้างไขมันในตับ และลดการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการกลูโคนีโอเจเนซิสและการสลายกรดอะมิโนในตับ ผลการศึกษาพบว่าลูกปลาอายุ 7 วันหลังฟัก และ 7 วันหลังเริ่มกินอาหารมีการตอบสนองทางเมแทบอลิซึมของคาร์โบไฮเดรตในทิศทางเดียวกันกับพ่อแม่พันธุ์ ซึ่งแสดงถึงความเป็นไปได้ของการถ่ายทอดผลของการปรับเปลี่ยนเมแทบอลิซึมจากพ่อแม่พันธุ์สู่ลูก อาหาร HC/LP ส่งผลต่อการเพิ่มการแสดงออกของยีน *tet* ที่เกี่ยวข้องต่อกระบวนการขจัดหมู่เมทิลบนดีเอ็นเอ และยีน *kdm4* ที่เกี่ยวข้องต่อกระบวนการขจัดหมู่เมทิลบนโปรตีนฮิสโตน ร่วมกับการลดการแสดงออกของยีน *dnmt* ที่เกี่ยวข้องต่อกระบวนการเติมหมู่เมทิลบนดีเอ็นเอ

โดยสรุปหลังจากอดอาหาร การ refeeding ปลานิลด้วยอาหารคาร์โบไฮเดรตสูงระยะสั้นส่งผลต่อการเปลี่ยนแปลงเมแทบอลิซึมของคาร์โบไฮเดรต และอีพิเจเนติกส์ และการให้อาหารคาร์โบไฮเดรตสูงในพ่อแม่พันธุ์เหนี่ยวนำให้เกิดการเปลี่ยนแปลงทางอีพิเจเนติกส์ และปรับเปลี่ยนเมแทบอลิซึมของคาร์โบไฮเดรตในทั้งพ่อแม่พันธุ์และลูกปลา ซึ่งบ่งชี้ถึงอิทธิพลข้ามรุ่นของเมแทบอลิซึมของคาร์โบไฮเดรตของพ่อแม่พันธุ์ในปลานิล

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2568 ลายมือชื่ออาจารย์ที่ปรึกษา _____

Sirijanya Thongchaitriwat : EFFECTS OF HIGH CARBOHYDRATE DIET ON CARBOHYDRATE METABOLISM AND EPIGENETIC MODIFICATIONS IN NILE TILAPIA (*Oreochromis niloticus*). THESIS ADVISOR : PROF. SURINTORN BOONANUNTANASARN, Ph. D.,149 PP.

Keyword: NILE TILAPIA/CARBOHYDRATE METABOLISM/REFEEDING/BROODSTOCK NUTRITION/EPIGENETICS

Nile tilapia (*Oreochromis niloticus*) efficiently utilize dietary carbohydrates (CHO). In tilapia farming, fish often experience fasting; therefore, farmers try to refeed them to compensate for growth reduction. It is important to investigate how fish respond to refeeding with different dietary CHO levels. In addition, it is necessary to examine how epigenetic modulation is affected by different dietary CHO levels during refeeding. Therefore, this study aimed to investigate the effects of fasting and short-term refeeding with high-CHO/low-protein (HC/LP) and low-CHO/high-protein (LC/HP) diets in juvenile and adult Nile tilapia on carbohydrate metabolism (Experiment I) and epigenetic modification (Experiment II). Additionally, this study examined the effects of HC feeding in broodstock on CHO metabolism and epigenetic modification (Experiment III).

In Experiment I, fish were subjected to a four-day fasting period and subsequently refed for four days with either the HC/LP or LC/HP diet. Refeeding with both diets effectively compensated for weight loss caused by fasting, increased plasma triglycerides, hepatic glycogen and triglyceride contents, and muscular triglycerides. It also elevated plasma protein and hepatic lipid contents in juveniles, and plasma cholesterol in adults. When comparing HC/LP and LC/HP diets, the HC/LP diet increased plasma glucose and promoted the accumulation of glycogen, triglycerides, and lipids in the liver, as well as muscular triglycerides. Additionally, HC/LP refeeding upregulated hepatic and muscular glycolytic genes and hepatic lipogenic and glucose transporter genes. HC/LP downregulated gluconeogenic and amino acid catabolism genes in the liver and muscle. Collectively, Nile tilapia showed clear responses to short-term refeeding with a high-carbohydrate diet on intermediary metabolism, which resembled those of long-term HC feeding.

In Experiment II, refeeding with both HC/LP and LC/HP diets altered epigenetic markers. Compared to the fasted state, both HC/LP and LC/HP refeeding reduced hepatic global DNA 5-mC oxidative derivatives in juveniles (5-hmdC) and adults (5-hmdC and 5-cadC). Both HC/LP and LC/HP refeeding induced hypermethylation and/or hyperacetylation in hepatic and muscular tissues. These refeeding conditions promoted the expression of genes encoding DNA- and histone-modifying enzymes. Notably, the HC/LP refeeding diet led to tissue-specific epigenetic regulation patterns, including hepatic H3K36 hypomethylation and H3K9 hypoacetylation in juveniles. The HC/LP refeeding diet influenced muscular DNA hypomethylation and H3K9 hypoacetylation in adults.

In Experiment III, the HC/LP diet induced muscular *glut4* expression, increased fecundity, but reduced egg weight and gonadosomatic index in female broodstock. In both male and female broodstock, the HC/LP diet elevated plasma glucose and triglycerides, decreased plasma protein, and altered nutrient composition in the liver and muscle. It upregulated genes associated with glycolysis (in the liver and muscle) and hepatic lipogenesis, and downregulated those involved in gluconeogenesis and amino acid catabolism in the liver. In addition, similar metabolic response trends were observed in offspring at seven days post-hatching and seven days after first feeding, suggesting a potential transgenerational effect of parental CHO metabolism. Moreover, feeding broodstock an HC/LP diet induced upregulation of *tet* (encoding a DNA demethylation enzyme) and *kdm4* (encoding the histone demethylase), but downregulated *dnmt* (encoding the DNA methylation enzyme).

In conclusion, the short-term refeeding with the HC diet modulated intermediary metabolism and epigenetic instability in Nile tilapia. Moreover, HC feeding in broodstock also induced epigenetic modification and modulated CHO metabolic responses in both parents and offspring, suggesting transgenerational effects of parental CHO metabolism in Nile tilapia

School of Animal Technology and Innovation Student's Signature Sirijanya
Academic Year 2025 Advisor's Signature

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LIST OF ABBREVIATIONS

°C	=	Degree Celsius
5-cadC	=	5-carboxyl-2'-deoxycytidine
5-fdC	=	5-formyl-2'-deoxycytidine
5-hmdC	=	5-hydroxymethyl-2'-deoxycytidine
5-mdC	=	5-methyl-2'-deoxycytidine
ADP	=	Adenosine diphosphate
alat	=	Alanine amino transferase
ANOVA	=	Analysis of variance
asat	=	Aspartate amino transferase
ATP	=	Adenosine triphosphate
CHO	=	Carbohydrate
daf	=	day after first-feeding
dC	=	deoxycytidine
dnmt	=	DNA methyltransferase
dph	=	day post hatching
fasn	=	Fatty acid synthase
g	=	gram
g6pca1	=	Hepatic glucose-6-phosphatase 1
g6pca2	=	Hepatic glucose-6-phosphatase 2
g6pd	=	Glucose-phosphate dehydrogenase
gck	=	Glucokinase
gdh	=	Glutamate dehydrogenase
glut4	=	Glucose transporter
gtf3c4	=	General Transcription Factor IIIC Subunit 4
H3	=	Histone 3
H3K36me3	=	Histone 3 lysine 36 trimethylation
H3K4me3	=	Histone 3 lysine 4 trimethylation
H3K9ac	=	Histone 3 lysine 9 acetylation

LIST OF ABBREVIATIONS (Continued)

H3K9me3	=	Histone 3 lysine 9 trimethylation
HC	=	High carbohydrate
hk1	=	Hexokinase 1
hk2	=	Hexokinase 2
HP	=	High protein
HSI	=	Hepatosomatic index
kat	=	lysine acetyltransferase
kdm	=	Lysine demethylase
kmt	=	Lysine methyltransferase
LC	=	Low carbohydrate
LP	=	Low protein
NFE	=	Nitrogen-Free Extract
NP	=	Nutritional programming
pck	=	Phosphoenolpyruvate Carboxykinase
pck1	=	Phosphoenolpyruvate carboxykinase cytosolic
pfklr	=	Hepatic Phosphofructokinase
pfkma	=	Muscular Phosphofructokinase a
pfkmb	=	Muscular Phosphofructokinase b
pk	=	Pyruvate kinase
pklr	=	Hepatic Pyruvate kinase
pkma	=	Muscular pyruvate kinase
riox1	=	lysine-specific demethylase and histidyl hydroxylase
rpm	=	round per minute
setd	=	SET domain-containing
sirt	=	sirtuin
suv39h1b	=	Suppressor of variegation 3-9 homolog 1b or histone lysine N-methyltransferase SUV39H1b
TAG	=	Triacylglycerol
tet	=	ten-eleven translocation (TET) methylcytosine dioxygenases

LIST OF ABBREVIATIONS (Continued)

μL = Microliter

CHAPTER I

INTRODUCTION

1.1 Introduction

Aquaculture plays a crucial role in global food security by increasing the supply of aquatic products. Fish and fish products are major sources of protein, energy, and functional nutrients worldwide. However, feed represents the largest proportion of aquaculture production costs, and rising feed prices directly affect profitability. Among feed ingredients, digestible carbohydrates (CHO) are the least expensive energy source, prompting extensive research on their optimal inclusion levels and protein-sparing effects, particularly in herbivorous and omnivorous species (Shiau and Peng, 1993; Singh et al., 2006; Li et al., 2013). In contrast, carnivorous species such as rainbow trout (*Oncorhynchus mykiss*) exhibit glucose intolerance when fed high-CHO diets (>20%) (Polakof et al., 2012; Kamalam et al., 2017). Moreover, energy expenditure and compensation according to food deprivation and refeeding which often occurs along life cycle of farmed-raised fish were also hypothesized to modulate CHO metabolism particularly for herbivorous and/or omnivorous fish. Although fasting and refeeding influence growth, body composition, liver metabolism, and blood parameters (Rios et al., 2006; Tian et al., 2013; Morshedi et al., 2017; Sakyi et al., 2020), their effects on carbohydrate utilization remain poorly understood.

Nowadays, epigenetics refers to the study of how environmental or behavioural factors can modify gene activity without altering the underlying DNA sequence (Waddington, 1957). Among these factors, nutritional status plays a key role in regulating metabolism, and this regulation may be mediated by epigenetic mechanisms—particularly DNA methylation/demethylation and histone modifications—in animals (Marandel et al., 2016; Liu et al., 2022; Tamaoki et al., 2018; Xu et al., 2012; Jiménez-Chillarón et al., 2012). For example, in mammals (Gibson et al., 2020; Hjort et al., 2017), DNA methylation was influenced by the metabolic cofactors during nutritional status, which could impact the changes in the dynamic balances of DNA methylation modulators. Furthermore,

nutritional status can establish a form of metabolic memory by reshaping the epigenetic landscape, in part by altering chromatin structure (Marandel et al., 2016). For example, in human cell cultures, glucose restriction induced changes in histone marks at gene promoter regions, leading to transcriptional alterations in target genes (Li et al., 2011).

Previous studies have demonstrated that specific dietary nutrients (e.g., refeeding, CHO, and/or protein) can influence the epigenetic landscape, affecting DNA methylation, histone modifications, and epigenetic modulators in both mammals (Rees et al., 2000), and fish (Marandel et al., 2016; Liu et al., 2022). In rainbow trout, refeeding after a period of fasting has been shown to alter the hepatic epigenetic landscape, including changes in histone modifications, DNA hypomethylation, and the transcript levels of associated epigenetic modulators (Marandel et al., 2016; Liu et al., 2022). Moreover, in trout, hepatic epigenetic remodelling induced by dietary CHO—both at the global level and at gluconeogenesis-related gene loci—has been suggested to play a key role in the nutritionally glucose-intolerant phenotype observed in this species (Marandel et al., 2016). In contrast, Nile tilapia, an omnivorous freshwater species, is regarded as a "highly adaptable user" of dietary CHO, efficiently utilizing it as a primary energy source (Bachman et al., 2015). While this metabolic flexibility has been well studied at physiological, biochemical, and transcriptional levels across different life stages (Bachman et al., 2015; Boonanuntanasarn et al., 2018a, 2018b; Kumkhong et al., 2020; Kumkhong et al., 2021; Srisakultiew et al., 2022) the underlying mechanisms—particularly at the epigenetic level—remain largely unexplored.

In previous studies of rainbow trout, although a glucose-intolerant phenotype is typically observed in juveniles stage (Polakof et al., 2012; Kamalam et al., 2017), female broodstock fed high-CHO diets tolerate carbohydrate levels up to 35% without adverse effects on reproduction (Callet et al., 2021). This stage-dependent adaptability highlights the potential for improving carbohydrate utilization. The concept of nutritional programming (NP) has been explored in Nile tilapia. NP refers to the introduction of a nutritional stimulus during early life stages to enhance the organism's ability to utilize specific nutrients when re-exposed to them later in life (Lucas, 1998; Panserat et al., 2019). In previous study, CHO-based NP in Nile tilapia has been shown to effectively improve the efficient utilization of HC diets up to approximately 66, which

have been achieved through early developmental stages (i.e., alevins, early feeding fry stages), highlighting developmental windows of metabolic plasticity (Kumkhong et al., 2020; Kumkhong et al., 2021; Srisakultiew et al., 2022). Nutritional programming can also be mediated through broodstock. In rainbow trout, maternal HC feeding has been shown to influence carbohydrate metabolism in offspring (Callet et al., 2021), suggesting that parental diet could serve as a strategy for improve carbohydrate utilization at early developmental stages in Nile tilapia.

1.2 Research objectives

The objectives of this study were:

1.2.1 To investigate the effects of short-term refeeding with different levels of dietary carbohydrate on intermediary carbohydrate metabolism in juvenile and adult Nile tilapia.

1.2.2 To investigate the effects of short-term refeeding with different levels of dietary carbohydrate on epigenetics modification in juvenile and adult Nile tilapia.

1.2.3 To investigate the effects of a high-carbohydrate diet in broodstock on intermediary metabolism in Nile tilapia offspring during early developmental stages.

1.3 Research hypotheses

1.3.1 Short-term refeeding with different levels of dietary carbohydrate after fasting could modulate intermediary carbohydrate metabolism in juvenile and adult Nile tilapia.

1.3.2 Short-term refeeding with different levels of dietary carbohydrate after fasting could influence epigenetic stability in juvenile and adult Nile tilapia.

1.3.3 A high-carbohydrate diet in broodstock could modulate intermediary carbohydrate metabolism and exert the modulation effect in Nile tilapia offspring during early developmental stages.

1.4 Scope of the study

1.4.1 To study the effects of short-term refeeding with different levels of dietary carbohydrate on body weight, plasma metabolites, hepatic and muscular nutrient

composition, and intermediary carbohydrate metabolism in juvenile and adult Nile tilapia.

1.4.2 To study the effects of short-term refeeding with different levels of dietary carbohydrate on global DNA (de)methylation, histone modification, and the molecular responses of epigenetic modulators in liver and muscle tissues of juvenile and adult Nile tilapia.

1.4.3 To evaluate the effects of a high-carbohydrate diet in broodstock on body weight, reproductive performance, plasma metabolites, hepatic and muscular nutrient composition, and intermediary carbohydrate metabolism in both female and male broodstock, as well as its consequent effects on growth performance, whole-body nutrient composition, and intermediary carbohydrate metabolism during the early developmental stages of Nile tilapia offspring.

1.5 Expected benefits

This study will enhance understanding of how Nile tilapia respond to dietary carbohydrate levels during short-term refeeding after fasting, particularly in relation to intermediary metabolism and epigenetic modifications at two key developmental stages—juvenile and adult. The findings will provide mechanistic insights into the interactions among nutritional status, macronutrient balance (carbohydrate and protein), metabolism, and epigenetic regulation. Such knowledge will contribute to developing cost-effective feeding strategies and optimized refeeding protocols that promote sustainable Nile tilapia production. Furthermore, investigating the effects of high-carbohydrate diets in broodstock on offspring performance may provide insights that improve least-cost feed in broodstock nutrition and hatchery management, thereby promoting sustainable Nile tilapia culture.

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CHAPTER II

LITERATURE REVIEW

2.1 Aquaculture and Nile tilapia production

Aquaculture has contributed to global food security by increasing the world's supply of aquatic products. Fish and fish products are among the most important sources of protein, energy, and functional foods worldwide. In 2022, global fish production was estimated at up to 182.8 million tons, with the highest yields originating from aquaculture with accounting for 94.4 million tons in both marine and inland waters, respectively. Approximately 164.6 million tons of this production were allocated for human consumption. According to annual estimates, global per capita fish consumption reached up to 20.7 kilograms. The remaining 20.8 million tons were designated for non-food purposes, primarily the production of fishmeal and fish oil.

In 2022, global aquaculture of finfish showed that the cichlid family (mainly tilapias) ranks third in world production of major aquaculture finfish species, with 6.5 million tons (10.8%), after catfishes with 6.6 million tons (10.8%) and carps, with the highest production at 31.7 million tons (51.6%) (FAO, 2024) (Figure 2.1). In addition, global aquatic animal production at the species level, the top ten species with the highest production in aquaculture and capture fisheries included whiteleg shrimp (*Penaeus vannamei*) with 6.8 million tons, cupped oysters nei (*Crassostrea* spp.) with 6.2 million tons, grass carp (*Ctenopharyngodon idellus*) with 6.2 million tons, Nile tilapia (*Oreochromis niloticus*) with 5.3 million tons, silver carp (*Hypophthalmichthys molitrix*) with 5.1 million tons, and anchoveta (Peruvian anchovy, *Engraulis ringens*) with 4.9 million tons. Note that aquaculture was the main source of production for the top five species and for eight of the top ten species of aquatic animals in 2022 (Figure 2.2).

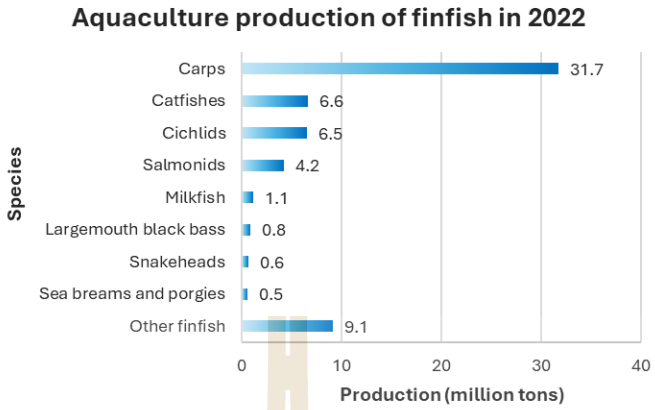

Figure 2.1 Aquaculture production of finfish in 2022 (FAO, 2024).

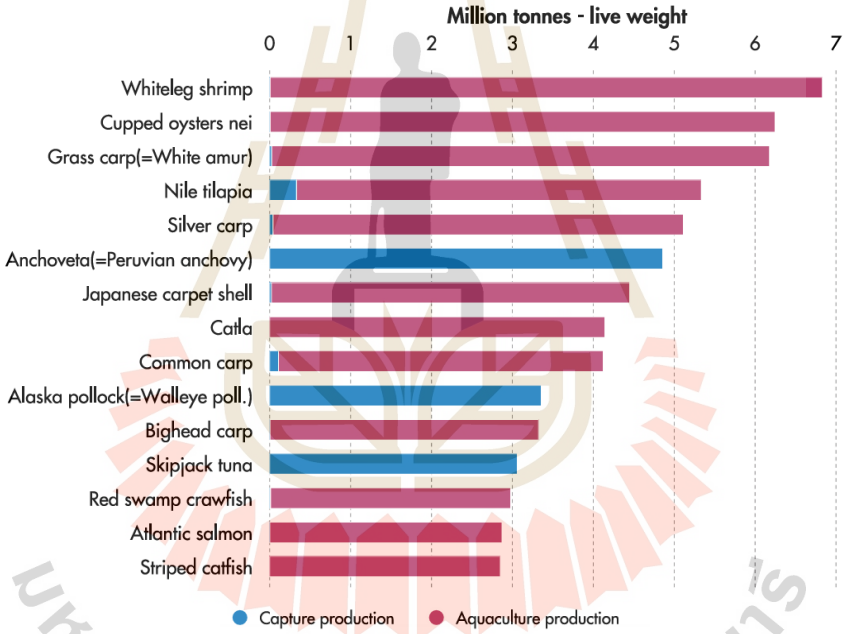

Figure 2.2 Production of main species items of aquatic animals by production sources (FAO, 2024).

Nile tilapia (*Oreochromis niloticus*) belongs to the family of cichlid (Figure 2.3 and Table 2.1), which is a freshwater fish native in parts of Africa, but it has been widely introduced worldwide for aquaculture due to its hardiness and adaptability. Nile tilapia is a fast-growing omnivorous fish, the body weight of Nile tilapia can reach up to 4.3 kg and has body length up to 60 centimeters, which males reach a larger size and grow

faster than females. Nile tilapia is a warm-water species that thrives in temperatures between 25 – 32°C. Nile tilapia has a compressed body with fusiform shape, cycloid scales, and interrupted lateral line. The tilapia body depth was counted as 36-50% of standard length. The spine and soft ray parts of the dorsal fin are continuous, which has 15 – 18 spines and 11 – 13 soft rays. The anal fin contains 3 spines and 9 – 11 soft rays. The vertebrae contain around 30-32. The most distinguishing characteristic is the presence of regular vertical stripes across the depth of the caudal fin together with truncated shape at all stages of life. The caudal fin shows a numerous black bar and a truncated shape. During the spawning season, the pectoral, dorsal, and caudal fins become reddish (Pullin and Lowe-McConnell, 1982; Beveridge and McAndrew, 2012).

Figure 2.3 Nile tilapia (*Oreochromis niloticus*).

Table 2.1 Taxonomy of the Nile tilapia.

Kingdom	**Animalia**
Phylum	Chordata
Class	Actinopterygii
Order	Cichliformes
Family	Cichlidae
Genus	*Oreochromis*
Species	*Oreochromis niloticus*

Reference: Linnaeus, 1758.

Among the large numbers of species of aquatic animals are harvest every year. In 2022, the great diversity in farmed aquatic species, only a small number of freshwater fish species dominate aquaculture production. Nile tilapia is one of the most widely farmed freshwater fish species, ranking third in global aquaculture production of

freshwater aquatic animals in 2022, after silver carp and grass carp (FAO, 2025). Nowadays, global tilapia production has shown an increasing trend year by year and is currently the fourth largest among aquatic animals, which reached a production volume of up to 5.1 million tons in 2023 (FAO, 2025) (Figure 2.4). In Thailand, Nile tilapia is the first important farmed fish which has a significant impact on aquaculture and food security. Nile tilapia production was provided for domestic consumption and export, which had high production up to two hundred thousand tons per year and reached up to 266 thousand tons in 2022 (Department of Fisheries, 2024). In terms of annual production of Nile tilapia in Thailand, production in 2022 appears to have decreased by 1.1% compared to 2021, due to high production costs from fish feed and the impacts of climate change (Department of Fisheries, 2024).

In general, the operating costs for Nile tilapia farming include significant expenses for fish feed, which is often the largest component, accounting for 50–70% of the total operating costs. The other major costs are fingerlings and labor, which account for 10–20% and 10–15% of the total operating costs, respectively. Since fishmeal is a primary protein source for aquafeeds, the decline in global fishmeal production has led to higher aquafeed prices. The high price of fish feed may have led farmers to consider shortening the rearing cycle of tilapia to reduce operational costs. Therefore, the development of high-quality, low-cost feed, together with the shortening of the cultivation period, may contribute to sustainable Nile tilapia production in Thailand.

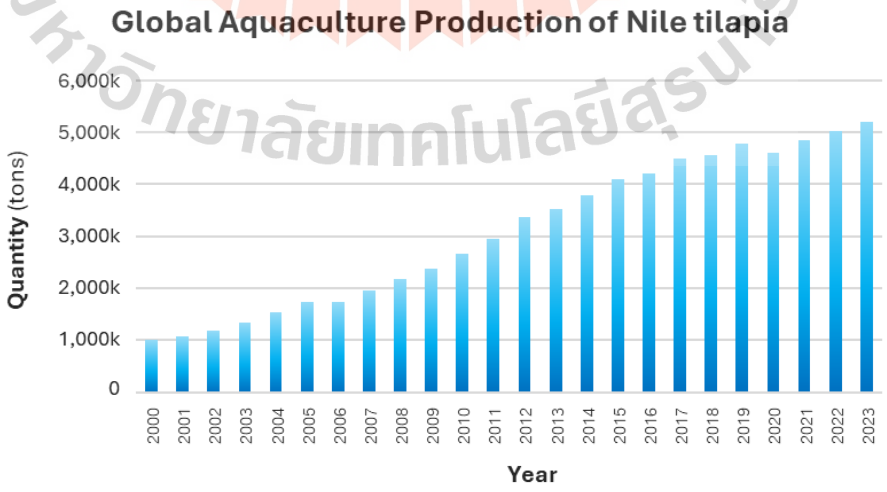

Figure 2.4 Global aquaculture production of Nile tilapia (FAO, 2025).

2.2 Aquaculture feed and function of nutrients

In general, fish nutrition is one of the most important factors in aquaculture. In aquafeed, fishmeal is a major protein source that provides a well-balanced amino acid profile and exhibits high digestibility and palatability for aquatic animals. In addition, fish oil is an important lipid source that contains high levels of essential fatty acids, which are crucial for both freshwater and marine species. However, the dramatic decline in global fishmeal and fish oil production, resulting from reduced capture fisheries production affected by climate change, has led to increased aquafeed prices. The cost of aquafeed represents 50–70% of the total operating costs of aquaculture, as well as in Nile tilapia production, and the price of commercial tilapia feed varies depending on the nutrient composition of the diet (Table 2.2). Decreasing feed costs through the development of dietary formulas, farming improvements, or other direct and/or indirect enhancements is a priority in the development of the aquaculture industry (Craig et al., 2017).

In aquaculture feeds, nutrients in the aquafeed are classified according to their functions: body-building, energy-providing, and protective or regulatory nutritive roles. The composition of proteins and amino acids in the diet is important for growth and body building in fish. In addition, the energy-providing sources include proteins, carbohydrates, and lipids, which play a crucial role in metabolic processes, physical activity, growth, and reproduction. Moreover, vitamins and minerals play an important role in the protection and regulation of normal metabolism, growth, immune function, and reproduction in fish. Among nutrients, research on carbohydrate use and metabolism has been extensively conducted, since efficient use of dietary carbohydrates can lead to cost-effective, high-quality feed.

In Nile tilapia, the ingredients used for practical diets and the commercial feeds are formulated in several variations according to the requirements of each growth phase, as shown in Table 2.3 and 2.4, respectively. According to the nutrient composition of commercial tilapia feed, protein content in the diet is not less than 30%, whereas dietary carbohydrates account for about 30–40%. Thus, the price of fish feed increases with the high protein content required at each growth phase. At the early life stage of Nile tilapia, protein is essential for growth, whereas dietary carbohydrates are important for maintaining energy balance and enhancing the protein-

sparing effect (Shiau and Peng, 1993). Therefore, further research is needed to develop feeds with reduced fishmeal content by substituting dietary carbohydrates.

Table 2.2 Commercial feed for Nile tilapia.

Proximate composition (g kg^{-1} dry weight)	Commercial 40% CP	Commercial 32% CP	Commercial 30% CP
Dry matter	909.46	956.02	937.75
Protein	402.31	336.97	314.04
Fat	88.51	35.94	33.74
Fiber	29.12	54.90	57.52
Ash	129.27	104.22	109.41
NFE[a]	260.25	423.99	423.03
Gross energy (kJ g^{-1})	16.30	17.87	16.86

[a] Nitrogen-free extract= Moisture – (crude protein + crude lipid + crude fiber + ash.

Table 2.3 The commercial diet for Nile tilapia on different stage.

Growth stage	Nutrient composition (%)					
	Moisture	Protein	Fat	Fiber	Ash	NFE[a]
Fry	11	40	5	3	8	33
Fingerlings	12	40	5	4	8	31
35 - 100 g	12	32	4	6	8	38
100 - 300 g	12	30	4	8	8	38
300 ～	12	25	4	8	10	41

[a] Nitrogen-free extract= Moisture – (crude protein + crude lipid + crude fiber + ash

Source; Aqua feed company, THAILAND.

2.3 Dietary carbohydrate and carbohydrate metabolism pathways

2.3.1 Dietary Carbohydrate

Carbohydrates are among the most inexpensive sources of energy and carbon for fish feeds. In general, a carbohydrate is an organic compound containing an aldehyde or ketone group and composed primarily of carbon, hydrogen, and oxygen

atoms. Carbohydrates have the general chemical formula $(CH_2O)_n$ and occur naturally in many organisms. Most carbohydrates are produced by plants through photosynthesis. Plants synthesize carbohydrates in three main forms: sugars, starches, and cellulose. Carbohydrates serve as nutrients that provide metabolic energy. Glucose is the major energy source for metabolism, functioning both as an immediate energy substrate and as a precursor for biosynthesis. When monosaccharides are not used by cells immediately, they are often converted into storage forms such as polysaccharides. In many animals, the storage form is glycogen, particularly in liver and muscle cells.

In aquaculture, dietary carbohydrates play an important role in aquafeed as a major and cost-effective source of energy. Optimizing the dietary carbohydrate level in fish diets allows proteins and lipids to be utilized more efficiently for growth and metabolic functions. Carbohydrates support various physiological processes, including providing readily available glucose for energy metabolism, sparing amino acids from catabolism for energy (Shiau and Peng, 1993), and enhancing feed utilization efficiency. However, the capacity of aquatic species to utilize carbohydrates varies among species. Herbivorous and omnivorous fish generally exhibit a greater ability to utilize dietary carbohydrates (particularly starch) as an energy source up to 50% of the diet, while carnivorous fish are poor users of carbohydrates, typically tolerating less than 25% of dietary carbohydrates inclusion (Kamalam et al., 2017). Therefore, understanding carbohydrate metabolism and optimizing its inclusion in aquafeed formulations are essential for enhancing growth performance, reducing feed costs, and promoting sustainable aquaculture production.

2.3.2 Carbohydrates metabolism

Carbohydrate metabolism refers to the biochemical processes involved in the digestion, absorption, synthesis, storage, and utilization of dietary carbohydrates. In general, carbohydrates are the main source of energy, and starch is a type of carbohydrate commonly used in animal feed, including that of aquatic species. When an animal consumes starch, α-amylase hydrolyzes its components, including amylose and amylopectin, yielding shorter oligosaccharides. Subsequently, various brush-border enzymes further hydrolyze these oligosaccharides into their constituent monosaccharides. The monosaccharides are then transported across the villi (Figure

2.5) (Krogdahl et al., 2005; NRC, 2011). Glucose is the main monosaccharide used as an energy source for muscle cells, the brain, and red blood cells. Transporters such as the sodium-dependent glucose transporter (SGLT1) and the sodium-independent glucose transporter (GLUT2) facilitate the movement of glucose from the intestinal lumen into the bloodstream. Afterward, glucose is utilized as an energy substrate, and any excess is stored as glycogen (through glycolysis pathways) and lipid in muscle and adipose tissues (through lipogenesis pathways), respectively. In the liver, which regulates both the synthesis and utilization of glucose, the excess is also stored as glycogen and lipid. The liver serves as the primary metabolic regulator of carbohydrate metabolism. In addition, when the animal faces an insufficiency of glucose for energy, glucose can be synthesized from non-carbohydrate sources (such as lactate, pyruvate, and amino acids) to produce energy through gluconeogenesis and amino acid catabolic pathways to maintain blood glucose levels. These processes are tightly regulated by hormones such as insulin and glucagon, ensuring a constant energy supply and metabolic balance in the body.

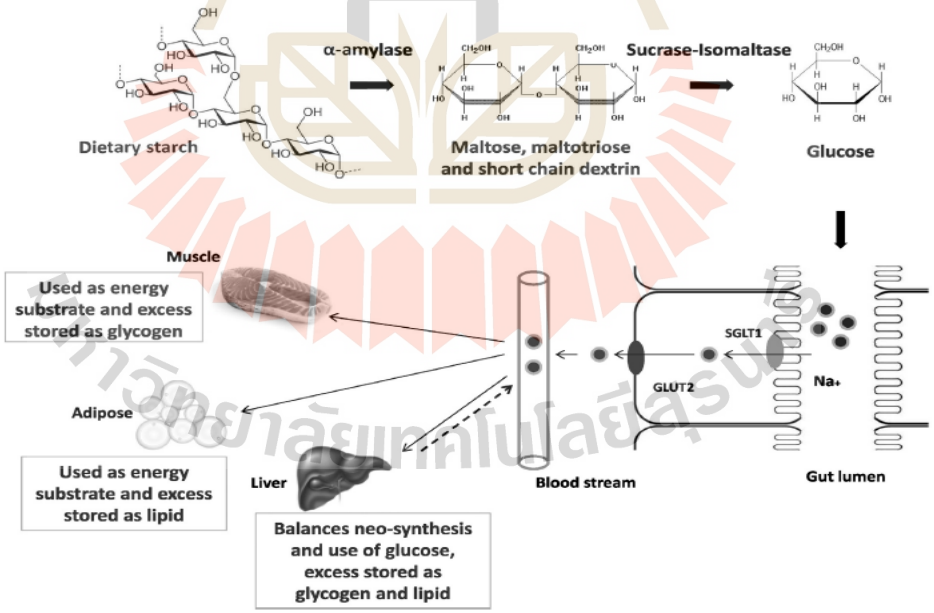

Figure 2.5 Digestion and absorption of dietary carbohydrates (Kamalam et al., 2017).

Glycolysis is the metabolic pathway that convert glucose into pyruvate in the cytoplasm, which can be occur in both anaerobic and aerobic conditions. In general, the steps of glycolysis are following 1) glucose will convert to glucose-6-phosphate by hexokinase (HK) or glucokinase (GCK), using ATP and a phosphate group; 2) glucose-6-phosphate is then converted to fructose-6-phosphate, an isomer, by phosphoglucose isomerase; 3) phosphofructose-kinase (PFK) then produces fructose-1,6-bisphosphate, using another ATP molecule; 4) phosphoenolpyruvate is generated from fructose 1,6-bisphosphate through a series of several enzymes; 5) pyruvate kinase (PK) catalyzes the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP), resulting in the conversion of phosphoenolpyruvate to pyruvate (Chandel, 2021) (Figure 2.6 and 2.7).

Gluconeogenesis is the metabolic pathway responsible for the biosynthesis of glucose from non-carbohydrate carbon substrates such as lactate, pyruvate, amino acids, and glycerol. This pathway occurs primarily in the liver and, to a lesser extent, in the kidney, within either the mitochondria or cytoplasm depending on the substrate utilized. In general, the steps of gluconeogenesis are following 1) pyruvate is converted to oxaloacetate by pyruvate carboxylase; 2) oxaloacetate is converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PCK); 3) phosphoenolpyruvate is then converted to glucose-6-phosphate through a series of enzymatic reactions; 4) glucose-6-phosphate is converted to glucose by glucose-6-phosphatase (G6PCA) (Nelson and Cox, 2008) (Figure 2.6 and 2.7).

Lipogenesis refers to the process of synthesizing lipids, specifically the de novo formation of fatty acids (FA) and neutral lipids. In addition, the excess glucose from dietary carbohydrate and glycolysis pathway in liver will convert into FA. The esterification process will be changed fatty acid into Acyl CoA, which could be converted into to be triacylglycerol (TAG) by fatty acid synthase (FASN) (Ojha et al., 2014) (Figure 2.6).

Amino acid catabolism can occur in both liver and muscle tissues. In muscle tissue, amino groups from amino acids are transferred to pyruvate to form alanine, which is then transported to the liver via the bloodstream. In the liver, alanine undergoes deamination, producing pyruvate and releasing ammonia. The resulting pyruvate is converted to glucose through gluconeogenesis, and this newly synthesized

glucose is subsequently released into the bloodstream and taken up by the muscle, where it is broken down again to produce pyruvate. Through this cyclic process, known as the alanine–glucose cycle, amino groups from muscle proteins are safely transferred to the liver for disposal, while the liver, in turn, supplies glucose back to the muscle. This mechanism allows ammonia to be transported in the non-toxic form of alanine and supports energy balance between muscle and liver tissues (Torres et al., 2023) (Figure 2.6).

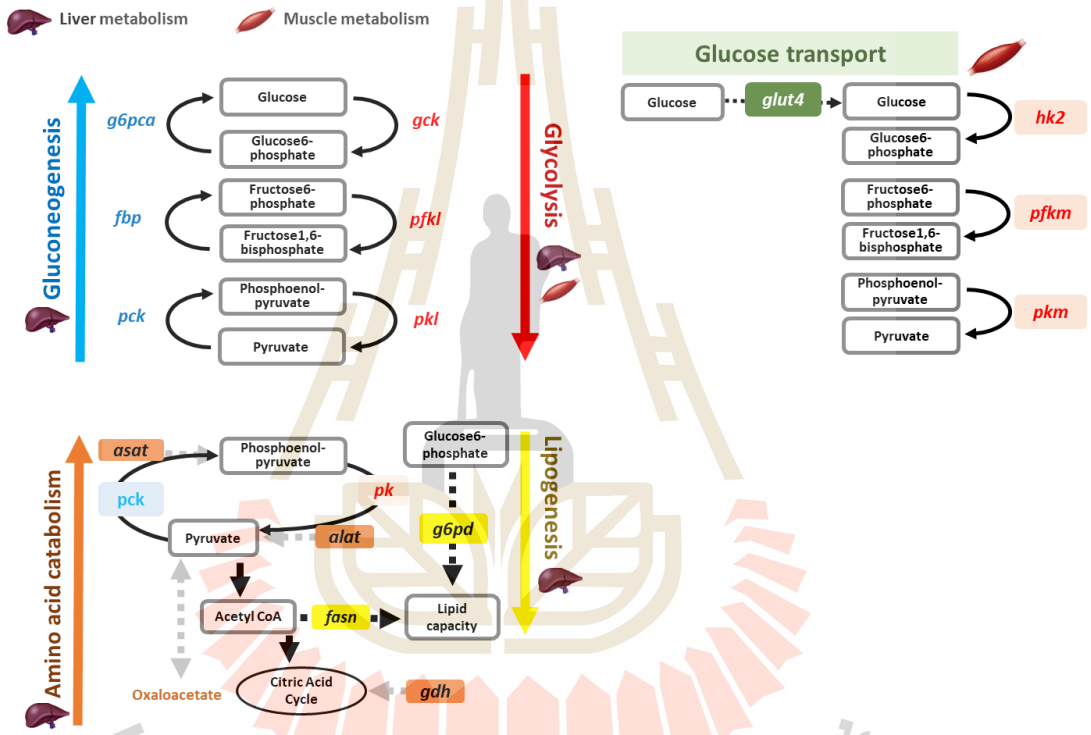

Figure 2.6 Carbohydrate metabolism and genes are involved in intermediaries metabolic pathway.

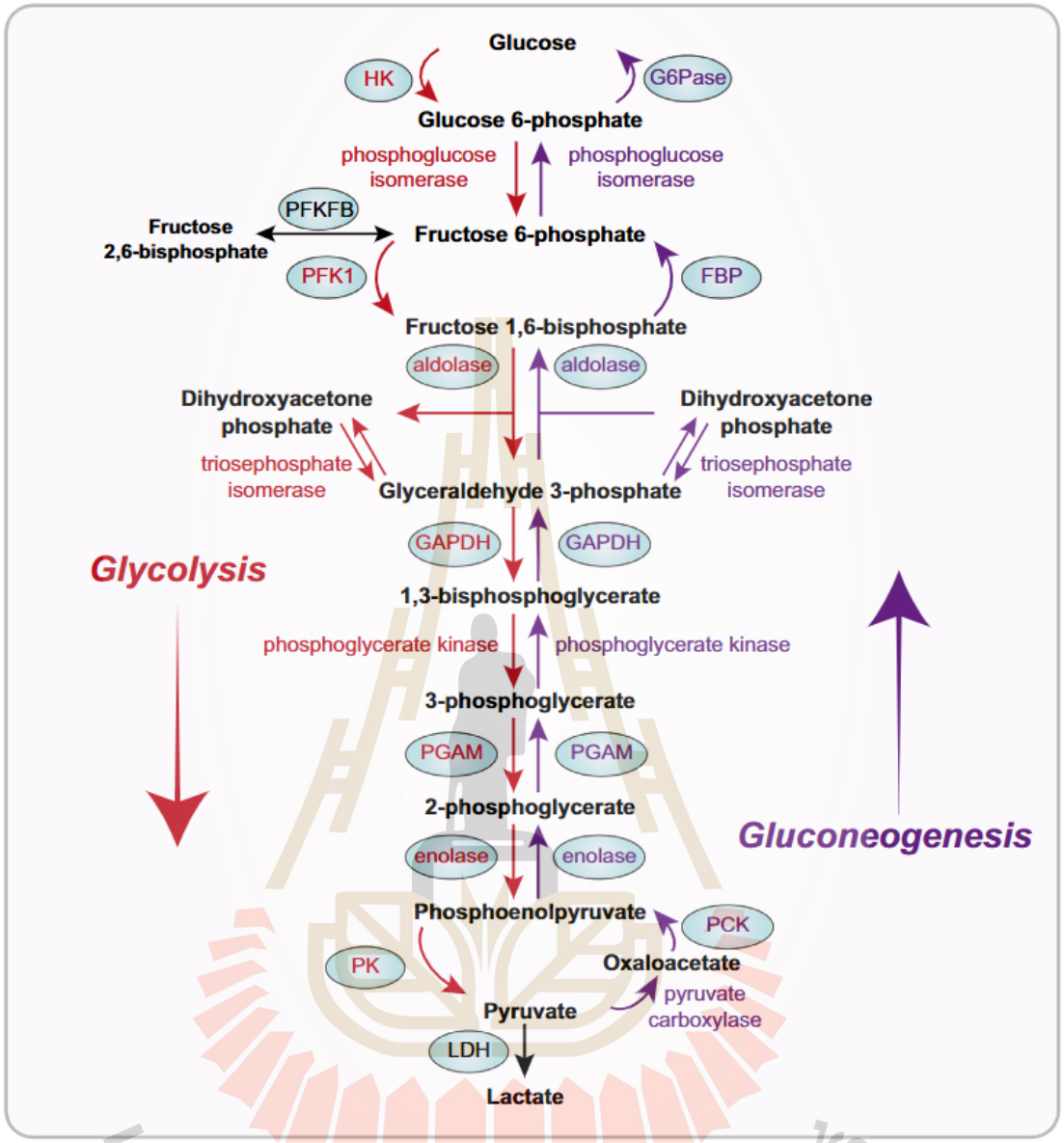

Figure 2.7. The overview of glycolysis and gluconeogenesis pathways (Bian et al., 2022).

2.3.3 Carbohydrate utilization in fish

Carbohydrate utilization in fish refers to the ability of fish to digest, absorb, and metabolize carbohydrates to obtain energy for growth and maintenance. Compared to terrestrial animals, most fish—especially carnivorous species—have a limited capacity to efficiently utilize dietary carbohydrates due to lower intestinal amylase activity and slower glucose clearance from the blood. However, herbivorous and omnivorous fish can utilize carbohydrates more effectively because of their higher

digestive enzyme activity and longer digestive tracts, which enhance carbohydrate breakdown and absorption. Once absorbed, glucose is metabolized through glycolysis and the citric acid cycle to produce energy, or it can be stored as glycogen in the liver and muscle for later use. The efficiency of carbohydrate utilization in fish depends on several factors, including species, diet composition, carbohydrate source and complexity, and environmental conditions.

In herbivorous fish, carbohydrate utilization is generally more efficient, allowing them to tolerate higher levels of dietary carbohydrates. However, excessive intake can still cause negative effects, such as inhibited growth, reduced protein utilization, and metabolic disorders. For example, grass carp (*Ctenopharyngodon idellus*) can utilize dietary carbohydrate from maize starch up to 38% and wheat starch up to 33%, representing the optimum levels for growth (Tian et al., 2012). Similarly, blunt snout bream (*Megalobrama amblycephala*) can utilize dextrin in the diet up to 29% (Li et al., 2013). Omnivorous fish also utilize dietary carbohydrates for energy, a capability more developed than in carnivorous species. Gibel carp (*Carassius auratus gibelio*) can tolerate dietary carbohydrate from α-starch and maize starch at levels of 28–30% (Tan et al., 2006). Nile tilapia (*Oreochromis niloticus*) can efficiently utilize maize grain and wheat bran at levels up to 48% in the diet (Ali and Al-Asgah, 2001), while hybrid tilapia (*O. niloticus* × *O. aureus*) can use maize starch at 22–46% of the diet (Wang et al., 2005). In contrast, carnivorous fish are poor utilizers of dietary carbohydrates. Rainbow trout (*Oncorhynchus mykiss*) can tolerate up to 18–27% gelatinized potato starch in the diet (Yamamoto, 2001), and European seabass can utilize up to 25% of precooked starch and wheat short in the diet (Pérez et al., 1997). Overall, carbohydrates represent the most cost-effective energy source in aquafeed, allowing proteins and lipids to be spared for growth and other metabolic functions. However, carbohydrate utilization varies according to the feeding habits and species of fish. Therefore, investigating strategies to improve carbohydrate utilization can contribute to the development of low-cost, high-quality aquafeeds and enhance the adaptability of fish to carbohydrate-rich diets, supporting sustainable aquaculture.

Nile tilapia, an economically important omnivorous fish, is characterized by high production and remarkable adaptability to diets containing high levels of carbohydrates as the primary energy source. Multidisciplinary nutritional research has

been conducted not only to expand tilapia production but also to address various farming challenges. Studies on glucose metabolism in Nile tilapia have shown that high-carbohydrate diets stimulate glycolysis and lipogenesis while suppressing gluconeogenesis and amino acid catabolism (Boonanuntanasarn et al., 2018a, 2018b). These experiments, carried out over several months, demonstrated that tilapia fed high-carbohydrate diets can efficiently metabolize glucose in the long term. In tropical aquaculture systems, tilapia are often cultured in open freshwater environments, where they experience periods of fasting and refeeding due to seasonal changes, heavy rainfall, bacterial infections, or transportation. Given Nile tilapia's efficient utilization of carbohydrates as a primary energy source, it is hypothesized that food deprivation followed by refeeding could significantly modulate glucose metabolism and carbohydrate utilization. Understanding how fasting and refeeding influence metabolic responses to diets with different carbohydrate levels is therefore essential for optimizing feeding strategies and improving sustainable aquaculture practices.

Table 2.4 Optimal level of dietary carbohydrate in farmed fish.

Fish species	Source of CHO	Feeding duration (days)	Optimum level	References
Herbivorous				
Grass carp (*Ctenopharyngodon idellus*)	Wheat starch	56	33	Tian et al. (2012)
	Maize starch	56	38	
Blunt snout bream (*Megalobrama amblycephala*)	Utilize dextrin	70	29	Li et al. (2013)
Rohu, Indian major carp (*Labreo rohita*)	Dextrin and starch	60	45	Mohapatra et al. (2003)
Omnivorous				
Gibel carp (*Carassius auratus gibelio*)	maize starch	56	30	Tan et al. (2006)
	α-starch and maize starch	56	28	
Nile tilapia (*Oreochromis niloticus*)	Maize grain and wheat bran	63	48	Ali and Al-asgah (2001)
	Dextrin	90	30	Boonanuntanasarn et al. (2018a)
	Dextrin	280	30	Boonanuntanasarn et al. (2018b)
Hybrid tilapia (*O. niloticus* × *O. aureus*)	Maize starch	56	22-46	Wang et al. (2005)

Table 2.4 Optimal level of dietary carbohydrate in farmed fish (continuous).

Fish species	Source of CHO	Feeding duration (days)	Optimum level	References
Carnivorous				
Atlantic salmon (*Salmo salar*)	Extruded wheat	90	9-22	Hemre et al. (1995)
Rainbow trout (*Oncorhynchuss mykiss*)	Gelatinized potato starch	56	18-27	Yamamoto (2001)
European seabass (*Dicentrachus labrax*)	Precooked starch and wheat shorts	90	25	Pérez et al. (1997)

Table 2.5 The effects of dietary high carbohydrates on intermediary carbohydrate metabolism in Nile tilapia.

Source of CHO	CHO levels (%)	Feeding duration (days)	Level of change		References
			Phenotypic traits	Metabolism	
Dextrin	50	90	↑ Plasma glucose, triglyceride, cholesterol ↓ Plasma BUN ↑ Hepatosomatic index ↑ Glycogen in liver and muscle	↓ Hepatic amino acid catabolism (*asat*)	Boonanuntanasarn et al. (2018a)
Dextrin	50	280	↑ Plasma glucose and triglyceride ↓ Plasma total protein and BUN ↑ Hepatosomatic index ↑ Glycogen in liver ↑ Crude fat in whole body	↑ Glycolysis in liver (*pklr*) and muscle (*pkma*) ↑ Hepatic lipogenesis (*fasn*) ↓ Hepatic gluconeogenesis (*g6pca1*, *g6pca2, pck1*) ↓ Hepatic amino acid catabolism (*alat, asat*)	Boonanuntanasarn et al. (2018b)

2.4 Nutritional status in fish

2.4.1 Rationale for fasting duration in fish

In recent years, the prices of fish feed have shown an increasing trend in aquaculture markets. As a result, it has become more challenging to achieve economic profitability when fish are reared using traditional feeding methods. Since feed represents the highest cost in aquaculture production, understanding nutrient requirements and applying appropriate feeding strategies are essential to minimize feed waste and improve profitability. Farmers often attempt to reduce feed costs by optimizing feeding management, such as lowering feeding levels (Cuvin-Aralar et al., 2012), which can also help improve water quality and reduce labor costs (Blanquet and Oliva-Teles, 2010). Several feeding strategies have been investigated, including feeding based on body weight, feed deprivation, and cyclic refeeding, where fish are temporarily fasted and then refed to satiation (Ali et al., 2003). Feed restriction or adjustment of feeding frequency has attracted considerable interest in aquaculture (Yengkokpam et al., 2013; Ren et al., 2015) because these practices can induce compensatory growth, characterized by accelerated growth during refeeding after a period of fasting, feed restriction, or environmental stress such as low temperature or low oxygen (Ali et al., 2003). Furthermore, Crampton (1991) demonstrated that daily feeding is not always necessary to achieve optimal growth performance, while Silva and Anderson (1995) reported that overfeeding can lead to poor growth and higher feed conversion ratios (FCR). In aquaculture systems, periods of food deprivation and refeeding often occur naturally due to environmental fluctuations, transportation, or pathogen infections, and these feeding cycles have a direct impact on energy metabolism, particularly carbohydrate metabolism. Therefore, understanding how fasting and refeeding influence carbohydrate metabolic responses is crucial for improving feeding management, enhancing fish welfare, and promoting more sustainable aquaculture practices.

2.4.2 The effects of fasting and refeeding in fish

Energy metabolism during fasting is dependent on glycogen, lipid and protein, and liver is a main organ to control these energy metabolisms (reviewed in Rui, 2014). Short-term fasting lead to breakdown of glycogen and induced gluconeogenesis (Pérez-Jiménez et al., 2007; Wang et al., 2019; Dai et al., 2022).

Prolonged fasting leads to mobilize muscle including lipolysis and protein, and gluconeogenesis is responsible for glucose production to maintain glucose homeostasis (Rios et al., 2006; Furné et al., 2012; Viegas et al., 2013). For instance, in juvenile Nile tilapia, short-term fasting led to decrease in hepatic glycogen, triglycerides and cholesterol. Nevertheless, short-term fasting increased resistance to infection of *Streptococcus agalactiae* (Wang et al., 2019). In addition, food deprivation affected growth performance, hematological and health status, and refeeding was demonstrated to recover these parameters (Sakyi et al., 2020). Moreover, Nile tilapia which was fasted for 14 days led to decrease body weight. Fasting also decreased triglyceride and glucose in serum as well as hepatosomatic index (HSI), hepatic fat and protein, although these effects were different according to fasting period. Refeeding recovered the reduction of these parameters with varying refeeding time (Tian et al., 2013). It was revealed that in European seabass (*Dicentrarchus labrax*), dietary protein content that was fed before fasting influenced metabolic changes during fasting, and metabolic responses were also different with refeeding with different dietary protein contents (Pérez-Jiménez et al., 2007).

Subsequently to fasting, energy metabolism during refeeding is also related to glucose and its related metabolism (Rios et al., 2006; Viegas et al., 2013). Indeed, these intermediate metabolic responses differed according to fish species as well as duration of fasting and refeeding. Previous study showed that food deprivation resulted in lower body weight, and refeeding led to compensation growth which varied according to fish species, duration of fasting and subsequently refeeding period as well as nutrient composition and/or regimes of refed feed. For example, red sea bream (*Pagrus major*) experienced fasting for either 1 or 2 weeks could achieve full compensatory growth in subsequently refeeding for 3 weeks (Oh et al., 2007). Dietary refeeding containing high protein and lipid diet effectively improved compensatory growth of juvenile olive flounder (*Paralichthys olivaceus*) (Cho and Heo, 2011). In Nile tilapia, refeeding for 5 weeks could achieve growth compensation of fasting for 1 week; however, this period of refeeding was not able to achieve compensatory growth for severity fasting for 2 or 4 weeks (Elbialy et al., 2022).

2.4.3 The effects of refeeding with dietary carbohydrates after fasting in fish

Since carbohydrates can serve as a primary source of energy for many fish species, understanding how dietary carbohydrates influence metabolic responses after fasting is crucial for elucidating carbohydrate utilization in fish. In European seabass, a subsequent 12-day refeeding period with a low-protein/high-carbohydrate (40.9% CP/30.6% NFE) diet after a 9-day fast increased plasma cholesterol, liver glycogen, and hepatic FAS and G6PDH activities, while the activity of hepatic FBPase decreased. Moreover, fish refed with a high-carbohydrate diet induced lipogenesis and suppressed gluconeogenesis in the liver of European seabass compared with the low-carbohydrate (48.9% CP/21.0% NFE) refeeding group (Pérez-Jiménez et al., 2007). These findings suggest that refeeding with a high-carbohydrate diet may influence metabolic adjustments by inducing lipogenesis and suppressing gluconeogenesis in European seabass (Pérez-Jiménez et al., 2007).

The rainbow trout is considered a strictly carnivorous fish that is highly adapted to the catabolism of dietary protein but exhibits low utilization of dietary carbohydrates, a characteristic referred to as glucose intolerance. In a previous study by Marandel et al. (2015), after a 4-day fast, trout fed a high-carbohydrate diet exhibited increased plasma glucose levels and induced hepatic glycolysis at both the molecular (*gck*) and enzymatic (GCK) levels, while the expression of genes related to gluconeogenesis (*g6pcb2*) remained elevated compared with the no-carbohydrate group. This finding suggests that the poor utilization of dietary carbohydrates in trout may be linked to their persistent hyperglycemia when fed a high-carbohydrate diet. Therefore, these results imply that short-term fasting and refeeding with a high-carbohydrate diet may significantly affect carbohydrate metabolism, providing insight into the underlying metabolic adjustments in Nile tilapia. In addition, changes in nutritional status (e.g., fasting, refeeding, or dietary carbohydrate intake) not only alter metabolic responses and biochemical pathways but may also induce epigenetic modifications, which could affect metabolic adaptation and long-term physiological responses in fish.

2.5 Epigenetic modification

2.5.1 Overview of epigenetic modification

Epigenetics is the branch of biology that studies the fundamental interactions between genes and their expression as influenced by environmental factors (nutrition or non-nutrition), which ultimately bring the phenotype into being (Waddington, 1957). Nowadays, epigenetics refers to the investigation of gene regulation and expression that are not associated with changes in DNA sequences. The three main mechanisms underlying epigenetic regulation are DNA methylation of cytosine or adenine residues, covalent modifications of histone proteins, alterations in chromatin structure and function, and the involvement of non-coding RNAs, as shown in Figure 2.8 (Axsom et al., 2021).

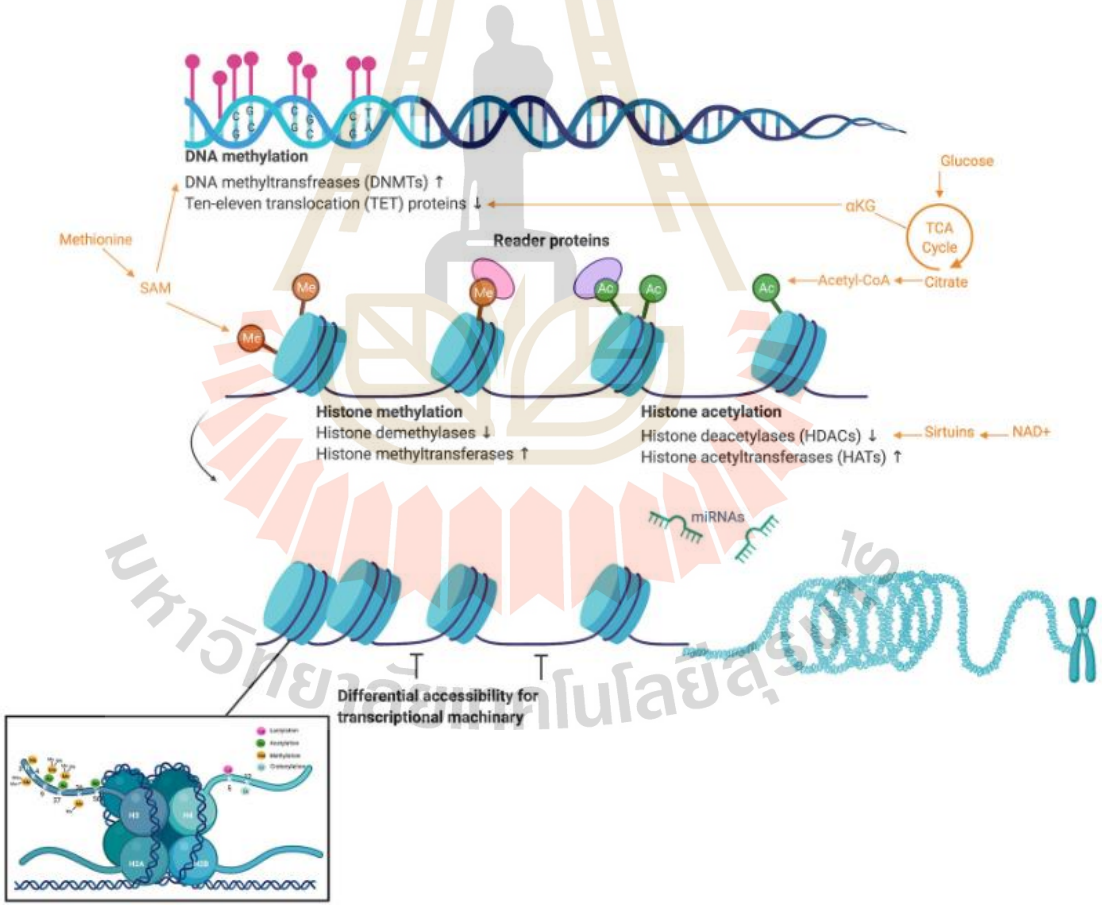

Figure 2.8 Overview of common epigenetic modifications, the activity of chromatin modifying enzymes, and the metabolites that act as substrate (Axsom et al., 2021).

2.5.1.1 DNA methylation and demethylation processes

DNA methylation is an epigenetic mechanism that occurs through the addition of a methyl group (CH_3) to the DNA strand, thereby modifying gene function and influencing gene expression. This process is catalyzed by DNA methyltransferase enzymes, which add a methyl group to cytosine bases within CpG islands (cytosine followed by guanine, or 5′-CG-3′). Moreover, methylation can also occur at non-CpG sites such as CpA, CpT, and CpC (Jang et al., 2017), with non-CpG methylation playing an important role in quiescent organs and in evolutionary or metabolic adaptations (Barres et al., 2011). The symmetrical presence of CpG methylation marks on both DNA strands enables the post-replicative maintenance of methylation patterns, making DNA methylation a key feature of epigenetic regulation (Lyko, 2018).

Conversely, DNA methylation dynamics involve both methylation and demethylation processes that act in balance. DNA demethylation refers to the removal of a methyl group from 5-methylcytosine (5-mC) through the oxidation of its derivatives. Two types of DNA demethylation have been described: (1) passive DNA demethylation and (2) active DNA demethylation. Passive DNA demethylation occurs when newly synthesized DNA strands lack methylation due to the inaction of the maintenance methyltransferase enzyme DNMT1 during successive rounds of DNA replication. In contrast, active DNA demethylation occurs independently of DNA replication through the oxidation of 5-mC into various cytosine derivatives, a process mediated by enzymes such as TET (ten-eleven translocation enzymes), TDG (thymine DNA glycosylase), and the base excision repair (BER) pathway. This active DNA demethylation was suggested to have been accomplished through the action of TETs (active demethylation pathway) which mediate iterative oxidation of 5-mC into derivatives 5–hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC), followed by TDG–dependent base excision repair or replication dependent dilution (He et al., 2011; Ito et al., 2010; Tahiliani et al., 2009), which lead to cytosine (Figure 2.9).

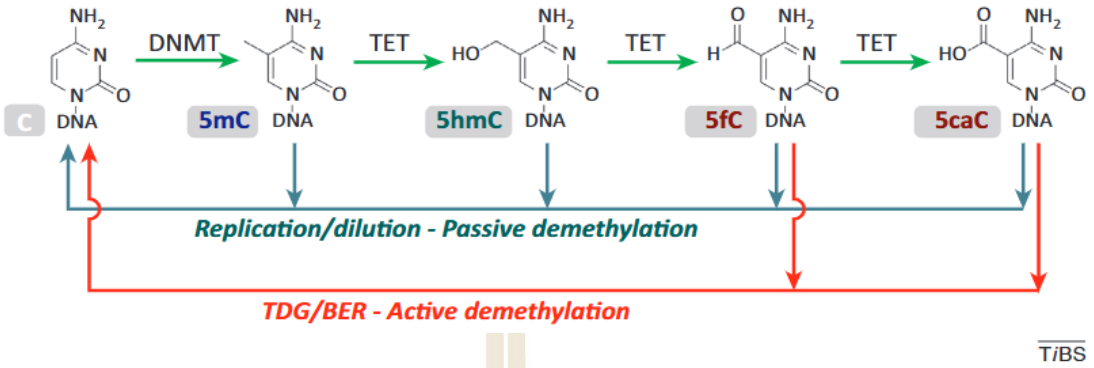

Figure 2.9. DNA methylation and demethylation are dynamically balanced (He et al., 2011).

2.5.1.2 Histone modification

Histones are a family of alkaline proteins that associate with DNA in the nucleus and facilitate its compaction. Histone proteins comprise five main types: H1, H2A, H2B, H3, and H4. Based on their functions, histones can be classified into core histones (H2A, H2B, H3, and H4) and a linker histone (H1). The histone octamer, composed of two copies each of H2A, H2B, H3, and H4, is wrapped by approximately 145–147 base pairs of double-stranded DNA to form a nucleosome, the fundamental subunit of chromatin.

Histone modification refers to covalent post-translational modifications (PTMs) of histone proteins, including methylation, phosphorylation, acetylation, and ubiquitylation, which occur mainly on their flexible N-terminal tails. These modifications are reversible and dynamically regulate chromatin structure and gene expression. Moreover, histone remodeling, similar to active DNA methylation mechanisms, is modulated by a variety of enzymes that act as “writers” and “erasers” of histone marks. These include histone methylation writers (histone lysine methyltransferases, KMTs), methylation erasers (histone lysine demethylases, KDMs), acetylation writers (histone lysine acetyltransferases, KATs), and acetylation erasers (histone deacetylases or sirtuins, SIRTs). Such enzymatic activities contribute to variations in gene expression not only by altering chromatin structure (from condensed heterochromatin to relaxed euchromatin) but also by recruiting specific proteins and chromatin-remodeling complexes (Bannister and Kouzarides, 2011; Kouzarides, 2007) (Figure 2.10).

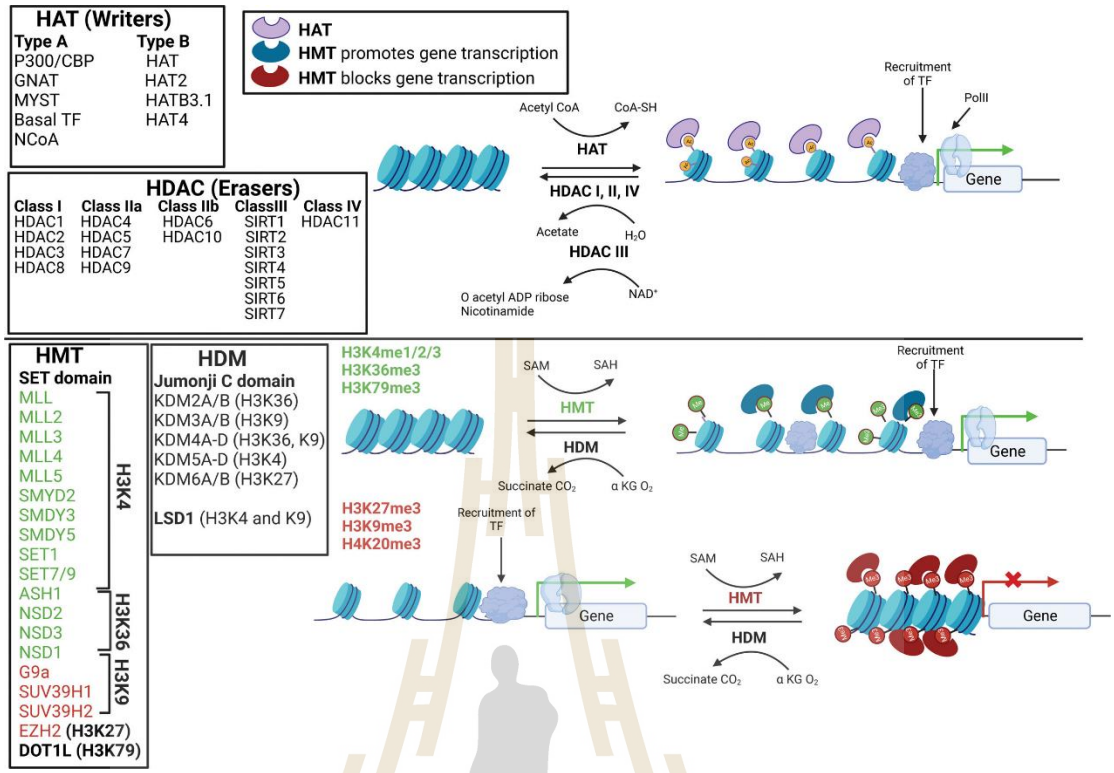

Figure 2.10. Different classes of histone-modifying enzymes and their effect on gene transcription (Ray et al., 2024).

2.5.1.3 Non-coding RNAs

Non-coding RNAs (ncRNAs) are RNA molecules transcribed from DNA that are not translated into proteins. ncRNAs are generally classified according to length into short ncRNAs (sncRNAs, less than 200 nucleotides [nt]) and long ncRNAs (lncRNAs, more than 200 nt) (Kapranov et al., 2007). The sncRNAs can be further divided into small interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs) (Diamantopoulos et al., 2018). For example, siRNAs are a class of double-stranded sncRNAs of approximately 22 nucleotides that originate from either cellular genes or pathogen-derived transcripts. They share similar functions with miRNAs, playing an important role in post-transcriptional gene regulation and RNA interference (RNAi). The main difference between siRNAs and miRNAs is that siRNAs usually target a single, specific mRNA, whereas miRNAs can regulate multiple mRNA targets (Lam et al., 2015).

2.5.2 Epigenetics and nutritional status

Epigenetics investigated how environmental or behavioral factors can alter gene activity without changing the DNA sequence (Waddington, 1957). Nutritional status is one of the environmental factors related to adjustment metabolism, which could be influenced by epigenetic mechanisms via DNA (de)methylation and/or histone modification in animals (Marandel et al., 2016; Liu et al., 2022; Tamaoki et al., 2018; Xu et al., 2012; Jiménez-Chillarón et al., 2012). For example, the fasted state could induce hypermethylation in mammals, which resulted in DNA and promoter regions of genes related to energy homeostasis (Gibson et al., 2020; Hjort et al., 2017). Moreover, the dynamic balances of DNA methylation modulators (DNMTs or TETs) were also affected by nutritional status. The DNA methylation process is mediated by the DNA methyltransferase family (DNMTs), whereas removal of the methyl group via active demethylation process was performed through the Ten-eleven translocation (TET) enzyme family by catalyzing DNA demethylation by oxidizing 5-methylcytosine (5mC) to 5-hmdC (Zhang et al., 2023; Tahiliani, 2009). In mice, the alteration of DNA methylation modulators (DNMTs or TETs) at protein and transcription levels was influenced by intermittent fasting and feed restriction, respectively (Selvaraji et al., 2022; Hahn et al., 2017).

Moreover, nutritional status could initiate a metabolic memory by remodeling the epigenetic landscape through chromatin structure (Marandel et al., 2016). Histone modifications (i.e., methylation or acetylation) are a type of epigenetic mechanism related to chromatin remodeling, which is important for regulating gene expression (Zhang et al., 2021). In human cell culture, glucose-restricting conditions induced the remodeling of histone marks at the gene promoter region, which resulted in alterations of the target gene at transcription levels (Li et al., 2011). In addition, alteration of histone marks via nutrition status might be influenced by histone lysine methylation modulators (e.g. KMTs and KDMs,) and histone lysine acetylation modulators (e.g. KATs and SIRTs), which plays a key role in histone lysine structure as well as in gene expression (Park et al., 2024; Gujral et al., 2020). For instance, fasting induced deacetylation status in mice, which might be affected by changes in histone deacetylases at molecular levels (Funato et al., 2011). In amphibians, the suppression

of *kat* genes was induced by feed deprivation even though histone modification remains stable (Tamaoki et al., 2018).

2.5.3 Nutrient–epigenetic interactions and the role of carbohydrates

Since epigenetic mechanisms are possibly responded to environmental factors. Dietary nutrients play a crucial role in providing the essential precursors and cofactors required for epigenetic modifications, particularly DNA methylation and histone modifications. The methylation of DNA depends on the availability of methyl groups donated by S-adenosylmethionine (SAM), which is generated through the one-carbon metabolism pathway involving nutrients such as methionine, choline, folate, and vitamins B6 and B12. A deficiency or imbalance in these nutrients can alter the cellular methylation potential, thereby influencing gene expression patterns (Anderson et al., 2012; Dominguez-Salas et al., 2019). Similarly, histone modifications, such as acetylation and deacetylation, are regulated by enzymes that rely on nutrient-derived cofactors. For instance, acetyl-CoA, a central metabolite derived from carbohydrate, lipid, and protein metabolism, acts as a substrate for histone acetyltransferases (HATs), while NAD^+, generated from niacin (vitamin B3), serves as a cofactor for sirtuin deacetylases (Kaelin and McKnight, 2013; Gut and Verdin, 2013).

Among macronutrients, dietary carbohydrates not only serve as an energy source but also play a central role in providing metabolic intermediates that influence epigenetic regulation (Figure 2.10), including DNA methylation and histone modifications. Through glycolysis and the tricarboxylic acid (TCA) cycle, carbohydrates contribute to the synthesis of acetyl-CoA, which acts as a key substrate for HATs that promote histone acetylation and transcriptional activation (Kaelin and McKnight, 2013; Gut and Verdin, 2013). Additionally, carbohydrate metabolism supplies intermediates to the one-carbon metabolism pathway, linking glucose-derived carbon flow to the production of SAM— the universal methyl donor for DNA and histone methylation (Locasale, 2013; Mentch and Locasale, 2016). The pentose phosphate pathway (PPP), derived from glucose metabolism, also generates NADPH, which supports redox balance and the activity of enzymes involved in chromatin remodeling (Etchegaray and Mostoslavsky, 2016). This interplay between nutrition, metabolism, and epigenetics highlights how dietary composition can affect gene expression and,

ultimately, phenotype and metabolic adaptation in organisms (Lillycrop and Burdge, 2011; Feil and Fraga, 2012).

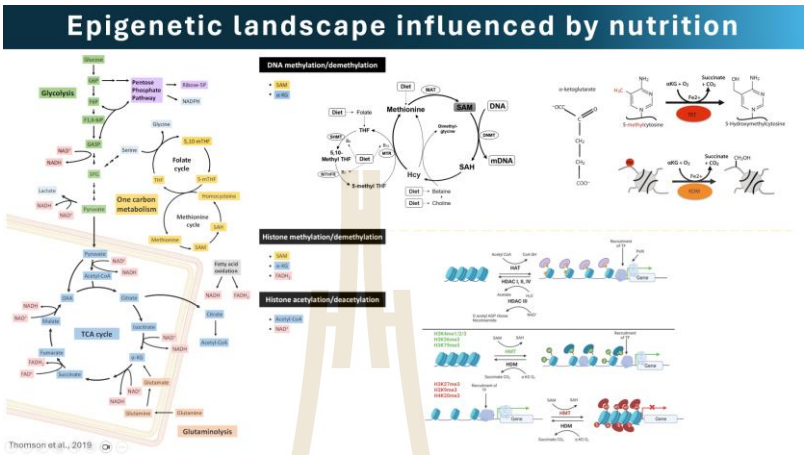

Figure 2.11 The overview of glucose metabolism and epigenetic regulation (Thomson et al. (2019); Ray et al. (2023).

In animals, previous studies demonstrated that dietary factors (i.e., refeeding, CHO and/or protein) also contributed to epigenetics stability, including DNA methylation, histone modification, and epigenetics modulators in vertebrate animals (Rees et al., 2000; Tamaoki et al., 2018), and as well as in fish (Marandel et al., 2016; Liu et al., 2022). In mammal, it was demonstrated that epigenetics changes are related to metabolic disorders particularly diabetes (Berger et al., 2009; Rosen et al., 2018). Moreover, in rats, insufficient dietary nutrients (i.e., protein) during pregnancy could induce hepatic DNA hypermethylation in their offspring (Rees et al., 2000). Moreover, several studies have been conducted to investigate the mechanisms underlying the limited ability of carnivorous fish to utilize dietary carbohydrates and to improve their capacity for high-carbohydrate diet utilization. In rainbow trout, subsequent refeeding after fasting could induce hepatic epigenetics landscape, which altered histone modification, DNA (de)methylation, and as well as in transcription levels (Marandel et al., 2016; Liu et al., 2022). Moreover, differences in refeeding nutrients could influence epigenetics modification independently, which results in rainbow trout refeeding with dietary low-protein or high-CHO that could exhibit a global DNA hypomethylation state in the liver (Liu et al., 2022).

In Nile tilapia, the remodeling of epigenetic stability through dietary factors has been demonstrated via nutritional programming, in which glucose injection during early development resulted in the persistent modulation of carbohydrate metabolism through DNA hypomethylation in liver and muscle tissues later in life (Kumkhong et al., 2020). However, detailed knowledge of the epigenetic mechanisms (e.g., DNA (de)methylation, histone modification, and molecular epigenetic modulators) underlying carbohydrate utilization in Nile tilapia remains limited. Therefore, understanding how dietary carbohydrates modulate these epigenetic mechanisms will provide valuable insights into the molecular basis of metabolic plasticity and nutrient adaptation in Nile tilapia, an omnivorous fish species.

Table 2.6 The effects of high-CHO diet on epigenetic regulation in fish.

Epigenetic regulation	Fish	Fish Stage	Tissue	CHO levels (%)	DNA methylation level	References
DNA methylation	Grass carp	Juvenile	Liver	45	Hypomethylation at promoter region of gluconeogenesis genes	Cai et al., 2020
	Nile tilapia (*Oreochromis niloticus*)	Juvenile with 2M glucose injection history	Liver	-	Hypomethylation	Kumkhong et al. (2020)
			Muscle	-	Hypomethylation	
	Rainbow trout (*Oncorhynchus mykiss*)	Juvenile	Liver	22	Hypomethylation	Craig and Moon (2013)
			Muscle	22	Hypomethylation	
		Juvenile	Liver	30	Hypomethylation	Marandel et al. (2016)
		Juvenile	Liver	30	Hypomethylation	Liu et al. (2021)
Histone modification	Rainbow trout (*Oncorhynchus mykiss*)	Juvenile	Liver	30	Hypoacetylation of H3K9	Marandel et al. (2016)

2.6 References

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CHAPTER III

SHORT-TERM REFEEDING WITH HIGH-CARBOHYDRATE DIET AFFECTS INTERMEDIARY CARBOHYDRATE METABOLISM IN JUVENILE AND ADULT NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

3.1 Abstract

This study aims to investigate the effects of short-term fasting and subsequent refeeding with high or low carbohydrate diets on the intermediary carbohydrate metabolism of juvenile and adult Nile tilapia (*Oreochromis niloticus*). Fish were fasted for four days and subsequently refed with either a low carbohydrate and high protein (LC/HP) or high carbohydrate and low protein (HC/LP) diet for four days. Our results showed that four days of refeeding with either one of the diets could compensate for weight loss due to fasting. Thus, we investigated the effects of a four-day-refeeding strategy and different carbohydrate refeeding diets on plasma metabolites, nutrient composition, and glucose and its related metabolism in the liver and muscle of adult fish. Refeeding had similar effects in adults and juveniles and induced modulations to the intermediary metabolism: 1) refeeding with the HC/LP diet elevated plasma glucose levels; 2) refeeding with both diets increased triglyceride levels in the plasma, liver, and muscle, but the effect of the HC/LP diet was superior; 3) refeeding elevated plasma cholesterol levels in adults, irrespective of diet; 4) refeeding with both diets increased hepatic lipid levels in juveniles, with stronger effects observed in those fed the HC/LP diet, and refeeding with the HC/LP diet elevated hepatic lipid levels in adults; 5) refeeding with both diets increased the plasma protein content, but the effect of the LC/HP diet was superior; 6) refeeding with the LC/HP diet increased hepatic protein content in adults; and 7) refeeding with both diets increased hepatic glycogen levels, but the effect of the HC/LP diet was superior. Additionally, in juveniles and adults, refeeding with the HC/LP diet upregulated the expression of glycolytic genes in the liver and muscle, lipogenic genes in the liver, and glucose transport genes. Moreover, refeeding with the HC/LP diet downregulated the expression of gluconeogenic and

amino acid catabolism genes in the liver and amino acid catabolism genes in the muscle. Collectively, the effect of short-term refeeding with a high carbohydrate diet on intermediary metabolism resembled that of long-term feeding, supporting the hypothesis that Nile tilapia, an omnivorous fish, is highly responsive to dietary carbohydrates.

Keywords: Nile tilapia; glucose metabolism; glycolysis; gluconeogenesis; lipid metabolism.

3.2 Introduction

In aquaculture, feed accounts for a majority of production costs, and a rise in feed prices has a direct impact on production costs. Among feedstuffs, digestible carbohydrates (CHO) are the cheapest to produce; therefore, intensive research and development has been conducted to drive the efficient use of CHO as an energy source. The optimum CHO levels and methods to increase dietary CHO levels to enable its protein-sparing effects during growth have been widely studied, particularly in herbivores and omnivores (Shiau and Peng, 1993; Singh et al., 2006; Li et al., 2013). Additionally, in carnivorous rainbow trout (*Oncorhynchus mykiss*), a glucose-intolerant phenotype is typically observed in juveniles fed a high CHO diet (more than 20%) (Polakof et al., 2012; Kamalam et al., 2017), however, female broodstock are adaptable to a 35% CHO diet without it having negative effects on their growth and reproductive cycle (Callet et al., 2021). These findings can not only be applied in other fish species but also provides basic knowledge of intermediary metabolism in fish which varies with feeding habits (Polakof et al., 2012). Moreover, energy expenditure and compensation according to food deprivation and refeeding which often occurs along life cycle of farmed-raised fish were also hypothesized to modulate CHO metabolism particularly for herbivorous and/or omnivorous fish. Indeed, intensive information of the effects of fasting and refeeding on CHO and its related metabolism is still limited although this nutritional status was demonstrated to influence several parameters including growth, liver size and composition, body composition, blood parameters and health status in various fish (Rios et al., 2006; Tian et al., 2013; Morshedi et al., 2016; Sakyi et al., 2020).

Nile tilapia is an economically important fish, and global tilapia aquaculture production reached 4.4 million tons in 2020 (FAO, 2022). The culture of Nile Tilapia is

estimated to increase annually. Therefore, multidisciplinary nutritional research has been conducted not only to expand tilapia production, but also to alleviate farming challenges. The tilapia is an omnivorous fish which is highly adaptable to high levels of dietary CHO as its main energy source. An investigation of glucose metabolism in Nile tilapia revealed that high dietary CHO induced glycolysis and lipogenesis and reduced gluconeogenesis and amino acid catabolism (Boonanuntanasarn et al., 2018a, 2018b). These experiments were conducted over several months and concluded that tilapia fed a high-CHO diet in the long term adequately metabolised glucose. In tropical areas, tilapia culture has been conducted in open freshwater environments in which fish often encounters fasting and refeeding states depending on seasonal variation, heavy rain, bacterial infection, and transportation. Since Nile tilapia is a good user of CHO as primary energy source, food deprivation and subsequently refeeding might hypothesized to largely modulate glucose metabolism and CHO use. Information on how fasting and refeeding influence metabolic responses to different CHO diet is required which would be beneficial to improve aquaculture practices.

Previous study demonstrated that short-term fasting (4 days) and subsequent refeeding (4-days) of dietary high CHO diets led to hyperglycaemia and subsequently remodelling of the hepatic epigenetic landscape in rainbow trout, a model of glucose-intolerant fish (Marandel et al., 2015). This information suggested that fasting and subsequently instantaneous refeeding might also obviously result in metabolic responses of CHO which enable underlying of CHO responses in tilapia, an omnivorous fish. Hence, the present study aims to investigate the short-term glucose metabolic response in Nile tilapia fed different CHO dietary level after a short fasting period. Since glucose and its related metabolism depends on the physiological stage, this study provided comparative investigation of CHO and its related metabolic responses at molecular level at two key life stages: juvenile and adult. In addition, the effects of different level of CHO refeeding on blood metabolites and muscle and hepatic composition were demonstrated, leading to the overall conclusion of intermediary carbohydrate metabolism.

3.3 Materials and methods

3.3.1 Experimental design, diet, and fish culture

All experimental procedures involving fish were approved by the Ethics Committee of the Suranaree University of Technology Animal Care and Use Committee (Approval No. SUT-IACUC-001/2023). The experimental plan for fasting and subsequent refeeding, which was completely randomised with six replicates (tanks), is shown in Figure 3.1. We conducted two separate experiments: one for juvenile and one for adult fish. For the first experiment, 60 juvenile fish (50–60 g) were randomly distributed in cement tanks (4 m^3, water depth: 0.8 m) under continuous aeration. For the second experiment, 34 adult fish (450–550 g) were randomly distributed in cement tanks (water depth: 0.8 m) under continuous aeration. A flow-through water change system was implemented by replacing one-third of the water in each tank weekly with dechlorinated water.

The experimental fish were acclimated to the experimental conditions for 14 days (hereinafter referred to as the initial phase) and hand fed twice daily (9:00 and 16:00) with a commercial diet (30% crude protein (CP) + 4% crude fat (CF)) administered at 3% of their body weight. Subsequently, the fish were fasted for four days.

For comparative investigation of the effects of instantaneous refeeding with different dietary CHO diet, two experimentally isoenergetic diets including high CHO and low protein (HC/LP) and low CHO and high protein (LC/HP) were formulated. The experimental diets and their proximate compositions, including moisture, CP, CF, crude fibre, and ash, which were analysed according to the standard method of the Association of Official Analytical Chemists (AOAC, 1990), are listed in Table 1. After fasting, the fish were divided into two groups for refeeding with two respective experimental diets for four days. During refeeding, tanks were divided into two partitions (replicates) for HC/LP and LC/HP refeeding. Fish (n = 6 partition replicates; juvenile; 22 fish/replication, adult: 13 fish/replication) were fed at 3% of their body weight with either the HC/LP or LC/HP diet for four days. Throughout the experimental period, air and water temperature were determined daily and ranged from 29 to 31°C and 27.0 to 28.0°C, respectively. Dissolved oxygen (DO) and pH were measured daily using a DO and pH meter, and their values were within acceptable ranges of 4.30–5.58 mg L-1 and 7.49–8.71, respectively. During the experimental period, fish deaths were

recorded to determine their survival rates, and no mortality was observed. Fish were weighed at the initial, 4-day fasting, and 4-day refeeding periods. Because the body weight loss of fasted fish was recovered after the 4-day refeeding period, we collected samples prior to and after the refeeding period.

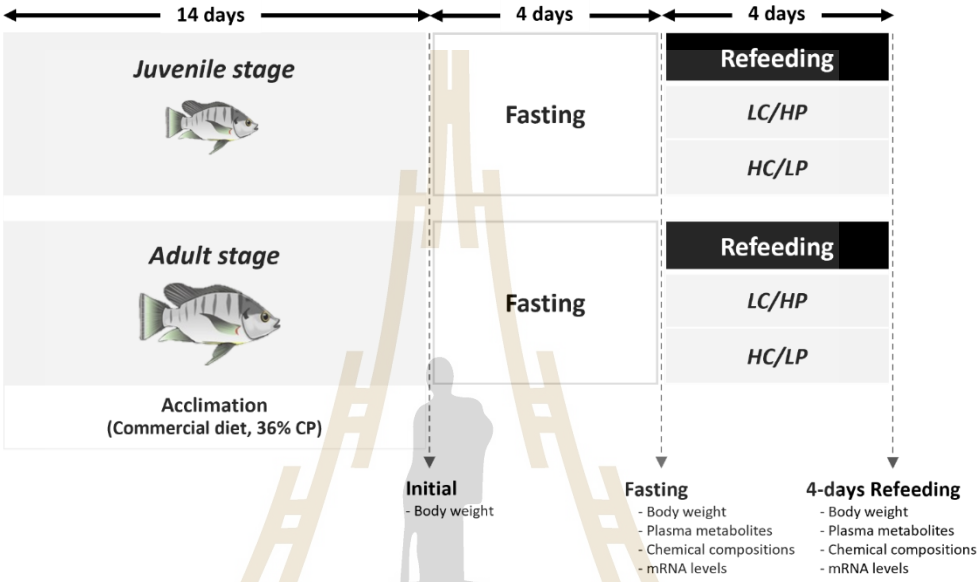

Figure 3.1 Experimental plan for fasting and refeeding juvenile and adult Nile tilapia. Fish were fed a commercial diet during the acclimation period. During the 4-day fasting and 4-day refeeding periods, fish were refed with either a low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet.

3.3.2 Fish sampling and blood collection

Samples were collected after the 4-day fasting and 4-day refeeding periods (Figure 3.1). After fasting for 4 days, fish (n = 6 tank replicates; juvenile; 4 fish/replication, adult: 2 fish/replication) were euthanized using 0.2% clove oil. For refeeding experiments, fish were sampled 5 h after the last meal based on the postprandial glycaemia curve of Nile tilapia determined in a previous study (Boonanuntanasarn et al., 2018a). Also, fish (n = 6 partition replicates; juvenile; 4 fish/replication, adult: 2 fish/replication) were euthanized using 0.2% clove oil. For each fish, blood samples were collected from the caudal vein using a hypodermic syringe

and mixed with K_2EDTA (1.5 mg mL^{-1} blood) as an anticoagulant, after which the K_2EDTA-treated blood samples were centrifuged at 12,500 rpm for 10 min at 4°C to obtain plasma and stored at −80 °C for biochemistry analysis. After bleeding, liver and epaxial muscle samples were collected, snap frozen in liquid nitrogen, and kept at −80°C for analysis of CP, crude lipids, triglyceride (TAG), and glycogen content, as well as metabolic gene expression. Whole fish and livers were weighed and the hepatosomatic index (HSI) was calculated.

3.3.3 Chemical composition, glycogen and triglyceride analysis

The chemical composition, including CP, CF, glycogen, and triglyceride (TAG), of the liver and muscle samples was analysed after four days of fasting and four days of refeeding. The CP and CF content (one fish/replicate; n = 6 replicates) were analysed according to the standard method of the AOAC (1990). Glycogen content was analysed (one fish/replicate; n = 6 replicates) according to the method described by Kirchner et al. (2003). In brief, samples (200 mg) were homogenised in 1 ml of 1M HCl. An aliquot was transferred to a new tube, and 5M KOH was added for neutralisation. The homogenised sample was centrifuged at 10 000xg at 4°C for 10 min, and the supernatant was used to measure free glucose content using a plasma glucose kit (Catalogue number: BLT00026, Erba Lachema s.r.o., Karásek Brno, Czech Republic), according to the manufacturer's instructions. Another aliquot was boiled at 100°C for 2.3 h to hydrolyse glycogen and was subsequently neutralised with 5M KOH. After neutralisation, the sample was centrifuged at 10 000xg at 4°C for 10 min. Total glucose (free glucose and glucose obtained from glycogen hydrolysis) was determined using a glucose kit (Catalogue number: BLT00026, Erba). The glycogen content in the tissues was obtained by calculating the amount of glucose after subtracting the total glucose from the free glucose levels. The TAG content (one fish/replicate; n = 6 replicates) was analysed using a TG Kit (Catalogue number: BLT00059; Erba), according to the manufacturer's instructions. In brief, 100 mg of grounded tissue was homogenised with 1 ml of 5% IGEPAL in deionised water containing 0.1-mm glass beads using the Bioprep-24 homogeniser. Samples were heated in a water bath at 90°C for 10 min and then cooled down to room temperature. After centrifugation at 10 000xg at 4°C for 10 min, the supernatant was transferred into a new tube and diluted with deionised water.

3.3.4 Blood chemistry analysis

Blood chemistry analysis was performed after four days of fasting and four days of refeeding. Determination of blood metabolites (two fish/replicate; n = 6 replicates), including glucose, TAG, cholesterol, and total protein, were performed as described in Kumkhong (2020). Plasma glucose levels were quantitatively measured according to Trinder's method (Trinder, 1969). Plasma TAG levels were determined using the glycerol-3-phosphate oxidase-sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (GPO-ESPAS) method as described by Bucolo and David (1973). Cholesterol levels were analysed using the cholesterol oxidase phenol + aminophenazone (CHOD-PAP) method according to Flegg (1973). Total plasma protein was analysed using the Biuret method (Gornall et al., 1949).

3.3.5 Total RNA extraction, cDNA synthesis, and real-time RT-qPCR analysis of metabolic gene expression

Expression of genes related to CHO metabolism was examined in the liver and muscle samples after four days of fasting and four days of refeeding (at each sampling point: one fish/replicate; n = 6 replicates). Total RNA was extracted from the liver (50 mg) and muscle (100 mg) samples using the TRIzol reagent (Catalogue number: 15596026, Invitrogen, Carlsbad, CA, USA). The quantity of total RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher, Madison, WI, USA), and the total RNA was verified using 1% agarose gel electrophoresis. cDNA synthesis was performed using 1 µg of total RNA and a SuperScript III RNAseH-Reverse transcriptase kit (Catalogue number: 18080093, Invitrogen) with random primers (Catalogue number: C1181, Promega, Charbonniéres, France), following the manufacturer's protocol. The primer sequences used for real-time RT-qPCR are listed in Table S1. Reverse transcription (duplicate for each sample) was performed, and each PCR assay (duplicate for each PCR reaction) was performed to analyse the mRNA levels.

To measure the mRNA levels of glucose metabolism-related genes in the liver and muscle tissue, quantitative real-time reverse-transcription polymerase chain reaction (real-time RT-qPCR) was performed. Hepatic glycolysis was evaluated by determining the expression of glucokinase (*gck*), phosphofructokinase (*pfklr*), and pyruvate kinase (*pklr*). To evaluate hepatic gluconeogenesis, the expression of glucose-6-phosphatase (*g6pca1* and *g6pca2*), cytosolic phosphoenolpyruvate carboxykinase

(*pck1*), and phosphoenolpyruvate carboxykinase (*pck2*) was determined. For lipogenesis in the liver, the expression of fatty acid synthase (*fasn*) and glucose-6-phosphate dehydrogenase (*g6pd*) was examined. The expression of several genes encoding enzymes involved in amino acid catabolism in the liver, including glutamate dehydrogenase (*gdh*), alanine aminotransferase (*alat*), and aspartate aminotransferase (*asat*), were analysed. In addition, the expression of CHO metabolism-related genes in the muscle was determined, including those related to glucose utilisation (glucose transporter, *glut4*), muscular glycolysis (hexokinase I/II, *hk1* and *hk2*; phosphofructokinase, pfkma and pfkmb; and pyruvate kinase, *pkma*), and amino acid catabolism (*alat, asat*, and *gdh*).

Real-time RT-qPCR to measure mRNA levels of glucose metabolism-related genes in the liver and muscle tissue was performed as described in Kumkhong (2020). Reverse transcriptase- and cDNA-template-free samples were used as negative controls for real-time RT-qPCR. Relative quantification of gene transcription was carried out using the Roche Applied Science E-method according to Pfaffl (2001). Relative ef1α expression was used to normalise the measured mRNA levels. The expression of ef1α was not significantly different among the experimental groups (data not shown). Throughout the analysis, PCR efficiency values ranged between 1.8 and 2.0, as determined from the slope of a standard curve using serial dilutions of cDNA.

3.3.6 Statistical analysis

The statistical model utilized was $yij = \mu + \alpha i + \varepsilon ij$, where yij was the response, μ was the general means, αi was nutritive status (fasted and LC/HP and HC/LP refed) effects and εij was the random error. All data were analysed using SPSS for Windows version 22 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed to analyse the differences among the 4-days fasting, 4-days HC/LP refeeding, and 4-days LC/HP refeeding groups. When significant differences were observed, Tukey's range test was performed to rank the treatment groups. The effects and differences were considered significant at $P<0.05$.

Table 3.1 Ingredients and chemical composition (g kg^{-1}) of the commercial diets (during acclimation) and refeeding diets, namely high protein and low carbohydrate (LC/HP) and low protein and high carbohydrate (HC/LP) diets.

Ingredients	Acclimation diet	Challenge diets	
	Commercial diet	LC/HP	HC/LP
Fish meal	-	880	180
Rice flour	-	0	700
Fish oil	-	0	70
Soybean oil	-	20	0
Gelatin	-	80	0
Di-calcium phosphate	-	0	30
Fish premix[a]	-	20	20
Proximate composition (g kg^{-1} dry weight)			
Dry matter	916.4	942.1	921.9
Protein	393.0	607.9	164.9
Fat	36.8	97.9	98.5
Fiber	77.7	5.2	4.6
Ash	137.0	241.6	92.9
NFE[b]	355.4	47.4	639.2
Gross energy (kJ g^{-1})	13.13	14.40	15.6

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein.

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

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

Table 3.2 List of primers used for RT-qPCR.

Genes	5'/3' Forward primer	5'/3' Reverse primer	SIZE (bps)	Accession numbers
Reference gene				
*ef1α**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	250	AB075952
Liver metabolism				
gck	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC	186	XM003451020
pfklr	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC	162	XM003447353
pklr	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA	164	XM005472622
g6pca1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA	195	XM003448671
g6pca2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA	245	XM013273429
pck1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA	162	XM003448375
pck2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT	202	XM019354843
fasn	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT	222	XM013276809
g6pd	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT	187	XM013275693
asat	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC	200	XM003451918
alat	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG	200	XM005476466
gdh	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC	203	XM003457465
Muscle metabolism				
glut4	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG	235	JN900493
hk1	CGTCGCTTAGTCCCAGACTC	TGACTGTAGCGTCCTTGTGG	235	XM019360229
hk2	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC	200	XM003448615
pfkma	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG	190	XM019349871
pfkmb	TTTGTGCATGAGGGTTACCA	CACCTCCAATCACACACAGG	208	XM003441476
pkma	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA	249	XM005447626

* Source: Yang et al. (2013).

3.4 Results

3.4.1 Effects of short-term fasting and refeeding with high- or low-CHO diet on body weight and plasma metabolites in juvenile and adult Nile tilapia

Juvenile and adult Nile tilapia were weighed after acclimatisation to the experimental conditions which was defined as the initial phase. The body weights of juvenile and adult Nile tilapia at the initial phase, after four days of fasting, and after four days of refeeding are shown in Figure 3.2. In juveniles, compared with their weight in the initial phase, four days of fasting led to a decrease in body weight ($P<0.05$), and subsequent refeeding for four days, irrespective of diet, recovered their body weight to that in the initial phase. In adult fish, compared with their weight in the initial phase, four days of fasting also resulted in a decrease in body weight ($P<0.05$) which was recovered after subsequent refeeding for four days, irrespective of diet. Because the body weight loss of fasted fish was recovered after the 4-day refeeding period, we collected samples prior to and after the refeeding period.

The glucose, triglyceride, cholesterol, and total protein levels in juvenile and adult Nile tilapia were investigated (Figure 3.3). Higher plasma glucose levels were observed in juvenile and adult fish fed the HC/LP diet ($P<0.05$), but not in those fed the LC/HP diet, than those in the fasting phase. In addition, refeeding with either the HC/LP or LC/HP diet elevated plasma triglyceride levels; this effect was greater in fish fed the HC/LP diet ($P<0.05$). In juveniles, there were no significant differences in cholesterol levels among experimental groups ($P>0.05$). However, compared with plasma protein levels in the fasting phase, refeeding with either the HC/LP or LC/HP diet increased plasma protein levels, and the LC/HP diet had a greater effect than the HC/LP diet ($P<0.05$). In adults, both the HC/LP and LC/HP diets increased plasma cholesterol levels, while only the LC/HP diet increased plasma protein levels ($P<0.05$).

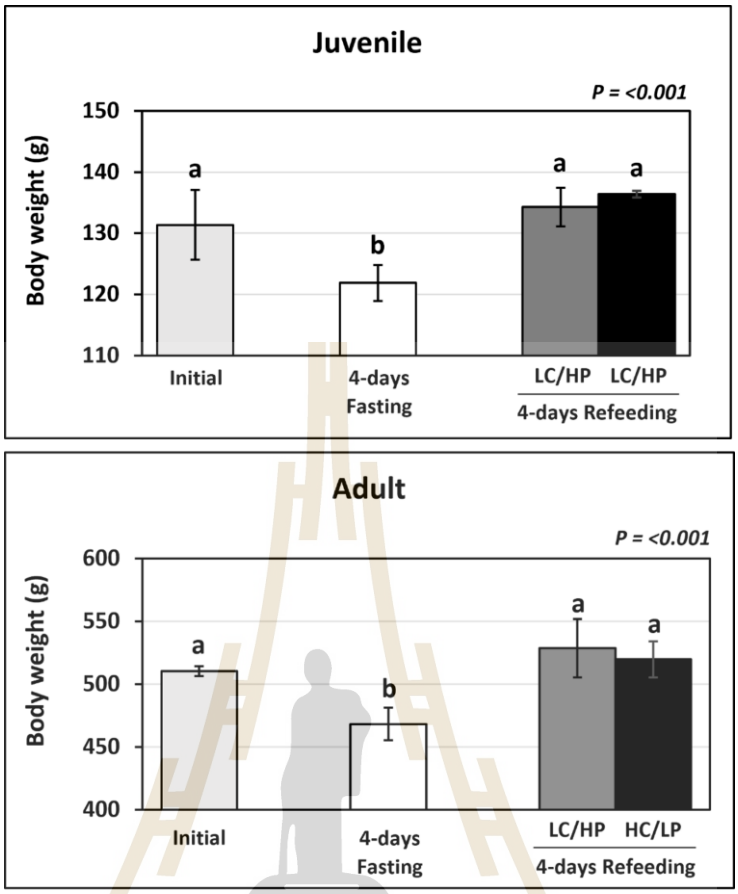

Figure 3.2 Body weight (mean ± SD, n = 6) of juvenile and adult Nile tilapia at initial, 4-day fasting, and 4-day refeeding periods. During the 4-day fasting and 4-day refeeding periods, fish were refed with either a low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet. Different lowercase letters indicate significant differences among nutritional status (P<0.05).

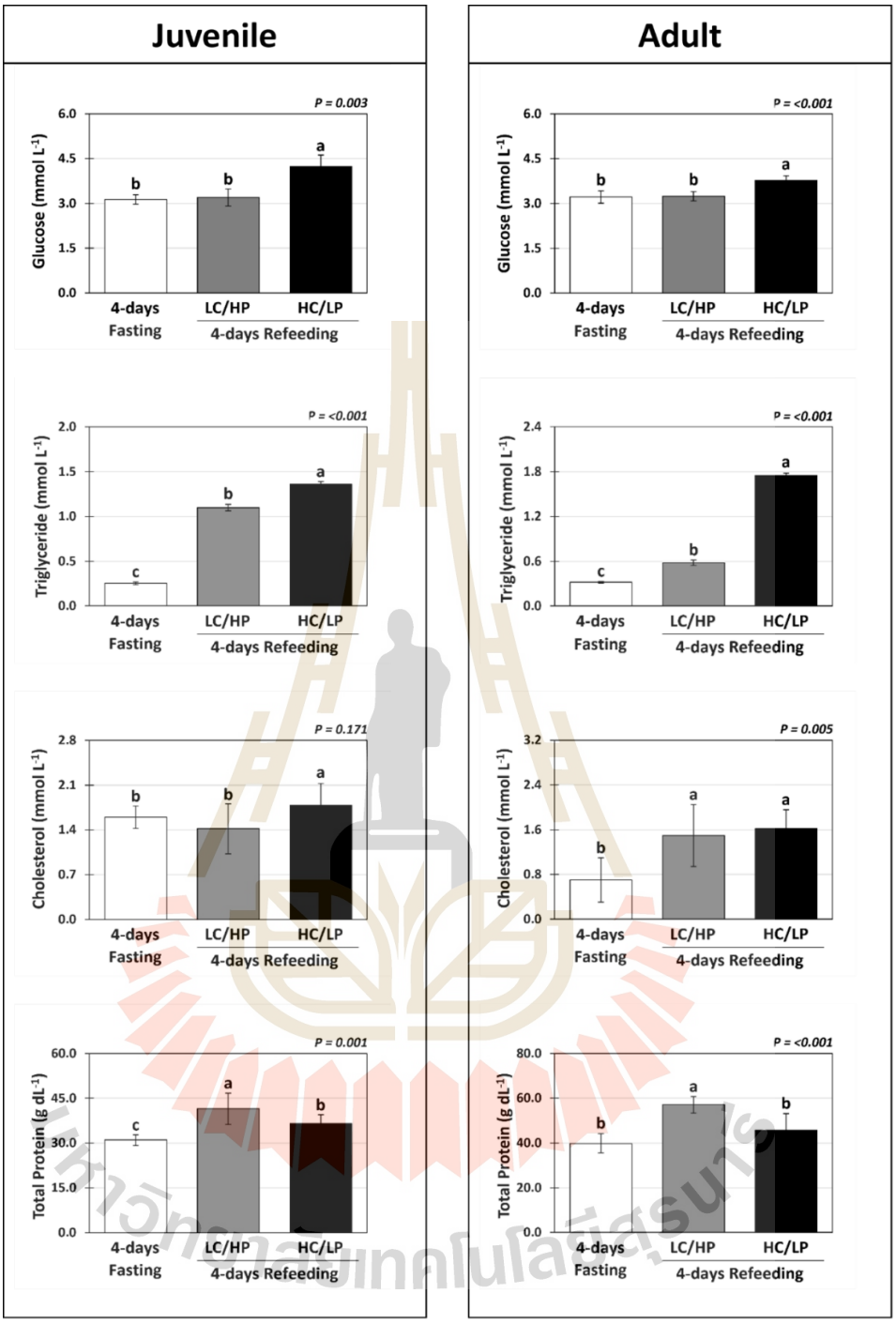

Figure 3.3 Plasma metabolites of Nile tilapia that were fasted for four days (fasting) and refed for four days (4-day refeeding) with either a low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet. Data represent means ± SD (n = 6 individuals per experimental group). Different lowercase letters indicate significant differences among nutritional status (P<0.05).

3.4.2 Effects of short-term fasting and refeeding with low- or high-CHO diets on liver composition and mRNA levels of genes involved in glucose metabolism in juvenile and adult Nile tilapia

The chemical composition of the liver samples of experimental Nile tilapia that were subjected to fasting and four days of refeeding are listed in Table 3.3. In juvenile fish, compared with those during fasting, refeeding with either HC/LP or LC/HP diets led to an increased hepatosomatic index (HSI) and increased hepatic lipid and glycogen levels ($P<0.05$); these effects were greater for the HC/LP diet ($P<0.05$). Elevation of hepatic triglyceride levels was found only in juvenile fish fed the HC/LP diet ($P<0.05$). In adult fish, compared with those during fasting, refeeding with either diets elevated hepatic glycogen and triglyceride levels; however, the effect of the HC/LP diet was superior ($P<0.05$). In addition, higher hepatic lipid and HSI levels were found in adult fish fed the HC/LP diet but not in those fed the LC/HP diet ($P<0.05$). Moreover, LC/HP refeeding resulted in significantly higher hepatic protein levels than those during fasting in adult fish ($P<0.05$), whereas there were no significant differences in hepatic protein levels between fasting and refeeding periods with either the HC/LP or LC/HP diet in juvenile fish ($P>0.05$).

The effects of fasting on the expression of genes related to glucose metabolism in the liver are shown in Table 3.4. In juveniles, compared with those during fasting, refeeding with the HC/LP diet upregulated *pklr* expression while refeeding with LC/HP downregulated *pfklr* ($P<0.05$) expression. Between the refeeding diets, fish fed the HC/LP diet had higher mRNA levels of glycolytic genes (*gck* and *pfklr*) ($P<0.05$). However, there were no significant differences in the expression of *pklr* between refeeding with HC/LP or LC/HP diets ($P>0.05$). Compared with those during fasting, HC/LP refeeding resulted in significantly lower expression of gluconeogenesis genes (*g6pca1, g6pca2, pck1*, and *pck2*) and higher expression of lipogenic genes (*g6pd* and *fasn*) ($P<0.05$). Note that refeeding with a HC/LP diet induced a higher expression of g6pd than refeeding with a LC/HP diet ($P<0.05$). Relative to fasting, refeeding modulated the expression of several genes involved in amino acid catabolism. Between the refeeding diets, the LC/HP diet induced the expression of *gdh*. There were no significant differences in *alat* and *asat* expression between the refeeding groups ($P>0.05$).

In adult fish, compared with those during fasting, refeeding with the HC/LP diet resulted in higher expression levels of hepatic glycolytic genes (*gck, pklr*, and *pfklr*) (P<0.05). Moreover, between the HC/LP and LP/HC diets, the HC/LP diet resulted in significantly higher *pklr* and *pfklr* expression than the LC/HP diet (P<0.05). Compared with that in fish subjected to fasting, the expression of gluconeogenic genes (*g6pca1* and *pck1*) was downregulated in adult fish refed with the HC/LP diet (P<0.05), while those refed with the LC/HP diet had upregulated *pck2* expression (P<0.05). However, there were no significant differences in *g6pca2* expression among the experimental groups (P>0.05). Elevation of hepatic lipogenic genes (*g6pd* and *fasn*) was observed only in adult fish refed the HC/LP diet (P<0.05). In addition, compared with that in fish subjected to fasting, there were no significant differences in the expression of amino acid catabolism genes (*alat, asat*, and *gdh*) in fish refed the LC/HP diet (P>0.05), while fish refed the HC/LP diet had significantly lower *alat* and *asat* expression levels (P<0.05). Fish fed the LC/HP diet had higher expression levels of *gdh* compared with those fed the HC/LP diet (P<0.05).

3.4.3 Effects of short-term fasting and refeeding with low- and high-CHO diets on muscle composition and mRNA levels of glucose metabolism-related genes in juvenile and adult Nile tilapia

The muscle composition of the fasted and refed juvenile and adult fish are shown in Table 3.3. In juvenile and adult fish, compared with those in the fasting group, there were no significant differences in muscular protein, lipid, and glycogen levels among the experimental groups (P>0.05). Compared with fish in the fasting group, refeeding with either a HC/LP or LC/HP diet resulted in increased muscular triglyceride levels in juveniles; however, those fed the HC/LP diet had higher muscular triglyceride levels (P<0.05). In adults, only those fed the HC/LP diet had increased muscular triglyceride levels compared with those in the fasting condition (P<0.05).

The expression of glucose metabolism-related genes in the muscles of juvenile and adult Nile tilapia during fasting and subsequent refeeding conditions are shown in Table 3.5. Compared with fish under fasting conditions, *glut4* was upregulated in fish under refeeding conditions, and the HC/LP diet induced higher *glut4* expression than the LC/HP diet (P<0.05). Compared with fasting, refeeding modulated the expression of several glycolytic genes in juveniles, such as the upregulation of *hk1,*

pfkma, and *pkma* and downregulation of *hk2* and *pfkmb*. In contrast, refeeding upregulated the expression of glycolytic genes in adults, particularly that of *hk1, pfkmb*, and *pkma*. Between the two refeeding diets, higher expression levels of several glycolytic genes (juveniles, *hk1* and *pfkma*; adults, *hk1, hk2*, *pfkma, pfkmb*, and *pkma*) were observed in fish refed the HC/LP diet. Compared with those during fasting, refeeding downregulated the expression of several genes related to muscular amino acid catabolism. Refeeding upregulated *alat* expression in juvenile fish fed the LC/HP diet. Comparison between the refeeding conditions at the molecular level revealed the elevation of muscular amino acid catabolism genes (*alat, asat* and *gdh*) in fish fed the LC/HP diet ($P<0.05$).

Table 3.3 Chemical compositions of Nile tilapia that were fasted for four days (fasting) and refed for four days (refeeding) either the low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet.

Parameters	Juvenile stage				Adult stage			
	Fasting	Refeeding		*P*-value[1]	Fasting	Refeeding		*P*-value
		LC/HP	HC/LP			LC/HP	HC/LP	
Liver compositions								
Hepatosomatic index (%)	1.19 ± 0.10^{c}	1.76 ± 0.15^{b}	3.56 ± 0.41^{a}	<0.001	1.92 ± 0.18^{b}	1.98 ± 0.57^{b}	2.95 ± 0.15^{a}	0.013
Protein (g kg^{-1})	72.32 ± 0.99	75.08 ± 3.20	74.15 ± 1.20	0.087	73.14 ± 1.48^{b}	75.57 ± 0.80^{a}	74.61 ± 1.08^{ab}	0.008
Lipid (g kg^{-1})	30.63 ± 0.97^{c}	34.74 ± 1.25^{b}	41.65 ± 2.19^{a}	<0.001	41.93 ± 1.51^{b}	41.81 ± 2.49^{b}	49.28 ± 1.41^{a}	<0.001
Glycogen (mg g^{-1} tissue)	18.68 ± 2.03^{c}	25.14 ± 2.92^{b}	37.3 ± 1.26^{a}	<0.001	25.44 ± 1.77^{c}	40.76 ± 2.66^{b}	50.17 ± 3.09^{a}	<0.001
Triglyceride (mg g^{-1} tissue)	2.33 ± 0.06^{b}	3.93 ± 0.24^{ab}	5.88 ± 0.29^{a}	0.001	4.02 ± 0.22^{c}	5.64 ± 0.18^{b}	8.41 ± 0.38^{a}	<0.001
Muscle compositions								
Protein (g kg^{-1})	181.52 ± 0.81	181.65 ± 1.17	182.90 ± 1.68	0.148	183.70 ± 1.52	185.18 ± 3.11	184.20 ± 3.69	0.678
Lipid (g kg^{-1})	8.45 ± 0.79	8.61 ± 0.78	8.38 ± 1.25	0.912	8.04 ± 0.51	8.55 ± 0.96	8.84 ± 0.55	0.171
Glycogen (mg g^{-1} tissue)	3.26 ± 0.80	3.30 ± 0.82	4.12 ± 0.86	0.164	5.47 ± 0.75	5.24 ± 0.34	5.96 ± 0.45	0.095
Triglyceride (mg g^{-1} tissue)	0.38 ± 0.07^{c}	0.50 ± 0.06^{b}	0.63 ± 0.08^{a}	<0.001	2.01 ± 0.03^{b}	2.14 ± 0.05^{b}	2.98 ± 0.14^{a}	0.001

[1]One-way ANOVA was used to determine the differences in nutritive compositions in liver and muscle obtained from juvenile and adult fish. Means with different superscripts in each row differ significantly ($P < 0.05$).

Table 3.4 mRNA levels of genes involved in intermediary glucose metabolism in liver tissue of Nile tilapia that were fasted for four days (fasting) and refed for four days (refeeding) either the low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet (mean ± SD, n=6).

Parameters	Juvenile stage				Adult stage			
	Fasting	Refeeding		*P*-value[1]	Fasting	Refeeding		*P*-value
		LC/HP	HC/LP			LC/HP	HC/LP	
Glycolysis								
gck	1.43 ± 0.89^{ab}	0.27 ± 0.04^{b}	1.91 ± 1.07^{a}	0.013	0.74 ± 0.14^{b}	1.35 ± 0.17^{ab}	3.28 ± 1.72^{a}	0.001
pklr	0.23 ± 0.01^{b}	0.64 ± 0.27^{ab}	1.51 ± 0.46^{a}	0.001	0.47 ± 0.01^{b}	0.42 ± 0.31^{b}	4.17 ± 2.54^{a}	0.003
pfklr	1.56 ± 0.36^{a}	0.59 ± 0.30^{b}	1.50 ± 0.47^{a}	0.001	1.17 ± 0.14^{b}	1.15 ± 0.37^{b}	2.63 ± 0.81^{a}	0.004
Gluconeogenesis								
g6pca1	2.10 ± 0.07^{a}	1.49 ± 0.35^{ab}	0.77 ± 0.26^{b}	0.001	1.30 ± 0.04^{a}	1.27 ± 0.59^{a}	0.65 ± 0.37^{b}	0.022
g6pca2	1.92 ± 0.08^{a}	1.22 ± 0.33^{ab}	0.44 ± 0.25^{b}	0.001	1.30 ± 0.04	1.88 ± 1.18	0.88 ± 0.54	0.099
pck1	6.46 ± 0.20^{a}	0.98 ± 0.68^{ab}	0.18 ± 0.13^{b}	0.001	2.13 ± 0.06^{a}	0.33 ± 0.23^{ab}	0.01 ± 0.01^{b}	0.001
pck2	2.19 ± 0.65^{a}	0.94 ± 0.40^{ab}	0.44 ± 0.20^{b}	0.003	0.81 ± 0.35^{b}	2.23 ± 1.25^{a}	1.95 ± 0.98^{ab}	0.044
Lipogenesis								
g6pd	0.34 ± 0.13^{b}	0.60 ± 0.41^{b}	2.22 ± 0.85^{a}	0.002	0.91 ± 0.07^{b}	0.26 ± 0.20^{b}	2.63 ± 1.47^{a}	0.001
fasn	0.002 ± 0.0^{b}	0.56 ± 0.53^{ab}	2.92 ± 0.91^{a}	<0.001	0.17 ± 0.01^{b}	0.14 ± 0.10^{b}	3.25 ± 1.87^{a}	0.001
Amino acid catabolism								
alat	2.02 ± 0.05^{a}	0.65 ± 0.40^{b}	0.66 ± 0.13^{b}	0.003	1.62 ± 0.10^{a}	0.96 ± 0.39^{ab}	0.48 ± 0.36^{b}	0.001
asat	3.10 ± 0.36^{a}	1.47 ± 0.93^{ab}	0.17 ± 0.09^{b}	0.001	1.77 ± 0.11^{a}	0.91 ± 0.52^{ab}	0.15 ± 0.05^{b}	0.001
gdh	0.87 ± 0.03^{b}	2.03 ± 0.56^{a}	0.73 ± 0.30^{b}	0.003	1.08 ± 0.05^{ab}	2.44 ± 1.09^{a}	0.44 ± 0.29^{b}	0.001

[1]One-way ANOVA was used to determine the differences in nutritive compositions in liver and muscle obtained from juvenile and adult fish. Means with different superscripts in each row differ significantly ($P < 0.05$).

Table 3.5 mRNA levels of genes involved in intermediary glucose metabolism in muscle tissue of Nile tilapia that were fasted for four days (fasting) and refed for four days (refeeding) with either the low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet (mean ± SD, n=6).

Parameters	Juvenile stage				Adult stage			
	Fasting	Refeeding		*P*-value[1]	Fasting	Refeeding		*P*-value
		LC/HP	HC/LP			LC/HP	HC/LP	
Glucose transport								
glut4	0.94 ± 0.03^{b}	1.37 ± 0.02^{ab}	1.53 ± 0.07^{a}	0.001	0.39 ± 0.01^{c}	0.75 ± 0.03^{b}	2.79 ± 0.06^{a}	<0.001
Muscular glycolysis								
hk1	0.76 ± 0.03^{c}	1.24 ± 0.03^{b}	1.43 ± 0.04^{a}	<0.001	0.90 ± 0.01^{b}	0.92 ± 0.04^{b}	1.53 ± 0.05^{a}	<0.001
hk2	1.40 ± 0.07^{a}	1.01 ± 0.03^{b}	1.07 ± 0.05^{b}	<0.001	1.08 ± 0.08^{b}	0.87 ± 0.03^{c}	1.25 ± 0.09^{a}	<0.001
pfkma	0.66 ± 0.02^{c}	1.07 ± 0.03^{b}	1.25 ± 0.03^{a}	<0.001	0.61 ± 0.01^{ab}	0.54 ± 0.01^{b}	0.66 ± 0.02^{a}	<0.001
pfkmb	1.37 ± 0.01^{a}	0.99 ± 0.01^{b}	1.15 ± 0.03^{ab}	<0.001	0.65 ± 0.01^{c}	1.08 ± 0.03^{b}	1.13 ± 0.03^{a}	<0.001
pkma	0.84 ± 0.02^{b}	1.06 ± 0.02^{ab}	1.38 ± 0.10^{a}	<0.001	0.74 ± 0.03^{c}	0.83 ± 0.03^{b}	1.04 ± 0.04^{a}	<0.001
Amino acid catabolism								
alat	1.16 ± 0.03^{b}	1.26 ± 0.03^{a}	0.98 ± 0.02^{c}	<0.001	1.47 ± 0.02^{a}	1.11 ± 0.03^{b}	0.42 ± 0.03^{c}	<0.001
asat	1.27 ± 0.18^{a}	1.04 ± 0.12^{a}	0.73 ± 0.25^{b}	0.001	1.26 ± 0.11^{a}	1.09 ± 0.18^{a}	0.80 ± 0.16^{b}	<0.001
gdh	1.65 ± 0.03^{a}	1.45 ± 0.05^{b}	0.94 ± 0.02^{c}	<0.001	1.02 ± 0.05^{a}	1.09 ± 0.04^{b}	0.70 ± 0.04^{c}	<0.001

[1]One-way ANOVA was used to determine the differences in nutritive compositions in liver and muscle obtained from juvenile and adult fish. Means with different superscripts in each row differ significantly ($P < 0.05$).

3.5 Discussion

Tilapia is a good user of CHO as an energy source, and thus responds sensitively to dietary CHO. Several studies have demonstrated the metabolic responses of Nile tilapia to a high-CHO diet throughout its life cycle, as well as the nutritional programming effects of CHO stimuli in early life (Boonanuntanasarn et al., 2018a, 2018b; Kumkhong et al., 2020a, 2020b, 2021; Srisakultiew et al., 2022). Considering that CHO could be a primary source of energy during refeeding after fasting, understanding how fish instantaneously respond to different types of dietary CHO refeeding after fasting would be interesting. In rainbow trout, 4-day refeeding of high dietary CHO after fasting showed significant epigenetic remodelling in liver, demonstrating that this instantaneous refeeding period had impact in metabolic disturbance (Marandel et al., 2015). This study provides information on how Nile tilapia adapted to short-term refeeding with different dietary CHO levels after fasting for plasma metabolites, nutrient composition in the liver and muscle, and CHO and its metabolic responses at the molecular level in different growth phase in Nile tilapia. Our results demonstrated differences in intermediary metabolic responses in Nile tilapia according to different CHO-refeeding diets, suggesting their susceptibility to the use of CHO as an energy source. These findings would be beneficial for further applications of refeeding diets to recover fish from short-term fasting, which often occurs during tilapia culture.

3.5.1 Effects of short-term fasting and refeeding with low- or high-CHO diet on body weight and plasma metabolites in juvenile and adult Nile tilapia

Previous studies have shown that food deprivation results in lower body weight, and refeeding leads to compensatory growth which varies according to fish species, the duration of fasting, and the subsequent refeeding period, as well as the nutrient composition of the refeeding diet and/or refeeding regime (Oh et al., 2007; Cho and Heo, 2011; Urbinati et al., 2004). For Nile tilapia, the effects of fasting and subsequent refeeding has been demonstrated. Refeeding for five weeks could compensate for growth after fasting for one week; however, this period of refeeding could not compensate for growth after severe fasting for two or four weeks (Elbialy et al., 2022). However, information on the influence of different levels of CHO in the diet (as an energy source) on CHO and its related metabolism is still limited. Previous study

demonstrated that Nile tilapia adapt well its metabolism to dietary CHO during a long-term nutritional study (Boonanuntanasarn et al., 2018), but nothing is known after a short term intake of CHO. In this context, a fasting-refeeding protocol with CHO is the best design to test the short-term effect of nutrient (CHO) intake, as shown previously with rainbow trout fasted 4 days and then refed 4 days (Marandel et al., 2015). Our results showed that four days of fasting reduced body weight, and four days of refeeding with either a LC/HP or HC/LP diet was adequate to compensate for growth lost during fasting; these results were similar between juvenile and adult fish. Therefore, we selected the 4-day refeeding period to investigate the instantaneous metabolic responses of Nile tilapia to different dietary CHO levels for compensatory growth.

3.5.2 Effects of short-term fasting and refeeding with low- or high-CHO diets on liver composition and mRNA levels of genes involved in glucose metabolism in juvenile and adult Nile tilapia

In fish, refeeding after fasting leads to the modulation of several intermediate metabolites in the plasma, including increased glucose, TAG, and cholesterol levels, depending on the fish species, as well as the duration of fasting and refeeding (Tian et al., 2013; Viegas et al., 2013; Yarmohammadi et al., 2012; Pérez-Jiménez et al., 2007). Different dietary refeeding conditions would have diverse effects on metabolic responses, particularly for intermediate metabolites in the plasma. Similarly, we observed that refeeding with different CHO levels had diverse effects in juveniles and adults: 1) Refeeding with a HC diet increased plasma glucose levels which was not observed in fish refed a LC diet; 2) Refeeding increased plasma TAG levels, irrespective of dietary CHO/protein contents; however, the effects of the HC diet on increased plasma TAG levels was superior to that of the LC diet; and 3) refeeding with a LC/HP diet increased plasma protein levels. Therefore, short-term refeeding with a HC diet had similar effects on plasma metabolites to that observed in Nile tilapia fed a high-CHO diet for the long term, including increased glucose and TAG levels (90 days), as well as reduced plasma protein levels (45 days) (Boonanuntanasarn et al., 2018a). In addition, long-term feeding of high dietary CHO for 40 weeks led to increased plasma glucose and TAG levels and decreased plasma protein levels in Nile tilapia (Boonanuntanasarn et al., 2018b). Overall, these findings suggest that refeeding dietary CHO content reflected plasma metabolites in Nile tilapia, which supports its sensitive responses to dietary CHO.

Previous studies have demonstrated that refeeding after fasting modulates the HSI and hepatic nutrient composition and that these modulations were affected by glucose homeostasis (Takahashi et al., 2011; Sakyi et al., 2020). Similarly, our results showed that, compared with the fasting state, short-term refeeding in juvenile and adult fish resulted in 1) an increased HSI, which was higher in fish fed the HC/LP diet than in those fed the LC/HP diet; 2) increased hepatic glycogen, lipid, and triglyceride contents, which was higher in fish fed with the HC/LP diet; and 3) increased hepatic protein levels in adults fed the LC/HP diet. These modulatory effects were also observed at the molecular level. The increase in plasma glucose levels and glycogen contents were linked to the upregulation of glycolytic genes in both adult (*pklr* and *pfklr*) and juvenile (*gck* and *pfklr*) fish refed with the HC diet, suggesting that refeeding induced glycolysis; this effect was greater in those fed the HC diet. In Nile tilapia, consistent and long-term feeding of high-CHO diets led to elevated hepatic glycogen levels and increased expression of some glycolytic genes (Boonanuntanasarn et al., 2018a, 2018b). Similarly, refeeding after fasting resulted in the recovery of glycogen content in European sea bass, gilthead seabream, and rainbow trout which was found to be linked with the induction of glycolytic glucokinase mRNA and/or enzyme activities (Viegas et al., 2013; Metón et al., 2004; Soengas et al., 2006). Our results suggest that the increase in hepatic lipid and TAG levels is correlated with the upregulation of lipogenic genes, for which stronger effects were observed in fish fed the HC/LP diet. Furthermore, at the molecular level, our findings suggest that refeeding lowers the expression of gluconeogenic genes (except for *g6pca2*, *pck1*, and *pck2* in adults) and amino acid catabolism-related genes (except for *alat* and *asat* in adults) and the HC/LP diet has stronger effects than the LC/HP diet. Similarly, long-term feeding with a high-CHO diet led to the downregulation of genes involved in gluconeogenesis and amino acid catabolism-related genes in adult Nile tilapia (Boonanuntanasarn et al., 2018a, 2018b). In rainbow trout, a carnivorous fish, feeding with a high-CHO diet led to the downregulation of several gluconeogenic genes; nevertheless, the upregulation of *g6pcb2* orthologues due to high-CHO feeding resulted in glucose-intolerant characteristics (Marandel et al., 2015). These findings suggested that both juvenile and adult Nile tilapia expeditiously responded refeeding HC diet for hepatic glycolysis and lipogenesis. Taken together, the refeeding status was related to CHO and its

metabolism, and at the molecular level, tilapia can efficiently metabolise the HC/LP diet even when administered in the short term.

3.5.3 Effects of short-term fasting and refeeding with low- and high-CHO diets on muscle composition and mRNA levels of glucose metabolism-related genes in juvenile and adult Nile tilapia

This study also investigated the effects of short-term refeeding on the chemical content, as well as CHO and its metabolism, in the muscle. Again, the effects appeared to be similar between juveniles and adults. Among the nutrient contents in the muscle, refeeding resulted in increased muscular TAG levels, and the effects of the HC/LP diet was superior to that of the LC/HP diet. These findings indicate that short-term HC/LP refeeding induces lipogenesis. Similarly, fasting and subsequent refeeding induced muscular lipid accumulation in Nile tilapia (Tian et al., 2013). At the molecular level, refeeding modulated gene expression with stronger effects observed in fish refed the HC/LP diet, including 1) the upregulation of *glut4* and several glycolytic genes in the muscle (except for *hk2* and *pfkmb* in juveniles and *pfkma* in adults) and 2) downregulation of genes related to amino acid catabolism. Overall, these findings suggest that short-term refeeding modulates glucose metabolism, including the induction of lipogenesis, glucose transport, and glycolysis, as well as the reduction of amino acid catabolism, and these effects were stronger in the HC/LP diet. Our results were similar to those observed in Nile tilapia and grass carp fed a long-term high-CHO diet (Boonanuntanasarn et al., 2018a, 2018b; Gaye-Siessegger et al., 2006; Cai et al., 2018; Xiong et al., 2014). Overall, the effects of short-term refeeding with a high- CHO diet appeared to only resemble the CHO metabolic response in Nile tilapia fed a long-term high- CHO diet, indicating that tilapia is highly susceptible to dietary CHO utilisation.

3.6 Conclusion

In conclusion, four days of fasting and subsequent refeeding could compensate for body weight loss in the juvenile and adult stages. These findings suggest that refeeding with a high-CHO diet induced glucose metabolism which led to the induction of glycolysis and lipogenesis as well as the suppression of gluconeogenesis and amino acid catabolism. The effects of short-term CHO refeeding diets resembled that of long-

term CHO refeeding diets, suggesting that Nile tilapia is a high responder to dietary CHO utilisation.

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CHAPTER IV

SHORT-TERM REFEEDING WITH DIFFERENT LEVELS OF DIETARY CARBOHYDRATE MODULATES EPIGENETIC MODIFICATIONS IN JUVENILE AND ADULT NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

4.1 Abstract

The Nile tilapia (*Oreochromis niloticus*) exhibits a strong metabolic response to dietary carbohydrates (CHO). Short-term refeeding after fasting with a high-carbohydrate (HC) diet has been shown to modulate CHO metabolism, but the role of epigenetic regulation in this response remains unclear. This study investigated how short-term refeeding with either a HC [639.2 g kg^{-1} diet]/low-protein [164.9 g kg^{-1} diet] (HC/LP) diet or a low-CHO [47.4 g kg^{-1} diet]/high-protein [607.9 g kg^{-1} diet] (LC/HP) diet influences global DNA methylation and demethylation, histone modifications, and mRNA levels of epigenetic regulators in the liver and muscle of juvenile and adult Nile tilapia. Following a 4-day fasting period, fish were refed for 4 days with either HC/LP or LC/HP diets. Compared to the fasted state, refeeding with either diet altered epigenetic markers by: (1) decreasing hepatic global DNA 5-mC oxidative derivatives—5-hmdC in juveniles, and both 5-hmdC and 5-cadC in adults; (2) inducing histone hypermethylation and/or hyperacetylation—H3K9ac (hepatic) and H3K36me3 (muscular) in juveniles, and H3K9me3 and H3K9ac (muscular) in adults; and **(3)** promoting expression of enzymes related to DNA hypermethylation (upregulated *dnmt*, downregulated *tet*) and histone hypermethylation/acetylation (upregulated *setd1b, kmt2, suv39h1b*; downregulated *kdm4, sirt5*). Diet-specific effects included hepatic H3K36 hypomethylation and H3K9 hypoacetylation in juveniles fed HC/LP, accompanied by upregulation of *kdm4b, kdm4c*, and *sirt5*. In adults, HC/LP refeeding induced muscular DNA hypomethylation and H3K9 hypoacetylation, associated with upregulation of *tet, sirt2*, and *sirt5*. Refeeding following fasting induced histone hypermethylation and/or hyperacetylation, while HC refeeding was particularly

associated with muscular global DNA hypomethylation and histone hypoacetylation/ methylation.

Keywords: refeeding; carbohydrate; 5-methylcytosine; histone; *Oreochromis niloticus*

4.2 Introduction

Epigenetics refers to the study of how environmental or behavioural factors can modify gene activity without altering the underlying DNA sequence (Waddington, 1957). Among these factors, nutritional status plays a key role in regulating metabolism, and this regulation may be mediated by epigenetic mechanisms—particularly DNA methylation/demethylation and histone modifications—in animals (Marandel et al., 2016; Liu et al., 2022; Tamaoki et al., 2018; Xu et al., 2012; Jiménez-Chillarón et al., 2012). For example, in mammals (Gibson et al., 2020; Hjort et al., 2017), DNA methylation was influenced by the metabolic cofactors during nutritional status, which could impact the changes in the dynamic balances of DNA methylation modulators (both writers, DNA methyltransferase family DNMTs, and erasers, the Ten-eleven translocation (TET) enzyme family). In mice, time-restricted feeding has been shown to alter DNMT and TET expression at both protein and transcript levels in the brain and liver (Selvaraji et al., 2022; Hahn et al., 2017). Furthermore, nutritional status can establish a form of metabolic memory by reshaping the epigenetic landscape, in part by altering chromatin structure (Marandel et al., 2016). Histone modifications, such as methylation and acetylation, are key epigenetic mechanisms involved in chromatin remodelling and regulation of gene expression (Zhang et al., 2021). For example, in human cell cultures, glucose restriction induced changes in histone marks at gene promoter regions, leading to transcriptional alterations in target genes (Li et al., 2011). Moreover, in teleost fish, although histone modifications at gluconeogenic gene loci were not affected by nutritional status or dietary carbohydrate (CHO) in juvenile rainbow trout, global hepatic hypermethylation of H3K9 during refeeding and global hepatic hyperacetylation under no-CHO conditions were observed (Marandel et al., 2016). Additionally, activation of *pepck* mRNA expression was associated with upregulation of hepatic H3K4me3 at the *pepck* promoter region, which may have contributed to hyperglycemia and anorexia in mandarin fish fed CHO-rich diets (You et

al., 2020). These histone modifications, along with active DNA methylation mechanisms, are regulated by a variety of enzymes: histone methylation writers (histone lysine methyltransferases, KMTs), erasers (histone lysine demethylases, KDMs), acetylation writers (histone lysine acetyltransferases, KATs), and erasers (histone deacetylases or sirtuins, SIRTs).

Previous studies have demonstrated that specific dietary nutrients (e.g., refeeding, CHO, and/or protein) can influence the epigenetic landscape, affecting DNA methylation, histone modifications, and epigenetic modulators in both mammals (Rees et al., 2000), and fish (Marandel et al., 2016; Liu et al., 2022). In rainbow trout, refeeding after a period of fasting has been shown to alter the hepatic epigenetic landscape, including changes in histone modifications, DNA hypomethylation, and the transcript levels of associated epigenetic modulators (Marandel et al., 2016; Liu et al., 2022). This hypomethylation is thought to occur via the active DNA demethylation pathway, mediated by TET enzymes, which iteratively oxidize 5-methylcytosine (5-mC) to generate the oxidative derivatives 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC), followed by thymine DNA glycosylase (TDG)-dependent base excision repair or replication-dependent dilution (He et al., 2011; Ito et al., 2010; Tahiliani et al., 2009), ultimately restoring cytosine. Specifically, the high-CHO (HC) and low-protein (LP) diets increased hepatic levels of 5-hydroxymethyl-2'-deoxycytidine (5-hmdC), while either the LP or HC diet independently decreased levels of 5-methyl-2′-deoxycytidine (5-mdC), compared with the control diet (Liu et al., 2022). It is increasingly important to consider these oxidative intermediates, as accumulating evidence suggests that they are dynamic, may possess regulatory functions (Kellinger et al., 2012; Song et al., 2013; Sun et al., 2014; Wang et al., 2015) and might even represent stable DNA modifications (Bachman et al., 2015).

Rainbow trout, a carnivorous fish, is often considered a model of "poor utilization" of dietary CHO. Hepatic epigenetic remodelling induced by dietary CHO—both at the global level and at gluconeogenesis-related gene loci—has been suggested to play a key role in the nutritionally glucose-intolerant phenotype observed in this species (Marandel et al., 2016). In contrast, Nile tilapia (*Oreochromis niloticus*), an omnivorous freshwater species, is regarded as a "highly adaptable user" of dietary CHO, efficiently utilizing it as a primary energy source (Bachman et al., 2015). While this

metabolic flexibility has been well studied at physiological, biochemical, and transcriptional levels across different life stages (Bachman et al., 2015; Boonanuntanasarn et al., 2018a, 2018b; Kumkhong et al., 2020; Kumkhong et al., 2021; Srisakultiew et al., 2022) the underlying mechanisms—particularly at the epigenetic level—remain largely unexplored. Understanding how fasting and refeeding with varying dietary CHO/protein (CHO/CP) ratios affect the epigenetic landscape in a species known for effective CHO utilization is critical. First, it could reveal mechanisms that underpin Nile tilapia's efficient dietary CHO use. Second, such insights may support improvements in CHO utilization in other aquaculture-relevant species, such as salmonids. Therefore, the present study aims to investigate how fasting and subsequent refeeding with diets differing in CHO/CP ratios influence the epigenetic landscape in Nile tilapia, focusing on DNA methylation (including oxidative derivatives) and histone modifications known to be CHO-responsive (Marandel et al., 2016), as well as the expression of associated epigenetic modulators (e.g., mRNA levels of writer and eraser enzymes). Since environmental adaptability may be linked to physiological stage—as previously shown in trout (Callet et al., 2020)—this study focuses on two key life stages: juvenile and adult.

4.3 Materials and methods

4.3.1 Experimental design, diet, and fish culture

All experimental procedures involving fish were approved by the Ethics Committee of the Suranaree University of Technology Animal Care and Use Committee (Approval No. SUT-IACUC-001/2023). The samples in this manuscript were obtained from a previously published article by Thongchaitriwat et al. (2024). The experimental plan for fasting and subsequent refeeding, which was randomised with six replicates (tanks), is shown in Figure 4.1. Nile tilapia efficiently utilizes CHO as an economical energy source to support protein-sparing growth (Shiau and Peng, 1993). Therefore, HC/LP and LC/HP diets are commonly used to investigate the effects of dietary CHO levels while maintaining a fixed gross energy content in the diet. In this study, refeeding diets including HC/LP and LC/HP diet were formulated (Table 4.1). The experimental diets and their proximate compositions, including moisture, CP, CF, crude fibre, and

ash, which were analysed according to the standard method of the Association of Official Analytical Chemists (AOAC, 1990), are listed in Table 4.1.

The experiment was conducted with six replicates of the pond per condition (i.e., LC/HP and HC/LP refeeding fish). During the experimental trial, 60 juvenile fish (50–60 g) were randomly distributed in cement tanks (4 m^2, water depth: 0.8 m) under continuous aeration. For the second experiment, 34 adult fish (450–550 g) were randomly distributed in cement tanks (water depth: 0.8 m) under continuous aeration. A flow-through water change system was implemented by replacing one-third of the water in each tank weekly with dechlorinated water.

Before the experimental trial, fish were acclimatized to the experimental conditions for 14 days, and fish were hand fed twice per day (9.00 and 16.00) with a commercial diet (36% crude protein [CP] + 4% crude fat [CF]) administered at 3% of their body weight. Subsequently, fish were randomly divided into two groups according to the experimental refeeding diets, including LC/HP and HC/LP diets (n = 6 replicates; juvenile; 22 fish/replication, adult: 13 fish/replication). After acclimation, fish were fed-deprived for 4 days (fasted). During refeeding, fish were refed at 3% of their body weight with either the HC/LP or LC/HP diet for four days.

Throughout the experimental period, air and water temperatures were determined daily and ranged from 29.0–31.0°C and 27.0–28.0°C, respectively. Dissolved oxygen (DO) and pH were measured daily using a DO and pH meter, and their values were within acceptable ranges of 4.30–5.58 mg L-1 and 7.49–8.71, respectively. During the experimental period, fish deaths were recorded to determine their survival rates, and no mortality was observed.

Table 4.1 Ingredients and chemical composition (g kg^{-1}) of the commercial diet (used during acclimation) and the refeeding diets: low-carbohydrate/high-protein (LC/HP) and high-carbohydrate/low-protein (HC/LP).

Ingredients	Acclimation diet	Refeeding diets	
	Commercial diet	LC/HP	HC/LP
Fish meal	-	880	180
Rice flour	-	0	700
Fish oil	-	0	70
Soybean oil	-	20	0
Gelatin	-	80	0
Di-calcium phosphate	-	0	30
Fish premix[a]	-	20	20
	Proximate composition (g kg^{-1} dry weight)		
Dry matter	916.4	942.1	921.9
Protein	393.0	607.9	164.9
Fat	36.8	97.9	98.5
Fiber	77.7	5.2	4.6
Ash	137.0	241.6	92.9
NFE[b]	355.4	47.4	639.2
Gross energy (kJ g^{-1})	13.13	14.40	15.6

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein.

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

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

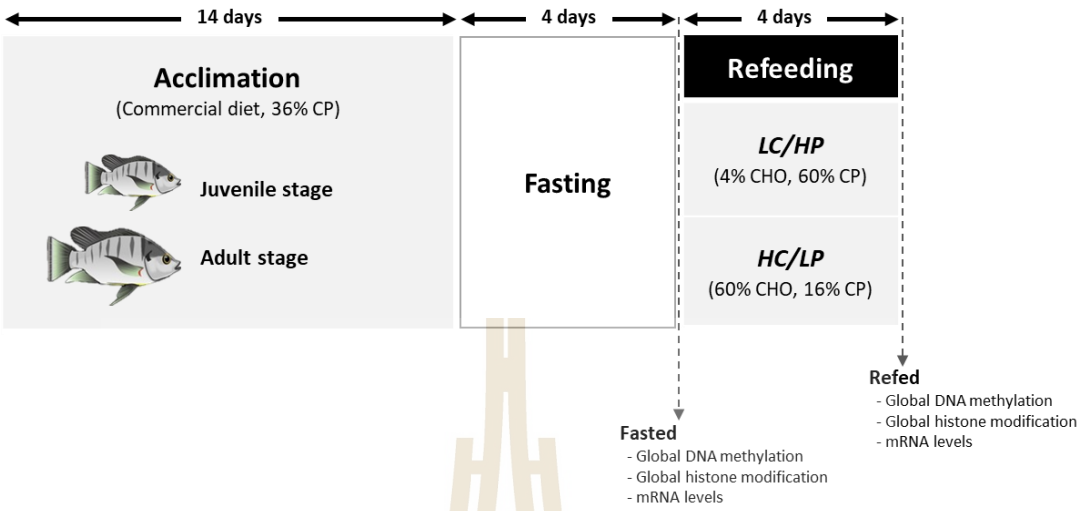

Figure 4.1 Experimental plan for fasting and refeeding juvenile and adult Nile tilapia. Fish were fed a commercial diet during the acclimation period. After fasting for 4 days, fish were subsequently refed for 4 days with either a low-carbohydrate/high-protein (LC/HP) or high-carbohydrate/low-protein (HC/LP) diets.

4.3.2 Fish sampling

Samples were collected after 4 days of fasting (fasted) and 4 days of refeeding periods (refed) (Figure 1). For refed conditions, fish were sampled based on the postprandial glycaemia curve of Nile tilapia determined in a previous study (Boonanuntanasarn et al., 2018b). At each sampling point, fish (n = 6 replicates; juvenile: 4 fish/replication, adult: 2 fish/replication) were euthanised using 0.2% clove oil. After fish bleeding, liver and epaxial muscle samples were collected, snap-frozen in liquid nitrogen, and stored at −80°C for analysis of global DNA demethylation, global histone modification, and mRNA levels of genes involved in epigenetic modifications.

4.3.3 Total RNA extraction, cDNA synthesis, and real-time RT-qPCR analysis of genes involved in epigenetic modifications

Expression of genes related to epigenetic modification was examined in the liver and muscle samples after four days of fasting (fasted), and four days of refeeding (refed) with either LC/HP or HC/LP diet (at each sampling point: two fish/replicate; n = 6 replicates). Total RNA was extracted from the liver (50 mg) and muscle (100 mg) samples using the TRIzol reagent (Catalogue number: 15596026,

Invitrogen, Carlsbad, CA, USA). The quantity of total RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher, Madison, WI, USA), and the integrity of the total RNA was verified by 1% agarose gel electrophoresis.

The primer sequences of gene-related epigenetic modulators and the reference gene (Yang et al., 2013) used for real-time RT-qPCR are listed in Table 4.2. To measure the mRNA levels of genes involved in epigenetic modification in liver and muscle tissue, quantitative real-time reverse-transcription polymerase chain reaction (real-time RT-qPCR) was performed. cDNA was synthesized from 1 µg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen, USA; cat. no. 18080044), RNaseOUT™ (Invitrogen, USA; cat. no. 10777019), dNTP mix (Promega, France; cat. no. U1511), and random primers (Promega, France; cat. no. C1181), following the manufacturer's protocol. Reverse transcription (duplicate for each sample) was performed, and each PCR assay (duplicate for each PCR reaction) was performed to analyse the mRNA level. Both hepatic and muscular tissues were examined for DNA methylation-related genes, including DNA methyltransferase type 1 (*dnmt1*) and DNA methyltransferase type 3 family members (*dnmt3aa*, *dnmt3ab*, *dnmt3ba*, and *dnmt3bb*). To evaluate the expressions of ten-eleven translocation (*tet1, tet2,* and *tet3*) involved in the deoxygenation of methyl-cytosine was determined. To investigate the regulation of histone modification of H3K4me3 writer, the genes that regulate histone lysine methyltransferase, including histone-lysine N-methyltransferase SETD1A (*setd1a*), histone-lysine N-methyltransferase SETD1B-A (*setd1ba*), lysine methyltransferase 2A (*kmt2a*), and histone-lysine N-methyltransferase 2B (*kmt2ba* and *kmt2bb*), were determined. Likewise, genes involved in the H3K4me3 eraser, including histone lysine demethylase 5 (*kdm5a, kdm5ba, kdm5bb,* and *kdm5c*) and bifunctional lysine-specific demethylase and histidyl hydroxylase (*riox1*), were also identified. In addition, H3K9me3 specific writer (histone lysine methyltransferase; *suv39h1b*) and H3K9me3 and H3K36me3 specific eraser (histone lysine demethylases 4; *kdm4aa, kdm4ab, kdm4b* and *kdm4c*) were investigated. The expression of SET domain-containing 2, a histone lysine methyltransferase (*setd2*) gene, involved in H3K36me3-specific writer, was determined. Moreover, the expression of genes involved in H3K9ac-specific writer (histone lysine acetyltransferase; *kat2a, kat2b, kat6a*, and general transcription factor IIIC subunit 4; *gtf3c4*) and H3K9ac-specific eraser (sirtuin; *sirt2* and *sirt5*) was detected. For the analysis of mRNA expression, the relative quantification of target gene expression was performed using the Roche Applied Science E-Method, as described by Pfaffl

(2001). The relative mRNA level of elongation factor 1 alpha (*ef1α*) was used for normalising the measured mRNA in each tissue, as its relative expression did not change significantly over the sampling process (data not shown). In all cases, PCR efficiency was measured from the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.0.

Table 4.2 List of primers used for RT-qPCR.

Genes		5'/3' Forward primer	5'/3' Reverse primer	SIZE (bps)	Accession number
Reference gene	*ef1**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	250	AB075952
DNA methylation writers	*dnmt1*	CTCACACTGCGCTGTCTTGT	ACAACGCTGAGAGAGCAAGC	188	XM_025906327.1
	dnmt3aa	CCAACAACCACGAGCAGGAA	TGCCGACAGTGATGGAGTCT	192	XM_005475084.4
	dnmt3ab	GCCGCAGCTTAGAGGACATC	CACACATGAGCACCTCTCGTC	189	XM_005477258.3
	dnmt3ba	GCTGCTGCAGATGCTACTGT	TTGCGCTGTTGTTGGCAAAG	186	XM_025901732.1
	dnmt3bb	TGCAGGAGTTCTTCGCCAAC	TGCCACATACTGACCCACCT	173	XM_025901790.1
DNA methylation eraser	*tet1*	CATCCAGTCCCAGCACAACC	CTCTATTTGGCGTGCGCTGA	194	XM_025897345.1
	tet2	GCAGCTGCCAACAAGAATGC	TGTTGCTGCTGCTGATGGAC	191	XM_005457001.3
	tet3	GCAAGCCAACCAACCAAACC	GATGTGTTGGCTCCGACCTG	177	XM_019365521.2
H3K4me3 writer (Histone Lysine methyltransferase)	*setd1a*	GGAACTCCGGTCTGGATGGT	CGAAGCTGCCCATCTGTGTT	172	XM_005468973.4
	setd1ba	AAGACAGGGAGGCAGCAGAA	CCTCAGGACTGGGAGGTCTG	198	XM_005470275.4
	kmt2a	AGAGCAGGAAAGCCAACAGC	CACTGGGCGTAGTTGTGGTC	178	XM_013274782.3
	kmt2ba	ACTCTGAGGGACCTGGAGGA	AGAGGAGGTGAAGCCGATCC	191	XM_013275905.3
	kmt2bb	GCTCCCGTCAGTGTGTCTTC	TCTGGCTCCAACCCAGTCAA	172	XM_013277028.3
H3K4me3 eraser (Histone lysine demethylases)	*kdm5a*	TCTGGCCACAGAGGAGTTGT	GTGACGTGGCTCTGCTGAAA	191	XM_005451728.4
	kdm5ba	TCTCAGAGCAGAGGGCATCC	GACCCGATGTCACACCTTGG	165	XM_003441348.2
	kdm5bb	CATCCCTGCCTACCTCCCAA	AAGGCTCCAGGTGGACTTGA	170	XM_003439103.5
	kdm5c	CTCTCCACCCTGGAGGCAAT	AGCTACCAGGCCCTCCAAAT	174	XM_005448517.4
	riox1	CCACCTGGCACACAAGGATT	TCCGGCTTCTACCACCACAT	192	XM_005475002.4
H3K9me3 specific writer	*suv39h1b*	TCCAACGCATGGCCTACAAC	CTTGATGTGCTGCAGTGTGC	197	XM_003459875.5
H3K9me3 and H3K36me3 eraser	*kdm4aa*	CGGATGCGAACCAAACCTCT	GGCTGGATCGACACCGTAAC	180	XM_005457300.3
	kdm4ab	TCTGTTCAGGGAGGCACACA	GCCTGTTGGCCCATCTGTTT	162	XM_005476068.4
	kdm4b	TGCTCGCTCTTCTGTCCGTA	AGCAGATCAGGAGGCTGGTT	196	XM_005453970.4
	kdm4c	CCTGCAGAGGAATGCAGTGG	GCACAGGTGCAATCTGGTGA	176	XM_005456806.2
H3K36me3 specific writer	*setd2*	AGGCAGCGATGACTTCAAGC	ATCTTGTGGCGTCCCACTCT	182	XM_019364854.2
H3K9ac specific writer	*kat2a*	CACTGACCCTGCTGCTATGC	GTAGGCCAACCAGCCACATC	173	XM_025906390.1
	kat2b	GGCCTTTCATGGAGCCTGTG	CTCGCTCTCTGGAGGGTTGT	188	XM_003444058.3
	kat6a	CATCCCGTCCACTGCTTTCC	CCTGTTCACGCTACCACCAC	173	XM_005472980.3
	gtf3c4	CTTGTGGCGGTTCAAGCTCT	GGCTCGCCTTCCTCTTTCAC	174	XM_003440231.5
H3K9ac specific eraser	*sirt2*	GCGAGTCTAGTCAGCAGGGT	CCCAGAAGATCAGCTAGAGCCA	197	XM_003449264.5
	sirt5	ATTTGCCCAGGTGTGAGCAG	GAGCAAACATGGCTGCAGGA	177	XM_003457306.5

* From Yang et al. (2013).

4.3.4 Global DNA methylation and its demethylation derivatives by HPLC-UV

4.3.4.1 Non oxidant DNA extraction

Ten micrograms of tissue were added to 800 µL of 1 M Guanidine Thiocyanate (GTH) buffer and 10 µL of 0.25 mg/ml Proteinase K was added. Then, add the antioxidants, including 8 µL of 0.1 mM deferoxamine, 32 µL of 16 mM histidine and 8 µL of 3 mM glutathione. Samples were incubated in a dry bath at 57°C for 1 hour and vortexed every 20 min. After incubation, samples were added 800 µL of Chloroform-alcohol isoamyl (24:1) and an antioxidant solution. Mixed samples were rotated on the rotating wheel for 15 minutes at room temperature. Then, samples were centrifuged at 10,000 rpm for 15 mins at room temperature. Then, 300 µL of the aqueous phase from the samples was collected and transferred to a new tube. Then, samples were added with 75 µL of 5 M NaCl and 937 µL of absolute ethanol. Samples were mixed and incubated at -20°C for 15 minutes to precipitate DNA fragments. After incubation, samples were centrifuged at 4°C and 10,000 rpm for 15 minutes. After that, the ethanol was discarded, and the DNA pellet was washed with 1 mL of 70% ethanol. Then, the samples were centrifuged at 4°C and 10,000 rpm for 10 minutes. DNA samples were discarded with 70% ethanol by pipetting, and then the samples were dried in a dry bath at 45°C for 10 minutes or until the pellet was completely dry. DNA samples were cooled for 5 minutes before adding 150 µL of distilled water to dissolve the pellet, as described in the publication by Liu et al. (2022). Then, DNA samples were treated with RNase cocktail (Invitrogen) to remove RNA contamination. The quantity of DNA was measured using a NanoDrop spectrophotometer (Thermo Fisher, Madison, WI, USA).

4.3.4.2 DNA hydrolysis

One microgram of DNA was degraded into single nucleosides using the DNA Degradase PlusTM kit (ZYMO RESEARCH) according to the manufacturer's procedures. The DNA samples were prepared for mixture, including 1 µg of DNA (measured by Nanodrop), 2.5 µL 10X DNA Degradase PlusTM Reaction Buffer, 1 µL of DNA Degradase PlusTM (5 units/µL), and ultrapure H2O to yield a total volume of 25 µL. The reaction will be mixed and incubated at 37°C for 2 hours, followed by heat inactivation at 70°C for 20 minutes, as described in the publication by Liu et al. (2022).

4.3.4.3 High-performance liquid chromatography-Ultraviolet (HPLC-UV)

Nucleosides were separated, detected, and quantified using an HPLC-UV technique (Alliance, Waters Corporation). The separation was performed using a Luna C8, 5 µm, 150 × 4.6 mm, 100 Å LC column (Phenomenex). The compositions of the mobile phase will include solvent A, 10 mM potassium phosphate buffer, pH 3.7, and solvent B, 100% methanol. Linear gradient elution will be as follows: 0–8.5 min, 98% A; 8.5–11.8 min, 97% A; 11.8–18.9 min, 73% A; 18.9–21.2 min, 65% A. The temperature of the column oven was set at 30°C. The wavelength of UV detection will be 277 nm. The standards of 2 2'-deoxycytidine (dC), 5-methyl-2'-deoxycytidine (5-mdC), 5-hydroxymethyl-2'-deoxycytidine (5-hmdC), 5-formyl-2'-deoxycytidine (5-fdC) and 5-carboethoxy-2'-deoxycytidine (5-cadC) will be made using products from Berry & Associates Inc. Injection was done with either 20 µL of hydrolysed samples or standards. The identification of nucleosides will be based on retention times, as reported by Liu et al. (2022).

4.3.4.4 Calculation of global 5-mdC and its other demethylation derivatives

The global level of dC, 5-mdC, 5-hmdC, 5-fdC and 5-cadC was calculated as a percentage of each molar quantity divided by the total of molar quantities of all detected cytidine forms. Take 5-mdC as an example, the percentage of 5-mdC was calculated using following equations: 5-mdC% = 100 x $Q_{5\text{-mdC}}$ / (Q_{dC} + $Q_{5\text{-mdC}}$ + $Q_{5\text{-hmdC}}$ + $Q_{5\text{-fdC}}$ + $Q_{5\text{-cadC}}$), and Q_{dC}, $Q_{5\text{-mdC}}$, $Q_{5\text{-hmdC}}$, $Q_{5\text{-fdC}}$, $Q_{5\text{-cadC}}$ values are the molar quantities of dC, 5-mdC, 5-hmdC, 5-fdC and 5-cadC, respectively.

4.3.5 Global Histone modifications

4.3.5.1 Histone protein extraction

One hundred micrograms of tissue were added 1,000 µl of Triton extraction buffer (TEB included PBS containing 0.5% Triton X-100, 5 mM sodium butyrate (NaBu) and Protease inhibitors) and use four beads of 2.8 mm ceramic beads and homogenization with bead beater (following the protocol liver: 5,000 rpm, 2x10 s, 15 s break, muscle: 5,500 rpm, 2x20 s, 15 s break). After that, samples were incubated on ice for 20 minutes. The homogenised mixture was transferred to a new tube and centrifuged at 2,000 rpm, 4°C, for 10 minutes. Thereafter, the supernatants were discarded, and the

pellet was resuspended in acid extraction buffer (AEB, which included 0.5 N HCl and 10% glycerol). Then, samples were incubated on ice for 30 minutes and vortexed briefly every 10 minutes. After incubation, the samples were centrifuged at 12,000 rpm for 5 min at 4°C. Next, the supernatant was transferred to a new tube, and subsequently, three volumes of cold acetone were added for protein precipitation at –20°C overnight. After protein precipitation, the protein pellets were collected via centrifugation at 12,000 rpm for 5 min at 4°C, and the samples were aspirated. Protein samples were washed with 1 ml of cold acetone, and the protein pellets were dried on ice. Protein samples were dissolved in distilled water and then warmed at 45–60°C for 1 hour to dissolve the pellets completely. The samples were aliquoted for protein quantification and stored at –80°C for further use.

4.3.5.2 Sample preparation

Samples were prepared with 5 µg of protein and mixed with 1X loading solution, together with 2-mercaptoethanol (BME). Samples were mixed and heated on a dry bath at 95–98°C for 5 minutes. Then, the samples were aliquoted and kept at –20°C for western blotting assays.

4.3.5.3 Western blotting and histone antibodies

Five micrograms of total protein were subjected to SDS-PAGE and western blotting using the specific antibodies on 15% gel (40% acrylamide, 2% bis-acrylamide, 1.5 M Tris-HCl pH 8.8, 10% SDS) for 100 min at 100 V and then 75 min at 100 mA, according to the publication of Marandel et al. (2016). Specific histone antibodies were used for Western blotting analysis. Prior to analysis, appropriate amounts of protein samples from muscle and liver, as well as antibody dilutions, were optimised and validated. Primary antibodies were used at a 1:1,000 dilution in Intercept™ (PBS) Blocking Buffer (LICORbio, USA; cat. no. 927-70001): anti-H3K4me3 (polyclonal; Diagenode, Belgium; cat. no. C15410003), anti-H3K9me3 (polyclonal; Diagenode, Belgium; cat. no. C15410056), anti-H3K9ac (polyclonal; Diagenode, Belgium; cat. no. C15410177), and anti-H3 (polyclonal; Abcam, UK; cat. no. ab1791). The secondary antibody, goat anti-rabbit IgG H&L (HRP) (polyclonal; Abcam, UK; cat. no. ab205718), was used at a 1:10,000 dilution in the same blocking buffer.

4.3.6 Statistical analysis

The statistical model used was $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where y_{ij} was the response, μ was the general mean, α_i was the effect of nutritive status (fed and fasted,

and LC/HP and HC/LP refed), and ε_{ij} was the random error. All data were analysed using SPSS for Windows, version 22 (SPSS Inc., Chicago, IL, USA). Normality of distributions was assessed using the Shapiro–Wilk test. Data were analysed using a Kruskal–Wallis non-parametric test, followed by a Tukey test as a post hoc analysis when data did not follow a normal distribution. In addition, if the data followed a normal distribution, a one-way analysis of variance (ANOVA) was performed. When significant differences were observed, Tukey's range test was performed to rank the treatment groups. Effects and differences were considered significant at $P < 0.05$.

4.4 Results

4.4.1 Effects of short-term refeeding with dietary low-or high-CHO on global DNA methylation landscape in juvenile and adult Nile tilapia

Figure 4.2 shows the modification of global DNA (de)methylation derivatives including 5-mdC, 5-hmdC, 5-fdC, 5-cadC, and dC in the liver and muscle of experimental fish. In juvenile fish, irrespective of dietary refeeding, decreased hepatic global 5-hmdC content compared to fasted fish ($P < 0.05$). However, there were no significant differences in global 5-mdC, 5-fdC, 5-cadC, and dC in the liver of juveniles between fasted and refed states ($P > 0.05$) (Figure 4.2A). In muscle, although muscular 5-mdC content between fasted and refed states in juveniles remains unchanged, the elevation of muscular 5-fdC derivative content was observed in juvenile refed with dietary HC/LP compared to LC/HP groups ($P < 0.05$) (Figure 4.2C).

In adults, the modification of global DNA (de)methylation derivatives in the liver and muscle is illustrated in Figures 4.2B and 4.2D, respectively. Compared to fasting, subsequently refeeding with either LC/HP or HC/LP diets decreased hepatic 5-hmdC content ($P < 0.05$), while a decrease of 5-cadC content was observed in only adult refed with HC/LP diet ($P < 0.05$). There were no significant differences in hepatic 5-mdC, 5-fdC, and dC contents between fasted and refed adult Nile tilapia ($P > 0.05$) (Figure 4.2B). In adult muscle, compared to fasted, although 5-mdC content was not affected by short-term refeeding ($P > 0.05$), a higher muscular 5-cadC content was observed in adult refed with either LC/HP or HC/LP diets ($P < 0.05$) (Figure 4.2D). Compared to fasted, dietary LC/HP increased muscular 5-hmdC and decreased dC contents ($P < 0.05$), while the muscular (de)methylation derivatives (except 5-

cadC) between fasted and HC/LP refeeding groups remain unchanged ($P > 0.05$). Between refed diets, dietary HC/LP decreased in muscular 5-mdC, while dC contents increased compared to adults refed with an LC/HP diet ($P < 0.05$). There were no significant differences in 5-hmdC, 5-fdC, and 5-cadC contents between LC/HP and HC/LP diets ($P > 0.05$).

4.4.2 Effects of short-term refeeding with dietary low- or high-CHO on expression of genes related to DNA (de)methylation in juvenile and adult Nile tilapia

This study evaluated hepatic mRNA levels of genes related to DNA (de)methylation in fasted juvenile and adult Nile tilapia (Table 4.3). Juveniles, irrespective of dietary refeeding, exhibited higher mRNA level of the hepatic *dnmt3bb* gene compared to fasted fish ($P < 0.05$). Except for *dnmt1* in LC/HP, short-term refeeding with the HC/LP diet showed the highest expression levels of hepatic DNA methylation writers (*dnmt1*, *dnmt3aa*, and *dnmt3ba*) compared to the fasted group ($P < 0.05$). For the DNA methylation eraser, the mRNA levels of the *tet1* and *tet2* genes was higher in fasted juveniles compared to refed fish ($P < 0.05$). Moreover, the mRNA level of *tet3* was significantly higher in fasted and HC/LP-refed fish compared with fish refed with the LC/HP diet ($P < 0.05$).

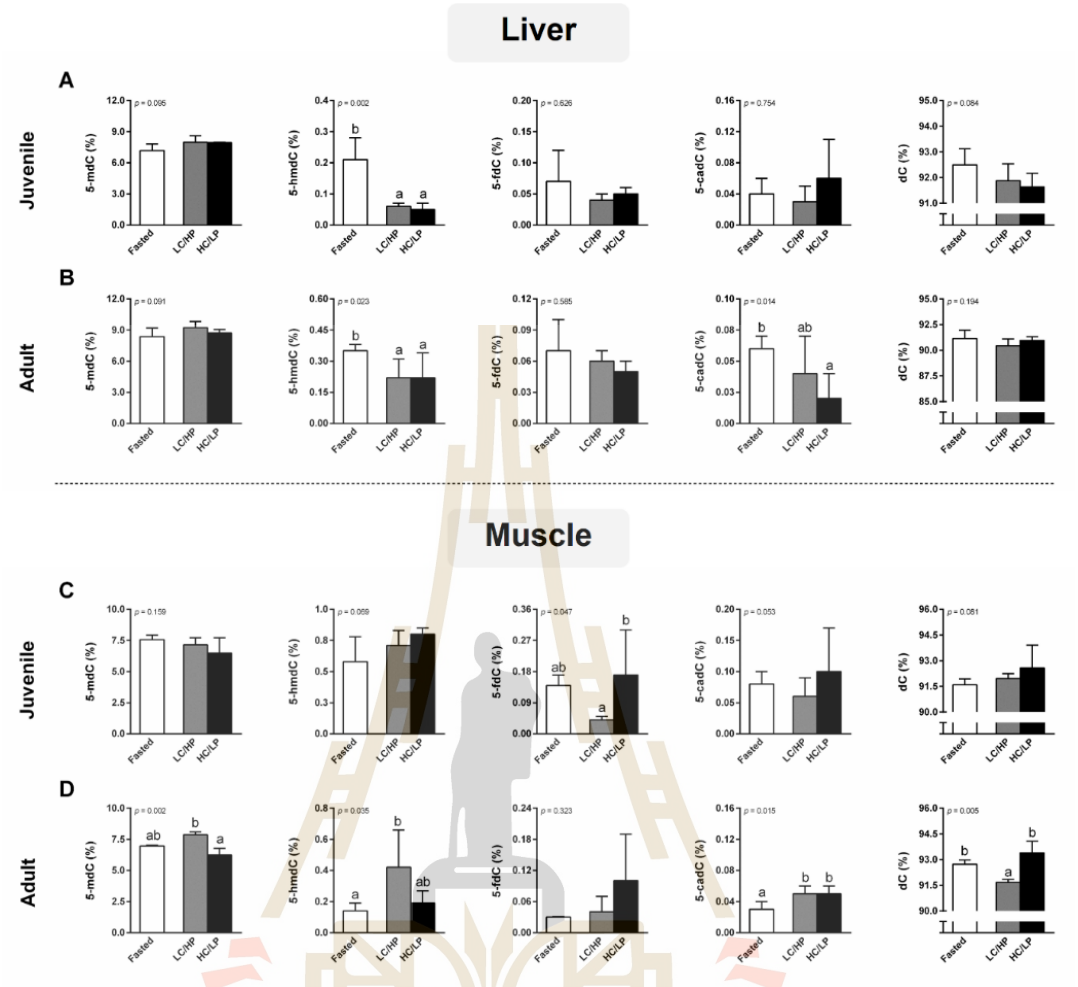

Figure 4.2 Global DNA methylation and their demethylation derivatives concentrations in liver of juvenile (A) and adult (B), and in muscle of juvenile (C) and adult (D) Nile tilapia. Fish were subjected to fasting for 4 days fast (fasted), followed by 4 days of refeeding (refed) with either a low-carbohydrate/high-protein (LC/HP) or high-carbohydrate/low-protein (HC/LP) diets. Data represent means ± standard deviation (SD; n = 6 individuals per experimental group). Different lowercase letters indicate significant differences among nutritional status ($P < 0.05$). 5-mdC, 5-methyl-2' -deoxycytidine; 5-hmdC, 5-hydroxymethyl-2' -deoxycytidine; 5-fdC, 5-formyl-2' -deoxycytidine; 5-cadC, 5-carboethoxy-2' -deoxycytidine; dC, 2'-deoxycytidine.

In adults, the regulation of genes related to DNA (de)methylation was affected by dietary LC/HP and HC/LP independently (Table 4.3). Compared to fasted, mRNA

levels of *the dnmt1* and *dnmt3aa* genes was lower only in fish refed with the LC/HP diet ($P < 0.05$). However, the mRNA level of *dnmt3ba* was higher in refeeding fish, whereas the mRNA level of the *dnmt3bb* gene was significantly higher in HC/LP refeeding fish compared to fasted ones ($P < 0.05$). When examining the diets, the mRNA level of *dnmt3ba* was higher in HC/LP-fed fish compared to the LC/HP group ($P < 0.05$). For the hepatic DNA methylation eraser, the mRNA level of *tet3* in adults was higher in fasted fish compared to refed ones ($P < 0.05$). However, compared to fasted, adults refed with the LC/HP diet showed significantly lower mRNA levels of the *tet1* and *tet2* genes ($P < 0.05$). After feed deprivation, the mRNA level of the *tet3* gene was significantly higher in HC/LP refeeding compared to LC/HP fish ($P < 0.05$). No significant difference was found in the mRNA levels of *dnmt1, dnmt3aa, dnmt3bb, tet1,* and *tet2* genes between HC/LP and LC/HP refeeding groups ($P > 0.05$).

At the molecular level in muscle tissue (Table 4.4), juvenile tilapia refed with either LC/HP or HC/LP diets exhibited significantly lower mRNA levels of DNA methylation writers (*dnmt1* and *dnmt3ba*) and erasers (*tet2* and *tet3*) compared to fasted fish ($P < 0.05$). In contrast, refeeding resulted in significantly higher mRNA levels of *dnmt3aa* and *dnmt3bb* relative to the fasted group ($P < 0.05$). Between the two refeeding diets, juveniles refed with the HC/LP diet showed significantly higher mRNA levels of DNA methylation writer genes (*dnmt1*, *dnmt3aa*, and *dnmt3bb*) and the eraser gene *tet3* compared to those refed with LC/HP ($P < 0.05$). No significant differences in *tet1* expression were observed among the fasted, LC/HP, and HC/LP groups ($P > 0.05$).

In adults, the alteration of genes related to muscular DNA (de)methylation was affected by dietary LC/HP and HC/LP independently (Table 4.4). Compared to the fasted state, mRNA levels of DNA methylation writer genes (*dnmt3aa* and *dnmt3bb*) was higher in fish refed with either LC/HP or HC/LP diets ($P < 0.05$). However, compared to fasted adults, those refed with the LC/HP diet showed significantly lower mRNA levels of *dnmt3ba, tet2,* and *tet3.* In contrast, the mRNA levels of *dnmt1* and *tet1* were significantly higher in adults refed with the HC/LP diet ($P < 0.05$). Between refeeding diets, the lower mRNA level of *dnmt3aa* and, together with higher mRNA levels of *dnmt3ba, dnmt33bb, tet1, tet2*, and *tet3* genes, were found in adult refed with HC/LP compared to the LC/HP refeeding group ($P < 0.05$).

Table 4.3 mRNA levels of genes involved in DNA (de)methylation in the liver of juvenile and adult Nile tilapia fasted for 4 days and refed for 4 days with either an LC/HP or an HC/LP diet (mean ± standard deviation [SD], n = 6).

Genes involved in		Juvenile stage				Adult stage			
Epigenetics modification		Fasted	LC/HP	HC/LP	*P*-value	Fasted	LC/HP	HC/LP	*P*-value
DNA methylation writers	*dnmt1*	0.40 ± 0.00^{a}	0.98 ± 0.03^{ab}	1.75 ± 0.09^{b}	0.001	1.78 ± 0.38^{b}	0.30 ± 0.02^{a}	1.31 ± 0.11^{ab}	0.002
	dnmt3aa	0.52 ± 0.05^{a}	0.62 ± 0.10^{a}	0.84 ± 0.08^{b}	<0.001	1.31 ± 0.16^{b}	0.38 ± 0.04^{a}	0.69 ± 0.03^{ab}	0.001
	dnmt3ab	nd	nd	nd		nd	nd	nd	
	dnmt3ba	0.22 ± 0.06^{a}	0.13 ± 0.01^{a}	0.67 ± 0.12^{b}	0.001	0.32 ± 0.05^{a}	0.55 ± 0.08^{b}	0.89 ± 0.16^{c}	<0.001
	dnmt3bb	0.21 ± 0.02^{a}	0.50 ± 0.08^{b}	0.67 ± 0.12^{b}	0.001	0.33 ± 0.05^{a}	0.58 ± 0.04^{ab}	0.90 ± 0.16^{b}	0.001
DNA methylation eraser	*tet1*	0.69 ± 0.15^{b}	0.35 ± 0.08^{a}	0.43 ± 0.12^{a}	0.001	1.16 ± 0.17^{b}	0.21 ± 0.03^{a}	0.31 ± 0.04^{ab}	0.001
	tet2	0.70 ± 0.10^{b}	0.42 ± 0.06^{a}	0.52 ± 0.14^{a}	0.001	1.58 ± 0.45^{b}	0.30 ± 0.09^{a}	0.70 ± 0.02^{ab}	0.001
	tet3	0.77 ± 0.01^{b}	0.50 ± 0.06^{a}	0.67 ± 0.10^{b}	<0.001	1.20 ± 0.15^{c}	0.45 ± 0.13^{a}	0.73 ± 0.18^{b}	<0.001

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein.

Means with different superscripts in each row differ significantly (P < 0.05).

Table 4.4 mRNA levels of gene involved in DNA (de)methylation in muscle of juvenile and adult Nile tilapia that were fasted 4 days and refed 4 days with either an LC/HP or an HC/LP diet (mean ± standard deviation [SD], n = 6).

Genes involved in		Juvenile stage				Adult stage			
Epigenetics modification		Fasted	LC/HP	HC/LP	*P*-value	Fasted	LC/HP	HC/LP	*P*-value
DNA methylation writers	*dnmt1*	2.11 ± 0.12^{c}	1.44 ± 0.08^{a}	1.75 ± 0.06^{b}	<0.001	1.01 ± 0.07^{a}	1.21 ± 0.05^{ab}	1.82 ± 0.28^{b}	0.001
	dnmt3aa	0.84 ± 0.03^{a}	1.29 ± 0.04^{b}	1.54 ± 0.03^{c}	<0.001	0.95 ± 0.02^{a}	1.39 ± 0.02^{c}	0.99 ± 0.04^{b}	<0.001
	dnmt3ab	nd	nd	nd		nd	nd	nd	
	dnmt3ba	1.87 ± 0.08^{b}	1.05 ± 0.12^{a}	1.17 ± 0.22^{a}	<0.001	1.13 ± 0.19^{b}	0.84 ± 0.13^{a}	1.12 ± 0.11^{b}	0.006
	dnmt3bb	0.83 ± 0.10^{a}	1.45 ± 0.04^{b}	1.79 ± 0.12^{c}	<0.001	0.60 ± 0.06^{a}	0.82 ± 0.03^{b}	1.28 ± 0.07^{c}	<0.001
DNA methylation eraser	*tet1*	1.39 ± 0.07	1.38 ± 0.05	1.45 ± 0.12	0.272	1.05 ± 0.01^{a}	1.00 ± 0.04^{a}	1.22 ± 0.04^{b}	<0.001
	tet2	1.59 ± 0.08^{b}	0.86 ± 0.02^{a}	0.98 ± 0.06^{a}	0.001	1.33 ± 0.04^{b}	0.76 ± 0.04^{a}	1.04 ± 0.09^{b}	0.001
	tet3	1.46 ± 0.05^{c}	0.91 ± 0.02^{a}	1.15 ± 0.03^{b}	<0.001	1.08 ± 0.05^{b}	0.76 ± 0.04^{a}	1.26 ± 0.13^{b}	0.001

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein.

Means with different superscripts in each row differ significantly ($P < 0.05$).

4.4.3 Effects of short-term refeeding with dietary low- or high-CHO on global histone modification landscape in juvenile and adult Nile tilapia

Global levels of selected histone modifications (H3K4me3, H3K9me3, H3K36me3, and H3K9ac) were measured in both liver and muscle of Nile tilapia (Figures 4.3 and 4.4). In the liver, compared to fasted, a higher and lower enrichment of global hepatic H3K9ac was detected in juveniles refed with LC/HP and HC/LP diets, respectively ($P < 0.05$) (Figure 4.3A). Between dietary refeeding conditions, a lower enrichment of global H3K36me3 and H3K9ac was exhibited in the liver of juveniles refed with HC/LP diet ($P < 0.05$). There were no significant differences in H3K4me3 and H3K9me3 in juvenile Nile tilapia among experimental conditions ($P > 0.05$). In adults, the global levels of hepatic H3K4me3, H3K9me3, H3K36me3, and H3K9ac remained stable between fasted and refed conditions ($P > 0.05$) (Figure 4.3B).

Compared to fasting, refeeding with either LC/HP and/or HC/LP modulated several histone marks (Figure 4.4). In juveniles, short-term refeeding with dietary HC/LP resulted in higher global enrichment of muscular H3K36me3 compared to fasted and refed groups with LC/HP ($P < 0.05$). There were no significant differences in muscular H3K4me3, H3K9me3, and H3K9ac in juveniles between fasted and refed groups ($P > 0.05$) (Figure 4.4A). In adults, compared to the fasted state, higher enrichment in muscular H3K9me3 and H3K9ac was exhibited in the LC/HP refeeding group ($P < 0.05$). Indeed, global enrichment of muscular H3K9ac was increased in adults refed with dietary LC/HP compared to the HC/LP diet ($P < 0.05$). There were no significant differences in muscular H3K4me3 and H3K36me3 in adults between fasted and refed conditions ($P > 0.05$) (Figure 4.4B).

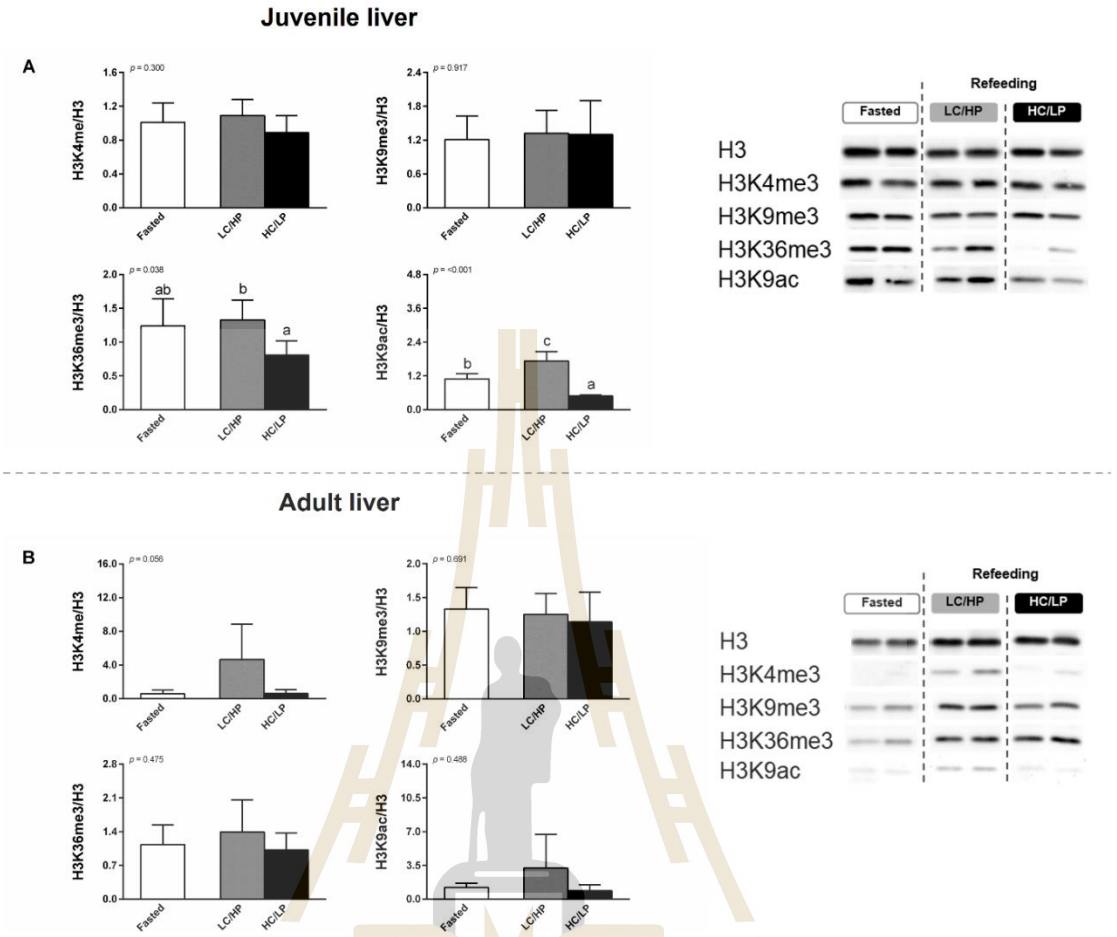

Figure 4.3 Global histone modification in liver of juvenile (A) and adult (B) Nile tilapia. Fish were subjected to fasting for 4 days (fasted), followed by refeeding for 4-days (refed) with either a low-carbohydrate/high-protein (LC/HP) or high-carbohydrate/low-protein (HC/LP) diets. Data represent means ± standard deviation (SD; n = 6 individuals per experimental group). Different lowercase letters indicate significant differences among nutritional status ($P < 0.05$).

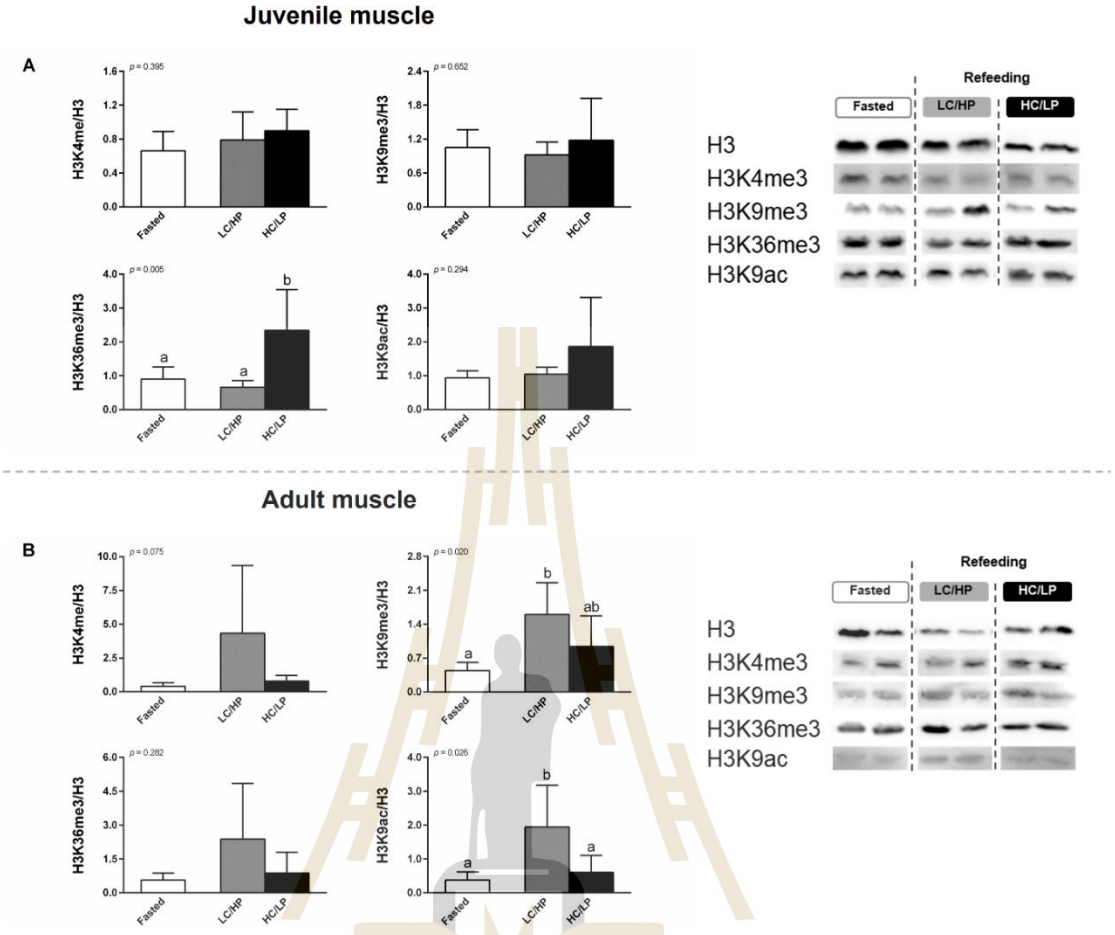

Figure 4.4 Global histone modification in muscle of juvenile (A) and adult (B) Nile tilapia. Fish were subjected to fasting for 4 days (fasted), followed by refeeding for 4 days (refed) with either a low-carbohydrate/high-protein (LC/HP) or high-carbohydrate/low-protein (HC/LP) diets. Data represent means ± standard deviation (SD; n = 6 individuals per experimental group). Different lowercase letters indicate significant differences among nutritional status ($P < 0.05$).

4.4.4 Effects of short-term refeeding with dietary low- or high-CHO on expression of genes related to histone modification in juvenile and adult Nile tilapia

After fasting, subsequent refeeding affects hepatic mRNA levels of genes related to histone modification in juveniles and adults independently (Table 4.5). In juvenile, fish refed with either LC/HP or HC/LP diets showed significantly lower mRNA levels of genes related to H3K4me3 writer (*setd1ba, kmt2a, kmt2ba,* and *kmt2bb*), H3K9ac writer (*kat6a*). In contrast, the higher mRNA levels of genes associated with H3K9me3 writer (*suv39h1b*), H3K9me3 and H3K36me3 eraser (*kdm4ab, kdm4c*), and H3K9ac writer (*kat2a, gtf3c4*) were observed in refeeding group when compared to fasted group ($P < 0.05$). Moreover, compared to fasted, the significantly lower mRNA levels of genes related to H3K4me3 (writer; *setd1a, kmt2bb*, and eraser; *kdm5ba*), H3K9me3 and H3K36me3 eraser (*kdm4aa*) in LC/HP refeeding group and significantly higher mRNA levels of genes related to H3K4me3 eraser (*kdm5a, kdm5ba, kdm5bb, kdm5c*, *riox1*), H3K9me3 and H3K36me3 eraser (*kdm4aa*, *kdm4b*), H3K9ac eraser (*sirt2, sirt5*) in HC/LP refeeding group were found in juvenile stage ($P < 0.05$). Between refeeding diets, the mRNA levels of genes related to H3K4me3 (writer; *setd1a,* and eraser; *kdm5a, kdm5ba, kdm5bb, kdm5c*), H3K9me3 writer (*suv39h1b*), H3K9me3 and H3K36me3 eraser (*kdm4aa, kdm4ab, kdm4b, kdm4c*), and H3K9ac (writer; *kat2b,* and eraser; *sirt5*) were higher in juvenile refed with dietary HC/LP compared with LC/HP diet ($P < 0.05$).

However, in adults, the mRNA levels of genes related to H3K4me3 (except *kdm5bb, kdm5c,* and *riox1* in HC/LP), H3K9me3 writer (except *suv39h1b* in HC/LP) and H3K36me3 eraser (except *kdm4ab* and *kdm4b* in HC/LP), H3K36me3 writer, and H3K9ac (except *kat2b, gtf3c4,* and *sirt5* in HC/LP) were lower in refed fish compared to fasted fish ($P < 0.05$). After four days of refeeding, the mRNA levels of genes related to H3K4me3 (writer; *setd1ba, kmt2bb*, and eraser; *kdm5a, riox1*), H3K9me3 and H3K36me3 eraser (*kdm4aa, kdm4b,* and *kdm4c*), H3K36me3 writer (*setd2*), and H3K9ac (writer; *kat2a, gtf3c4*, and eraser; *sirt2*) were significantly higher in HC/LP fish compared to LC/HP fish ($P < 0.05$). No significant differences were observed in the mRNA levels of *setd1a*, *kmt2a*, *kmt2ba*, *kdm5ba*, *kdm5bb*, *kdm5c*, *suv39h1b*, *kdm4ab*, *kat2b*, *kat6a*, and *sirt5* between HC/LP and LC/HP diets ($P > 0.05$).

In muscle, subsequent refeeding affects the muscular mRNA levels of genes related to histone modification in juveniles and adults differently (Table 4.6). In juvenile, compared to fasted, except the higher mRNA levels of *riox1* and *kat2a* genes, the lower mRNA levels of genes related to H3K4me3 (except *kdm5a* and *kdm5ba* in HC/LP), H3K9me3, H3K36me3, and H3K9ac were observed in fish refed with either HC/LP or LC/HP diets ($P < 0.05$). After refeeding, mRNA levels of genes related to H3K4me3 (writer; *setd1ba, kmt2ba,* and eraser; *kdm5ba, riox1*), H3K9me3 and H3K36me3 eraser (*kdm4aa,* and *kdm4b*), and H3K9ac writer (*kat2a, kat6a,* and *gtf3c4*) were significantly higher in fish refed with HC/LP compared to LC/HP fish ($P < 0.05$). However, the significant differences in mRNA levels of *setd1a, kmt2a, kmt2bb, kdm5a, kdm5bb, kdm5c, suv39h1b, kdm4c, setd2, kat2b, sirt2,* and *sirt5* genes between HC/LP and LC/HP were not observed ($P > 0.05$).

In adults, alterations in muscular mRNA levels of histone modification were affected by dietary HC/LP and LC/HP independently (Table 4.6). Compared to fasted, lower mRNA levels of *kdm5a, kdm5bb, kdm4aa,* and *sirt5* genes, together with higher mRNA levels of *riox1, suv39h1b*, and *kat2a* genes, were observed in refeeding fish ($P < 0.05$). Moreover, compared to fasted, mRNA levels of genes related to H3K4me3 (writer; *kmt2a, kmt2ba*, and eraser; *kdm5c*) and H3K9ac (writer; *kat2b, kat6a, gtf3c4*, and eraser; *sirt2*) were lower in LC/HP refeeding fish, whereas the significantly higher in mRNA levels of genes related to H3K4me3 writer (*setd1ba* and *kmt2bb*), H3K9me3 and H3K36me3 eraser (*kdm4b*), and H3K9ac writer (*gtf3c4*) were observed in only adult refed with HC/LP diet ($P < 0.05$). There were no significant differences in *setd1a, kdm5ba, kdm4c,* and *setd2* genes between fasted and refed conditions ($P > 0.05$). At the end of refeeding, higher muscular mRNA levels of genes related to H3K4me3 (writer; *setd1ba, kmt2a, kmt2ba, kmt2bb*, and eraser; *kdm5a, kdm5ba, riox1*), H3K9me3 writer (*suv39h1b*), H3K9me3 and H3K36me3 eraser (*kdm4aa, kdm4b*), and H3K9ac (writer; *kat6a, gtf3c4,* and eraser; *sirt2, sirt5*) were observed in adult refed with HC/LP compared to LC/HP diet ($P < 0.05$). However, there were no significant differences in the mRNA levels of *kdm5bb, kdm5c, kdm4c, setd2*, and *kat2b* between fish refed with HC/LP and LC/HP diets ($P > 0.05$).

Table 4.5 mRNA levels of gene involved in histone modifications in liver of juvenile and adult Nile tilapia that were fasted 4 days and refed 4 days with either an LC/HP or an HC/LP diet (mean ± standard deviation [SD], n = 6).

Genes involved in Epigenetics modification		Juvenile stage				Adult stage			
		Fasted	LC/HP	HC/LP	*P*-value	Fasted	LC/HP	HC/LP	*P*-value
H3K4me3 writer	*setd1a*	0.63 ± 0.03^{b}	0.47 ± 0.04^{a}	0.68 ± 0.07^{b}	0.003	1.94 ± 0.46^{b}	0.29 ± 0.11^{a}	0.68 ± 0.22^{a}	0.001
	setd1ba	0.46 ± 0.03^{b}	0.38 ± 0.07^{a}	0.35 ± 0.05^{a}	0.014	2.21 ± 0.40^{c}	0.35 ± 0.14^{a}	0.88 ± 0.31^{b}	<0.001
	kmt2a	1.01 ± 0.10^{b}	0.39 ± 0.08^{a}	0.48 ± 0.05^{a}	<0.001	1.52 ± 0.17^{b}	0.64 ± 0.21^{a}	0.88 ± 0.10^{a}	<0.001
	kmt2ba	0.96 ± 0.02^{b}	0.57 ± 0.02^{a}	0.57 ± 0.10^{a}	0.009	1.80 ± 0.25^{b}	0.55 ± 0.25^{a}	0.81 ± 0.16^{a}	<0.001
	kmt2bb	0.84 ± 0.09^{b}	0.67 ± 0.09^{a}	0.75 ± 0.04^{ab}	0.010	2.06 ± 0.36^{c}	0.53 ± 0.20^{a}	0.94 ± 0.11^{b}	<0.001
H3K4me3 eraser	*kdm5a*	0.46 ± 0.07^{a}	0.51 ± 0.02^{a}	1.18 ± 0.12^{b}	0.003	1.89 ± 0.07^{c}	0.18 ± 0.05^{a}	0.64 ± 0.12^{b}	<0.001
	kdm5ba	0.82 ± 0.08^{b}	0.64 ± 0.10^{a}	1.32 ± 0.10^{c}	<0.001	2.71 ± 0.99^{b}	0.36 ± 0.06^{a}	1.02 ± 0.22^{a}	0.001
	kdm5bb	0.34 ± 0.05^{a}	0.25 ± 0.04^{a}	0.64 ± 0.11^{b}	<0.001	1.03 ± 0.29^{b}	0.11 ± 0.05^{a}	0.38 ± 0.09^{ab}	0.001
	kdm5c	0.30 ± 0.04^{a}	0.25 ± 0.06^{a}	0.42 ± 0.07^{b}	0.001	0.81 ± 0.32^{b}	0.14 ± 0.06^{a}	0.35 ± 0.08^{ab}	0.001
	riox1	0.09 ± 0.04^{a}	0.29 ± 0.09 ^{ab}	0.84 ± 0.38^{b}	0.001	0.80 ± 0.33^{b}	0.07 ± 0.05^{a}	0.42 ± 0.28^{b}	0.002
H3K9me3 specific writer	*suv39h1b*	0.19 ± 0.04^{a}	0.67 ± 0.06^{b}	0.96 ± 0.06^{c}	<0.001	1.01 ± 0.28^{b}	0.24 ± 0.07^{a}	0.41 ± 0.11^{ab}	0.001
H3K9me3 and H3K36me3 eraser	*kdm4aa*	0.45 ± 0.02^{b}	0.36 ± 0.03^{a}	0.53 ± 0.02^{c}	<0.001	1.90 ± 0.13^{c}	0.25 ± 0.04^{a}	0.73 ± 0.06^{b}	<0.001
	kdm4ab	0.25 ± 0.02^{a}	0.32 ± 0.05^{b}	0.49 ± 0.03^{c}	<0.001	1.10 ± 0.06^{b}	0.17 ± 0.01^{a}	0.51 ± 0.02^{ab}	0.002
	kdm4b	0.35 ± 0.04^{a}	0.37 ± 0.03^{a}	0.75 ± 0.05^{b}	<0.001	1.22 ± 0.08^{b}	0.24 ± 0.05^{a}	1.25 ± 0.09^{b}	<0.001
	kdm4c	0.26 ± 0.02^{a}	0.30 ± 0.02^{b}	0.56 ± 0.04^{c}	<0.001	0.84 ± 0.05^{c}	0.18 ± 0.03^{a}	0.59 ± 0.03^{b}	<0.001
H3K36me3 specific writer	*setd2*	0.45 ± 0.04	0.40 ± 0.01	0.44 ± 0.05	0.097	1.43 ± 0.03^{c}	0.29 ± 0.03^{a}	0.44 ± 0.02^{b}	<0.001
H3K9ac specific writer	*kat2a*	0.22 ± 0.05^{a}	0.45 ± 0.03^{b}	0.39 ± 0.09^{b}	<0.001	0.88 ± 0.21^{c}	0.27 ± 0.09^{a}	0.58 ± 0.14^{b}	<0.001
	kat2b	0.47 ± 0.10^{ab}	0.33 ± 0.13^{a}	0.58 ± 0.19^{b}	0.034	1.24 ± 0.46^{b}	0.37 ± 0.12^{a}	0.62 ± 0.22^{ab}	0.003
	kat6a	0.70 ± 0.04^{b}	0.26 ± 0.02^{a}	0.22 ± 0.11^{a}	<0.001	1.04 ± 0.18^{b}	0.28 ± 0.09^{a}	0.38 ± 0.15^{a}	<0.001
	gtf3c4	0.40 ± 0.03^{a}	0.51 ± 0.05^{b}	0.52 ± 0.10^{b}	0.009	0.78 ± 0.21^{b}	0.33 ± 0.17^{a}	0.59 ± 0.20^{b}	0.004
H3K9ac specific eraser	*sirt2*	0.17 ± 0.02^{a}	0.33 ± 0.05^{ab}	1.24 ± 0.46^{b}	0.001	1.14 ± 0.04^{c}	0.06 ± 0.02^{a}	0.32 ± 0.03^{b}	<0.001
	sirt5	0.44 ± 0.02^{a}	0.34 ± 0.07^{a}	0.62 ± 0.03^{b}	0.002	1.03 ± 0.15^{b}	0.12 ± 0.02^{a}	0.35 ± 0.06^{ab}	<0.001

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein.
Means with different superscripts in each row differ significantly ($P < 0.05$).

Table 4.6 mRNA levels of gene involved in histone modifications in muscle of juvenile and adult Nile tilapia that were fasted 4 days and refed 4 days with either an LC/HP or an HC/LP diet (mean ± standard deviation [SD], n = 6).

Genes involved in Epigenetics modification		Juvenile stage				Adult stage			
		Fasted	LC/HP	HC/LP	*P*-value	Fasted	LC/HP	HC/LP	*P*-value
H3K4me3 writer	*setd1a*	1.57 ± 0.09^{b}	1.25 ± 0.07^{a}	1.24 ± 0.05^{a}	<0.001	1.16 ± 0.04	1.21 ± 0.04	1.21 ± 0.16	0.262
	setd1ba	1.78 ± 0.10^{c}	1.38 ± 0.06^{a}	1.57 ± 0.08^{b}	<0.001	1.01 ± 0.03^{a}	1.00 ± 0.05^{a}	1.17 ± 0.06^{b}	<0.001
	kmt2a	1.51 ± 0.04^{b}	0.79 ± 0.08^{a}	0.78 ± 0.05^{a}	<0.001	0.98 ± 0.06^{b}	0.73 ± 0.05^{a}	0.94 ± 0.09^{b}	<0.001
	kmt2ba	1.34 ± 0.05^{c}	0.86 ± 0.03^{a}	0.99 ± 0.03^{b}	<0.001	0.98 ± 0.01^{b}	0.82 ± 0.03^{a}	0.96 ± 0.09^{b}	0.005
	kmt2bb	1.88 ± 0.12^{b}	1.13 ± 0.11^{a}	1.19 ± 0.04^{a}	<0.001	1.04 ± 0.06^{a}	0.96 ± 0.08^{a}	1.30 ± 0.11^{b}	<0.001
H3K4me3 eraser	*kdm5a*	1.86 ± 0.30^{b}	0.94 ± 0.04^{a}	1.18 ± 0.07^{ab}	0.001	1.36 ± 0.08^{c}	1.02 ± 0.04^{a}	1.14 ± 0.09^{b}	<0.001
	kdm5ba	1.19 ± 0.07^{b}	0.96 ± 0.03^{a}	1.23 ± 0.06^{b}	<0.001	0.72 ± 0.04^{ab}	0.65 ± 0.05^{a}	0.78 ± 0.09^{b}	0.006
	kdm5bb	1.80 ± 0.16^{b}	0.94 ± 0.09^{a}	0.98 ± 0.02^{a}	0.003	1.15 ± 0.05^{b}	0.86 ± 0.03^{a}	0.88 ± 0.03^{a}	<0.001
	kdm5c	1.42 ± 0.05^{b}	1.07 ± 0.13^{a}	1.07 ± 0.03^{a}	0.006	1.01 ± 0.15^{b}	0.78 ± 0.15^{a}	0.88 ± 0.03^{ab}	0.020
	riox1	0.82 ± 0.02^{a}	1.42 ± 0.06^{b}	1.88 ± 0.08^{c}	<0.001	0.85 ± 0.03^{a}	0.92 ± 0.04^{b}	1.25 ± 0.05^{c}	<0.001
H3K9me3 specific writer	*suv39h1b*	1.66 ± 0.23^{b}	0.82 ± 0.08^{a}	1.01 ± 0.08^{a}	0.001	0.71 ± 0.09^{a}	1.07 ± 0.07^{b}	1.71 ± 0.05^{c}	<0.001
H3K9me3 and H3K36me3 eraser	*kdm4aa*	1.35 ± 0.06^{c}	0.90 ± 0.05^{a}	1.06 ± 0.04^{b}	<0.001	1.09 ± 0.03^{c}	0.65 ± 0.03^{a}	0.90 ± 0.02^{b}	<0.001
	kdm4b	1.22 ± 0.02^{c}	0.93 ± 0.01^{a}	0.97 ± 0.04^{b}	<0.001	0.82 ± 0.03^{a}	0.83 ± 0.04^{a}	1.23 ± 0.02^{b}	<0.001
	kdm4c	1.60 ± 0.09^{b}	1.17 ± 0.04^{a}	1.18 ± 0.03^{a}	<0.001	0.98 ± 0.03	0.97 ± 0.06	1.08 ± 0.09	0.051
H3K36me3 specific writer	*setd2*	1.51 ± 0.08^{b}	1.34 ± 0.05^{a}	1.41 ± 0.04^{a}	0.001	1.17 ± 0.05	1.17 ± 0.05	1.26 ± 0.09	0.185
H3K9ac specific writer	*kat2a*	0.75 ± 0.03^{a}	1.45 ± 0.05^{b}	1.72 ± 0.09^{c}	<0.001	0.72 ± 0.01^{a}	1.17 ± 0.02^{b}	1.19 ± 0.01^{b}	<0.001
	kat2b	1.87 ± 0.13^{b}	0.67 ± 0.04^{a}	0.68 ± 0.06^{a}	0.003	1.35 ± 0.13^{b}	0.58 ± 0.02^{a}	0.95 ± 0.11^{ab}	0.001
	kat6a	1.60 ± 0.11^{c}	0.93 ± 0.05^{a}	1.08 ± 0.04^{b}	<0.001	1.21 ± 0.02^{b}	0.73 ± 0.02^{a}	0.95 ± 0.06^{b}	0.001
	gtf3c4	1.60 ± 0.14^{c}	0.96 ± 0.05^{a}	1.11 ± 0.09^{b}	<0.001	1.02 ± 0.05^{b}	0.88 ± 0.06^{a}	1.31 ± 0.07^{c}	<0.001
H3K9ac specific eraser	*sirt2*	1.18 ± 0.04^{b}	0.95 ± 0.03^{a}	0.96 ± 0.10^{a}	0.003	0.96 ± 0.02^{b}	0.71 ± 0.03^{a}	0.94 ± 0.04^{b}	<0.001
	sirt5	1.39 ± 0.08^{b}	0.93 ± 0.06^{a}	0.99 ± 0.12^{a}	<0.001	0.99 ± 0.04^{c}	0.78 ± 0.03^{a}	0.88 ± 0.05^{b}	<0.001

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein.

Means with different superscripts in each row differ significantly ($P < 0.05$).

4.5 Discussion

As originally proposed by Waddington, epigenetics refers to the study of how environmental or behavioural factors can alter gene expression without changing the underlying DNA sequence (Waddington, 1957). Numerous studies have shown that nutritional factors—such as refeeding, CHO, and protein intake—can influence the epigenetic landscape through mechanisms like DNA (de)methylation and histone modifications in mammals (Gibson et al., 2020; Rees et al., 2000), amphibians (Tamaoki et al., 2018) and fish (Marandel et al., 2016; Liu et al., 2022). In rainbow trout, although it is a poor user of CHO as an energy source, both nutritional status and high dietary CHO intake were shown to affect epigenetic regulation, leading to changes such as global DNA hypomethylation, hypoacetylation of H3K9ac, and remodeling of epigenetic modulators (Marandel et al., 2016; Liu et al., 2022). Previous study showed that refeeding for 4 days following a 4-day fast induced changes in intermediary metabolism in both juvenile and adult stages of Nile tilapia (Thongchaitriwat et al., 2024). Furthermore, the effects of short-term refeeding with HC/LP diet on intermediary metabolism resembled the responses observed with long-term intake of HC diet, including the induction of glycolysis and lipogenesis, along with the suppression of gluconeogenesis and amino acid catabolism (Thongchaitriwat et al., 2024), suggesting a strong responsiveness to dietary CHO regardless of life stage. The present study builds upon these findings by investigating how the epigenetic landscape in Nile tilapia responds to short-term dietary CHO refeeding following a fasting period. Specifically, this study examined DNA (de)methylation, histone modifications, and the expression of related epigenetic modulators at the molecular level in liver and muscle tissues across different developmental stages. The results were analysed in relation to the fish's nutritional status and different refeeding diets, with comparisons made among fasted, LC/HP, and HC/LP groups.

4.5.1 Short-term refeeding with different dietary carbohydrate levels influenced global DNA (de)methylation and DNA methylation modulators in Nile tilapia

The DNA methylation landscape and its oxidative derivatives are highly responsive to environmental changes, particularly nutritional status and dietary composition (e.g., refeeding, CHO, protein). For instance, in rats, a LP maternal diet

during pregnancy led to DNA hypermethylation in the offspring's liver (Rees et al., 2000). In fish, refeeding—especially with HC diets—has been shown to induce hepatic global DNA hypomethylation in juvenile rainbow trout (Marandel et al., 2016; Liu et al., 2022). Previous work in Nile tilapia demonstrated a strong ability to utilise HC diets during short-term refeeding, as reflected in phenotypic responses (increased hepatic glycogen and triglyceride content) and molecular adjustments (suppressed hepatic amino acid catabolism) in both juvenile and adult stages (Thongchaitriwat et al., 2024). Building on this, the present study investigated how short-term fasting and subsequent refeeding with varying CHO levels affect the cytosine methylation landscape—including its oxidative derivatives—in juvenile and adult Nile tilapia. We also examined the mRNA levels of key enzymes involved in DNA methylation and demethylation. These included the DNA methyltransferase (DNMT) family, which regulates both de novo methylation and maintenance methylation (Lyko, 2018; Uysal et al., 2017), and the Ten-eleven translocation (TET) family, which catalyses active DNA demethylation through the stepwise oxidation of 5-mC to 5-hmC, 5-fC, and 5-caC (Tahiliani et al., 2009; Zhang et al., 2023).

In the liver, refeeding after fasting resulted in a decrease in 5-mC oxidative derivatives—specifically, 5-hmdC in both juveniles and adults, and 5-cadC in adults. However, no significant differences in hepatic 5-mdC levels were observed across nutritional statuses. Similarly, Liu et al. (2022) reported a reduction in 5-hmC following a four-day fasting and four-day refeeding period in trout. However, in that study, it was accompanied by a decrease in 5-mdC and an increase in dC, highlighting species-specific differences in the epigenetic response to nutritional changes. Additionally, our results suggest that the effects of refeeding varied slightly across different developmental stages. In juveniles, unlike in adults, a general reduction in *tet* mRNA expression following refeeding was consistent with the observed decrease in 5-mC oxidative derivatives. Despite stable 5-mdC and dC levels in both age groups, the expression of *dnmt* genes was responsive to both nutritional status and dietary composition, though the pattern varied by gene. Similar findings have been reported in trout (Marandel et al., 2016; Liu et al., 2022), where the authors suggested that more complex regulatory mechanisms, including post-transcriptional or protein-level controls, may be involved.

In muscle tissue, unlike in the liver, changes in nutritional status induced alterations in DNA (de)methylation derivatives, and most of these effects were primarily influenced by the composition of the refeeding diet (except 5-cadC in adults). In adult fish, refeeding with the HC/LP diet led to DNA hypomethylation—as evidenced by a decrease in 5-mdC—compared to the LC/HP-fed group. This was accompanied by increased dC levels and decreased 5-hmdC. This pattern was specific to adults and represents the first report of DNA methylation dynamics in the muscle of Nile tilapia. It suggests that, as previously shown in trout (Liu et al., 2022), muscle tissue in tilapia undergoes active DNA demethylation in response to dietary formulation. The observed differences in DNA methylation remodelling between growth stages may reflect developmental plasticity and/or adaptability to dietary CHO utilisation. For instance, in rainbow trout, Callet et al. (2020) demonstrated that broodstock had a greater capacity to utilise HC diets than juveniles-a difference attributed to growth stage-specific energy demands and gluconeogenic balance. Overall, our findings suggest that dietary composition during refeeding can influence epigenetic stability, with HC/LP diets promoting global DNA hypomethylation in adult muscle. At the molecular level, increased *tet1* mRNA expression in adults fed the HC/LP diet corresponded with decreased 5-mdC and 5-hmdC levels and increased dC, supporting its role in active demethylation. Other DNA methylation-related genes (writers and erasers) responded to nutritional status and/or dietary composition but did not exhibit a direct correlation with global methylation patterns in either juveniles or adults. As previously suggested, regulation may occur at the post-transcriptional or protein level. In conclusion, refeeding and dietary HC appear to affect epigenetic stability in tilapia muscle through global DNA (de)methylation and associated modulators. Further investigation into the enzymatic activity of these modulators is warranted. However, this will require distinguishing between DNA methylation-related proteins with high sequence similarity in order to define their specific roles and expression profiles.

4.5.2 Short-term refeeding with different dietary carbohydrate levels influenced global histone modification and histone modulators in Nile tilapia

Within the epigenetic landscape, histone modifications serve as key regulatory mechanisms that alter chromatin structure and, consequently, influence gene expression. These modifications are responsive to environmental cues, particularly

nutritional status and dietary composition. For example, in amphibians, fasting followed by refeeding induced histone modifications—such as hypomethylation of H3K9 and H3K36—in the intestines of African clawed frogs (Tamaoki et al., 2018). Similarly, in fish, Marandel et al. (2016) explained that refeeding fasted juvenile rainbow trout resulted in hepatic hypermethylation of H3K9, whereas the acetylation of H3K9 was influenced by dietary CHO.

In this study, we examined four histone modifications previously associated with metabolic disorders and dysregulation of glucose metabolism: permissive marks (H3K4me3, H3K9ac, H3K36me3) and the repressive mark H3K9me3 (Tu et al., 2015). Histone modifications are reversible and regulated by the dynamic balance between writer and eraser enzymes involved in methylation and acetylation (Hyun et al., 2017), processes known to be influenced by diet and feeding status. Our findings showed that histone modifications were primarily affected by dietary composition in both liver and muscle tissues across juvenile and adult stages. In juveniles, hepatic hyperacetylation of H3K9 was observed in fish fed the LC/HP diet, while muscular hypermethylation of H3K36 occurred in those fed the HC/LP diet. However, compared to fasting, hepatic H3K9ac levels were reduced in HC/LP-fed juveniles. In adults, while hepatic histone marks were unaffected by dietary treatments, muscular hypermethylation and hyperacetylation of H3K9 were evident in fish refed with the LC/HP diet compared to those given the HC/LP diet. These results indicate that even short-term refeeding with differing nutritional compositions can significantly influence global histone acetylation and methylation patterns in Nile tilapia, in a life stage-dependent manner. Notably, Marandel et al. (2016) reported that hepatic hypoacetylation of H3K9 was observed in juvenile rainbow trout refed with HC/LP diet compared to those on LC/HP diet. Taken together, our study suggests that dietary nutrient composition during refeeding can alter the histone modification landscape in Nile tilapia, with HC diets tending to induce hypomethylation or hypoacetylation of histone marks, particularly in juvenile stages.

Regarding genes encoding histone modification writers and erasers, only a few showed expression patterns consistent with the observed changes in histone marks. These genes were mainly associated with the erasure of H3K36me3 and H3K9ac. Specifically, the expression profiles of hepatic *kdm4b* and *kdm4c* in juveniles, as well as muscular *kdm4c* in adults, were consistent with the observed decrease in H3K36me3 levels in the respective tissues and developmental stages. Additionally, the increased

expression of *sirt2* and *sirt5* in the liver of juveniles and the muscle of adults fed the HC/LP diet aligned with the reduction in H3K9ac levels. These findings suggest that even short-term refeeding with different dietary formulations can modulate the expression of histone-modifying enzymes in Nile tilapia, potentially contributing to changes in histone marks at the protein level. However, while many of the genes analysed were influenced by nutritional status and/or dietary composition, their mRNA expression changes did not always correspond to observed alterations in histone modification levels. This highlights the likelihood that regulation also occurs at the post-transcriptional or protein activity level. Taken together, our findings suggest that both refeeding status and HC diet may influence histone modification dynamics in Nile tilapia by modulating histone methylation and acetylation regulators. Taken together, our findings suggest that both refeeding status and HC diet may influence histone modification dynamics in Nile tilapia by modulating histone methylation and acetylation regulators. Future studies assessing enzyme activity are needed to clarify these mechanisms, particularly given the potential for regulation at the protein level despite similar amino acid sequences among modulators.

4.6 Conclusion

In conclusion, the epigenetic landscape of Nile tilapia is sensitive to short-term fasting and refeeding with LC or HC diets, in both juvenile and adult stages. Refeeding following fasting led to a reduction in global hepatic 5-mC oxidative derivatives, and dietary composition during refeeding impacted histone methylation and acetylation patterns. Notably, a HC/LP refeeding diet induced DNA hypomethylation in adult muscle and influenced histone mark hypomethylation and hypoacetylation in Nile tilapia, highlighting the importance of dietary CHO/protein balance in shaping epigenetic responses during different life stages.

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CHAPTER V

EFFECTS OF HIGH-CARBOHYDRATE DIETS IN BROODSTOCK NILE TILAPIA ON REPRODUCTIVE PERFORMANCE, INTERMEDIARY METABOLISM, EPIGENETIC REGULATION AND ON CARBOHYDRATE METABOLISM IN THEIR OFFSPRING

5.1 Abstract

Nile tilapia are capable of utilizing dietary carbohydrates (CHO) as an energy source; however, information regarding the metabolic responses related to dietary CHO in Nile tilapia broodstock remains limited. Therefore, this study aimed to investigate the effects of different dietary CHO levels on reproductive performance and carbohydrate metabolism in both female and male broodstock. Two dietary treatments were formulated: a high-carbohydrate/low-protein (HC/LP) diet and a low-carbohydrate/high-protein (LC/HP) diet, which were fed to mature females (six replicates) and males. The HC/LP diet affected reproductive performance in females, including a decrease in egg weight and gonadosomatic index (GSI) but an increase in fecundity, compared with the LC/HP diet ($P<0.05$). In both females and males, blood metabolites, hepatic and muscular composition, as well as carbohydrate-related metabolic responses at the molecular level, reflected dietary CHO feeding, as evidenced by: (1) increased plasma glucose and triglyceride levels and decreased plasma protein levels; (2) increased hepatosomatic index; (3) increased lipid, glycogen, and triglyceride contents in the liver but decreased protein content; (4) increased muscular glycogen and triglyceride in females; (5) increased lipid but decreased protein contents in male muscle; (6) upregulation of genes related to glycolysis and lipogenesis and downregulation of genes associated with gluconeogenesis and amino acid catabolism in the liver; (7) upregulation of *glut4* (only in females) and genes related to muscle glycolysis ($P< 0.05$). Moreover, feeding broodstock a HC/LP diet downregulated DNA methylation writer (*dnmt*) and upregulated genes related to DNA demethylation (*tet*) and histone H3K9me3 and H3K36me3 erasers (*kdm4*). In addition, a similar trend in intermediary carbohydrate metabolic responses was observed in offspring at seven

days post-hatching and seven days after first feeding, suggesting that parental carbohydrate metabolism may influence offspring through intergenerational transmission. In summary, dietary carbohydrates modulated reproductive performance, carbohydrate metabolism, and epigenetic stability in Nile tilapia broodstock, which subsequently influenced carbohydrate metabolism in their offspring.

Keywords: Glycolysis, Gluconeogenesis, Amino acid catabolism, Lipogenesis, Nile tilapia

5.2 Introduction

With the rising global demand for animal-source foods—including those derived from both aquaculture and terrestrial livestock—there is a corresponding increase in the need for animal feed. Livestock and aquaculture industries compete for limited feed resources, driving efforts to develop cost-effective, high-quality diets by incorporating alternative feed ingredients and optimizing nutrient utilization (Sandström et al., 2022). Among these nutrients, carbohydrates (CHO) are considered the most economical energy source and are commonly included in practical commercial diets to reduce feed costs. Aquafeeds typically contain higher protein levels than terrestrial animal feeds (NRC, 2000; NRC, 2011; NRC, 2012; FAO, 2022), making protein-sparing strategies a major focus of nutritional research. Consequently, numerous studies have investigated the optimal inclusion levels of carbohydrates to enhance protein retention and improve growth performance, which often depends on species-specific metabolic capacities (Wilson, 1994; Jia et al., 2022; Yang et al., 2023). Due to the wide variety of feeding habits among fish, carnivorous species often exhibit limited capacity to utilize CHO as an energy source, owing to glucose intolerance. In contrast, omnivorous and herbivorous species demonstrate a greater ability to metabolize carbohydrates efficiently (Polakof et al., 2012; Kamalam et al., 2017). Indeed, appropriate levels of digestible CHO can elicit protein-sparing effects and support growth in species such as carp and tilapia (Wu et al., 2016; Boonanuntanasarn et al., 2018a,b).

Globally, tilapia represents the second most economically important aquaculture species after carp. Its rapid growth rate and tolerance to diverse environmental conditions have contributed to its widespread adoption in aquaculture systems (FAO, 2024). Among tilapia species, *Oreochromis niloticus* (Nile tilapia) dominates global

production, and extensive multidisciplinary research has been conducted to optimize its farming. Feed costs account for approximately 50–70% of total production costs in tilapia farming, prompting intensive research into feed formulation and nutrient metabolism to develop cost-effective, nutritionally balanced diets.

As an omnivorous species, Nile tilapia can utilize high levels of dietary carbohydrates as an energy source, making carbohydrate inclusion a promising strategy for reducing feed costs. Numerous studies have documented the metabolic responses of tilapia to high-carbohydrate (HC) diets, including the stimulation of glycolysis and lipogenesis, and the suppression of gluconeogenesis and amino acid catabolism under both long-term feeding and short-term refeeding following fasting (Boonanuntanasarn et al., 2018a,b; Thongchaitriwat et al., 2024). For example, Azaza et al. (2015) reported optimal growth performance in fry fed a diet containing 32% starch over 45 days, while Wang et al. (2005) demonstrated that juvenile hybrid tilapia could utilize dietary starch levels up to 46%. Boonanuntanasarn et al. (2018a) further showed that while a 32% CHO diet promoted growth, excessively high (50%) or low (14%) CHO levels resulted in poor growth performance during adulthood. Long-term feeding from first feeding through adulthood confirmed that a 32% CHO level provided the most favorable growth outcomes (Boonanuntanasarn et al., 2018b). These findings highlight the capacity of Nile tilapia to efficiently utilize carbohydrates throughout its life cycle. While several studies have investigated the effects of dietary protein and lipid on the reproductive performance of Nile tilapia (El-Sayed et al., 2005; El-Sayed et al., 2008), limited information is available on how dietary carbohydrate influences reproductive traits and intermediary metabolism in broodstock. Given the significance of broodstock nutrition on progeny development, further research is warranted to evaluate the impact of dietary carbohydrates across the life cycle of Nile tilapia.

To improve carbohydrate utilization efficiency, the concept of nutritional programming (NP) has been explored in Nile tilapia. Nutritional programming refers to the introduction of a nutritional stimulus during early life stages to enhance the organism's ability to utilize specific nutrients when re-exposed to them later in life (Lucas, 1998; Panserat et al., 2019). The effects of CHO-based NP vary among species; while limited effects have been reported in rainbow trout (Geurden et al., 2013), CHO-based NP in Nile tilapia has been shown to significantly improve the efficient utilization of HC diets—up

to approximately 66%. Effective CHO programming interventions have been achieved through glucose injection into the yolk sac and dietary HC feeding during early fry stages, highlighting developmental windows of metabolic plasticity (Kumkhong et al., 2020a,b; Kumkhong et al., 2021; Srisakultiew et al., 2022). Nutritional programming can also be mediated through broodstock. In rainbow trout, maternal HC feeding has been shown to influence carbohydrate metabolism in offspring (Callet et al., 2021). Therefore, investigating the effects of dietary HC in both male and female broodstock could provide valuable insights not only into CHO metabolism and reproductive performance but also into its potential application as a nutritional programming strategy.

Epigenetics is defined as a heritable alteration of gene regulation or expression which does not involve changes to the DNA sequence (Waddington, 1957). Nutritional status and/or nutrients are demonstrated to be a factor can modulate epigenetic stability such as DNA methylation and histone modifications (Berger et al., 2009; Rosen et al., 2018). In mammals, diabetes is associated with the stabilization of epigenetic regulation (Ling and Rönn, 2019). Early NP such as excess nutrition during pregnancy can lead to epigenetic changes that affect fetal susceptibility to metabolic diseases such as obesity in adulthood (Ramirez-Alarcon et al., 2019). In rainbow trout, 4 days fasting and refed with HC diet could lead hepatic DNA hypomethylation and histone hypoacetylation (Marandel et al., 2016). DNA hypomethylation in liver and muscle was observed which was proposed to link with NP of glucose overload in yolk reserve in larvae on modulating of CHO metabolism responses in Nile tilapia (Kumkhong et al., 2020b). In addition, HC feeding in female broodstock modulated expression of genes involved in DNA (de)methylation in liver of juvenile offspring (Callet et al., 2021).

In this study, we aimed to investigate the effects of a high-carbohydrate diet on intermediary carbohydrate metabolism in both male and female Nile tilapia broodstock. Specifically, we evaluated the impact of dietary HC on reproductive performance, including fecundity and larval size. Moreover, considering the potential for nutritional programming through broodstock, we examined carbohydrate-related metabolic responses at the molecular level in both broodstock and their offspring. Since CHO may be linked to epigenetic regulation, this study examined the effects of a high-CHO diet on several genes associated with global DNA (de)methylation and histone modifications in broodstock Nile tilapia.

5.3 Materials and methods

5.3.1 Experimental design, diet, and fish culture

All experimental procedures involving fish were approved by the Ethics Committee of the Suranaree University of Technology Animal Care and Use Committee (Approval No. SUT-IACUC-001/2023). Table 5.1 presents the experimental diets, including the LC/HP (low-carbohydrate/high-protein) and HC/LP (high-carbohydrate/low-protein) formulations. The proximate compositions, including moisture, crude protein (CP), crude fat (CF), crude fiber, and ash, were analyzed according to the standard methods of the Association of Official Analytical Chemists (AOAC, 1990) and are shown in Table 1. The experimental fish were cultured under experimental conditions following commercial aquaculture practices. The commercial diet used for acclimating the fish and nursing the fry is also presented in Table 5.1.

A completely randomized design with two dietary treatments (HC/LP and LC/HP) and six female replicates was employed in this study. The experimental plan for broodstock and offspring feeding, as well as the sampling scheme, is illustrated in Figure 5.1. According to common aquaculture practice for natural breeding, Nile tilapia broodstock (1 male:2 females) are generally cultured together in earthen ponds and fed commercial diets (Table 1). In this study, experimental male and female broodstock Nile tilapia (Oreochromis niloticus) were obtained from the Suranaree University of Technology Farm (SUT Farm), Nakhon Ratchasima, Thailand.

The breeding pond was an earthen pond (5 m × 10 m; water depth 0.8 m) that was divided into two equal sections (5 m × 5 m; water depth 0.8 m) for the HC/LP and LC/HP groups. Twelve males (body weight [BW]: 920 ± 16.7 g) and twenty-four females (BW: 609.6 ± 28.4 g) were used in total, with six males and twelve females assigned to each section. Before the experimental trial, fish were acclimated to the experimental conditions for one week using a commercial diet. Subsequently, broodstock were fed either the HC/LP or LC/HP diet for five weeks. Fish were fed twice daily (09:00 and 15:00) at 1.5% of their body weight. The experiment was not continued long-term, as a notable impact of the HC/LP diet on fertilization rate was observed. A flow-through water exchange system was employed, in which one-third of the water in each pond was replaced twice per week with dechlorinated water.

Fertilized eggs were examined at 14 and 28 days after feeding. The eggs were counted and weighed, and healthy fertilized eggs from six females ovulating at the same developmental stage (six replications) were collected and transferred to hatching trays with circulating water. Because the fertilized eggs obtained at 28 days were small, further analyses were conducted only on the eggs obtained from broodstock at 14 days after feeding. After hatching, the larvae at seven days post-hatching (dph), when yolk reserves were completely absorbed, were transferred to cages (0.4 m × 0.4 m × 0.6 m) placed in cement ponds (2 m × 2 m × 0.8 m; water depth 45 cm) and fed a commercial diet (Table 1) at 30% of their body weight. The fry at seven days after first feeding (daf) were sampled for the evaluation of carbohydrate metabolism and related parameters.

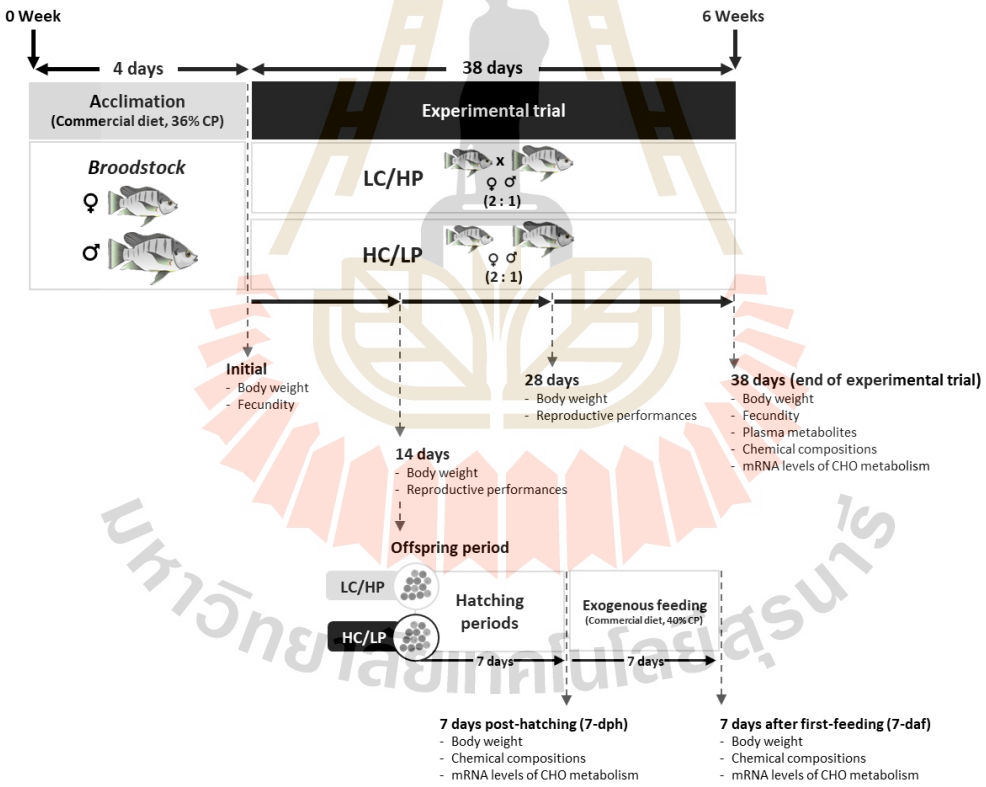

Figure 5.1 Experimental plan of broodstock and offspring Nile tilapia. Fish were fed with a commercial diet during acclimation period. During the stimulus in broodstock, female and male were fed with either a low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet.

Table 5.1 Ingredients and chemical composition (g kg^{-1}) of the commercial diets (diets during nursing) and for stimulus diets.

Ingredients	Commercial diet		Experimental diets	
	Broodstock acclimation (36% protein)	Offspring nursing (40% protein)	LC/HP	HC/LP
Fish meal	-	-	88	18
Rice flour	-	-	0	70
Fish oil	-	-	0	7
Soybean oil	-	-	2	0
Gelatin	-	-	8	0
Di-calcium phosphate	-	-	0	3
Fish premix[a]	-	-	2	2
Proximate composition (% from wet weight)				
Dry matter	91	89	94.2	90.2
Protein	36	40	57.3	15.3
Fat	3.4	8	9.2	9
Fiber	7.1	4.2	0.5	0.4
Ash	12.6	12.2	22.8	8.6
NFE[b]	32.5	24.3	4.5	56.8
Gross energy (kJ g^{-1})	13.13	14.42	14.40	15.6

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

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

5.3.2 Fish sampling

In this study, offspring from the first ovulation (after the 14-day feeding trial) were sampled. Offspring at seven days post-hatching (7 dph) and seven days after first feeding (7 daf) were collected for the analysis of intermediary glucose metabolism. For sampling, 7-dph (200 larvae per replication) and 7-daf (100 fry per replication) groups were collected and snap-frozen for proximate composition, glycogen, and triglyceride analyses. In addition, 7-dph (50 larvae per replication) and 7-daf (50 fry per replication) samples were collected for total RNA extraction for subsequent quantitative reverse-transcription PCR (qRT-PCR) analysis.

At the end of the experimental period (six weeks), six females (from 14 days after feeding, which had ovulated at the same egg stage) and three males were sampled five hours after the last meal, based on the postprandial glycaemia peak of Nile tilapia (Boonanuntanasarn et al., 2018a). The fish were euthanized using 0.2% clove oil. Blood samples were collected from the caudal vein using a hypodermic syringe, mixed with K_2EDTA (1.5 mg mL^{-1} of blood) as an anticoagulant, and centrifuged at 10,000 × g for 5 min at 4°C to obtain plasma. The plasma was stored at −80°C until used for blood chemistry analyses.

After blood collection, the liver was removed and weighed to calculate the hepatosomatic index (HSI). Liver and epaxial muscle samples were then rapidly frozen in liquid nitrogen and stored at −80°C for subsequent analyses of nutritive composition, total RNA extraction, and carbohydrate-related metabolism. In addition, ovaries and testes from broodstock were collected and weighed to determine the gonadosomatic index (GSI).

5.3.3 Blood chemistry analysis

Determination of blood metabolites in broodstock (females, n = 6; males, n = 3 per condition) included glucose, triglycerides (TAG), cholesterol, total protein, and blood urea nitrogen (BUN). Plasma glucose was quantitatively analyzed using the Trinder method (Barham and Trinder, 1972). Plasma TAG was determined using the glycerol-3-phosphate oxidase–sodium N-ethyl-N-(3-sulfopropyl)-m-anisidine (GPO-ESPAS) method described by Bucolo and David (1973). Cholesterol concentration was measured using the cholesterol oxidase–phenol + aminophenazone (CHOD-PAP) method described by Flegg (1973). Total protein was analyzed using the Biuret method (Gornall et al., 1949). BUN was determined using a modified indophenol colorimetric method (Weatherburn, 1967).

5.3.4 Chemical composition, glycogen and triglycerides analysis

Broodstock tissues (liver and muscle; females, n = 6; males, n = 3 per condition) and whole-body samples of their offspring (n = 6 per HC/LP and LC/HP group) were analyzed for chemical composition, including crude protein, crude fat, glycogen, and triglycerides (TAG), according to the methods of AOAC (1990).

Glycogen content was analyzed according to the method of Kirchner et al. (2003), with modifications. Briefly, samples (200 mg) were homogenized in 1 mL of 1 M HCl, and an aliquot of the homogenate was transferred to a new tube. The homogenate was neutralized by adding 5 M KOH and subsequently centrifuged at 10,000 × g for 10 min at 4°C. Free glucose in the supernatant was measured using a plasma glucose kit (catalog no. BLT00026; Erba Lachema s.r.o., Karásek, Brno, Czechia) according to the manufacturer's instructions.

Another aliquot of the homogenate was then boiled at 100°C for 2.5 h to hydrolyze glycogen. After boiling, the homogenates were cooled to room temperature and neutralized with 5 M KOH. Following centrifugation (10,000 × g, 10 min, 4°C), the total glucose concentration (sum of free glucose and glucose derived from glycogen hydrolysis) in the supernatant was determined. Glycogen content was calculated as the difference between total glucose and free glucose concentrations.

For the determination of TAG content, samples (100 mg) were homogenized with 1 mL of 5% IGEPAL in deionized water containing 2.8-mm glass beads using a Bioprep-24 homogenizer. Samples were heated in a water bath at 90°C for 10 min and then cooled to room temperature. Subsequently, samples were centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was transferred to a new tube and diluted with deionized water. TAG concentration was measured using a triglyceride kit (catalog no. BLT00059; Erba Lachema s.r.o., Karásek, Brno, Czechia) following the manufacturer's instructions.

5.3.5 Total RNA extraction, cDNA synthesis, and real-time RT-qPCR analysis of genes involved in carbohydrate metabolism and epigenetic modifications

Total RNA was extracted from the liver and muscle of broodstock (females, *n* = 6; males, *n* = 3 per condition) and from whole-body samples of offspring at 7 days post-hatching (7 dph) and 7 days after first feeding (7 daf) (*n* = 6 per HC/LP

and LC/HP group). RNA extraction was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from liver (50 mg), muscle (100 mg), and whole-body fry (100 mg) samples. The quantity and purity of total RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA), and RNA integrity was verified by 1% agarose gel electrophoresis.

Complementary DNA (cDNA) synthesis was carried out using the SuperScript III RNase H–Reverse Transcriptase kit (Invitrogen, USA) with random primers (Promega, Charbonnières, France). cDNA was synthesized from 1 μg of total RNA (in duplicate for each sample) according to the manufacturer's protocol.

Quantitative real-time reverse transcription polymerase chain reaction (real-time RT-qPCR) was performed to determine the expression of genes related to carbohydrate (CHO) metabolism and its associated pathways. At the molecular level, genes related to glucose and its associated metabolic pathways were analyzed. These included hepatic glycolysis genes [glucokinase (*gck*), phosphofructokinase (*pfklr*), and pyruvate kinase (*pklr*)]; hepatic gluconeogenesis genes [glucose-6-phosphatase (*g6pca1*, *g6pca2*), cytosolic phosphoenolpyruvate carboxykinase (*pck1*), and mitochondrial phosphoenolpyruvate carboxykinase (*pck2*)]; lipogenesis genes [fatty acid synthase (*fasn*) and glucose-6-phosphate dehydrogenase (*g6pd*)]; amino acid catabolism genes in liver [glutamate dehydrogenase (*gdh*), alanine aminotransferase (*alat*), and aspartate aminotransferase (*asat*)]; glucose utilization gene [glucose transporter (*glut4*)]; and muscular glycolysis genes [hexokinase I/II (*hk1*, *hk2*), phosphofructokinase (*pfkma*, *pfkmb*), and pyruvate kinase (*pkma*)].

To investigate epigenetic modifications at molecular levels, we evaluated the alteration of genes related to DNA methylation writers (DNA methyltransferase; *dnmt1, dnmt3aa, dnmt3ab, dnmt3ba*, and *dnmt3bb*) and DNA methylation eraser (ten-eleven translocation; *tet1, tet2*, and *tet3*). The histone modification of H3K4me3 included H3K4me3 writer (histone-lysine N-methyltransferase related SET domain; *setd1a* and *setd1ba*, histone-lysine methyltransferase; *kmt2a, kmt2ba* and *kmt2bb*) and H3K4me3 eraser (histone lysine demethylase; *kdm5a, kdm5ba, kdm5bb* and *kdm5c*; bifunctional lysine specific demethylase and histidyl hydroxylase; riox1). For H3K9me3. H3K9me3 specific writer (histone lysine methyltransferase; *suv39h1b*) and H3K9me3 specific eraser (histone lysine

demethylases; *kdm4aa, kdm4ab, kdm4b* and *kdm4c*) were quantified. In addition, expression of gene related to H3K36me3 specific writer (SET domain containing 2, histone lysine methyltransferase; *setd2*) was examined. Moreover, for H3K9ac, two groups of genes related H3K9ac specific writer (histone lysine acetyltransferase; kat2a, kat2b, and kat6a, general transcription factor IIIC subunit 4; *gtf3c4*) and H3K9ac specific eraser (sirtuin; *sirt2* and *sirt5*) were determined.

Primer sequences used for real-time RT-qPCR are listed in Tables S1 and S2. Quantification of mRNA levels was conducted using the Roche Applied Science E-Method according to Pfaffl (2001). The elongation factor 1 alpha (*ef1α*) transcript, which did not vary among conditions or tissues, was used as the internal reference gene for normalization (data not shown). In all cases, PCR efficiency was calculated from the slope of a standard curve based on serial dilutions of cDNA, with efficiency values ranging between 1.8 and 2.0.

Table 5.2 List of primers used for qRT-PCR analysis of genes related to carbohydrate and intermediary metabolism in muscle, liver, and whole-body tissues.

Genes	5'/3' Forward primer	5'/3' Reverse primer	SIZE (bps)	Accession numbers
Reference gene				
*ef1α**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	250	AB075952
Liver metabolism				
gck	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC	186	XM003451020
pfklr	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC	162	XM003447353
pklr	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA	164	XM005472622
g6pca1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA	195	XM003448671
g6pca2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA	245	XM013273429
pck1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA	162	XM003448375
pck2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT	202	XM019354843
fasn	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT	222	XM013276809
g6pd	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT	187	XM013275693
asat	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC	200	XM003451918
alat	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG	200	XM005476466
gdh	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC	203	XM003457465
Muscle metabolism				
glut4	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG	235	JN900493
hk1	CGTCGCTTAGTCCCAGACTC	TGACTGTAGCGTCCTTGTGG	235	XM019360229
hk2	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC	200	XM003448615
pfkma	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG	190	XM019349871
pfkmb	TTTGTGCATGAGGGTTACCA	CACCTCCAATCACACACAGG	208	XM003441476
pkma	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA	249	XM005447626

* From Yang et al. (2013).

Table 5.3 List of primers used for qRT-PCR analysis of genes related to epigenetic modifications in muscle and liver tissues.

Genes		5'/3' Forward primer	5'/3' Reverse primer	SIZE (bps)	Accession number
Reference gene	*ef1**	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	250	AB075952
DNA methylation writers	*dnmt1*	CTCACACTGCGCTGTCTTGT	ACAACGCTGAGAGAGCAAGC	188	XM_025906327.1
	dnmt3aa	CCAACAACCACGAGCAGGAA	TGCCGACAGTGATGGAGTCT	192	XM_005475084.4
	dnmt3ab	GCCGCAGCTTAGAGGACATC	CACACATGAGCACCTCTCGTC	189	XM_005477258.3
	dnmt3ba	GCTGCTGCAGATGCTACTGT	TTGCGCTGTTGTTGGCAAAG	186	XM_025901732.1
	dnmt3bb	TGCAGGAGTTCTTCGCCAAC	TGCCACATACTGACCCACCT	173	XM_025901790.1
DNA methylation eraser	*tet1*	CATCCAGTCCCAGCACAACC	CTCTATTTGGCGTGCGCTGA	194	XM_025897345.1
	tet2	GCAGCTGCCAACAAGAATGC	TGTTGCTGCTGCTGATGGAC	191	XM_005457001.3
	tet3	GCAAGCCAACCAACCAAACC	GATGTGTTGGCTCCGACCTG	177	XM_019365521.2
H3K4me3 writer (Histone Lysine methyltransferase)	*setd1a*	GGAACTCCGGTCTGGATGGT	CGAAGCTGCCCATCTGTGTT	172	XM_005468973.4
	setd1ba	AAGACAGGGAGGCAGCAGAA	CCTCAGGACTGGGAGGTCTG	198	XM_005470275.4
	kmt2a	AGAGCAGGAAAGCCAACAGC	CACTGGGCGTAGTTGTGGTC	178	XM_013274782.3
	kmt2ba	ACTCTGAGGGACCTGGAGGA	AGAGGAGGTGAAGCCGATCC	191	XM_013275905.3
	kmt2bb	GCTCCCGTCAGTGTGTCTTC	TCTGGCTCCAACCCAGTCAA	172	XM_013277028.3
H3K4me3 eraser (Histone lysine demethylases)	*kdm5a*	TCTGGCCACAGAGGAGTTGT	GTGACGTGGCTCTGCTGAAA	191	XM_005451728.4
	kdm5ba	TCTCAGAGCAGAGGGCATCC	GACCCGATGTCACACCTTGG	165	XM_003441348.2
	kdm5bb	CATCCCTGCCTACCTCCCAA	AAGGCTCCAGGTGGACTTGA	170	XM_003439103.5
	kdm5c	CTCTCCACCCTGGAGGCAAT	AGCTACCAGGCCCTCCAAAT	174	XM_005448517.4
	riox1	CCACCTGGCACACAAGGATT	TCCGGCTTCTACCACCACAT	192	XM_005475002.4
H3K9me3 specific writer	*suv39h1b*	TCCAACGCATGGCCTACAAC	CTTGATGTGCTGCAGTGTGC	197	XM_003459875.5
H3K9me3 and H3K36me3 eraser	*kdm4aa*	CGGATGCGAACCAAACCTCT	GGCTGGATCGACACCGTAAC	180	XM_005457300.3
	kdm4ab	TCTGTTCAGGGAGGCACACA	GCCTGTTGGCCCATCTGTTT	162	XM_005476068.4
	kdm4b	TGCTCGCTCTTCTGTCCGTA	AGCAGATCAGGAGGCTGGTT	196	XM_005453970.4
	kdm4c	CCTGCAGAGGAATGCAGTGG	GCACAGGTGCAATCTGGTGA	176	XM_005456806.2
H3K36me3 specific writer	*setd2*	AGGCAGCGATGACTTCAAGC	ATCTTGTGGCGTCCCACTCT	182	XM_019364854.2
H3K9ac specific writer	*kat2a*	CACTGACCCTGCTGCTATGC	GTAGGCCAACCAGCCACATC	173	XM_025906390.1
	kat2b	GGCCTTTCATGGAGCCTGTG	CTCGCTCTCTGGAGGGTTGT	188	XM_003444058.3
	kat6a	CATCCCGTCCACTGCTTTCC	CCTGTTCACGCTACCACCAC	173	XM_005472980.3
	gtf3c4	CTTGTGGCGGTTCAAGCTCT	GGCTCGCCTTCCTCTTTCAC	174	XM_003440231.5
H3K9ac specific eraser	*sirt2*	GCGAGTCTAGTCAGCAGGGT	CCCAGAAGATCAGCTAGAGCCA	197	XM_003449264.5
	sirt5	ATTTGCCCAGGTGTGAGCAG	GAGCAAACATGGCTGCAGGA	177	XM_003457306.5

* From Yang et al. (2013)

5.3.6 Statistical analysis

The statistical model utilized was $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where y_{ij} was the response, μ was the general means, α_i was dietary (LC/HP and HC/LP) effects and ε_{ij} was the random error. All data were analyzed using SPSS for Windows version 22 (SPSS Inc., Chicago, IL, USA). An independent sample t-test was performed to analyze the

differences between the LC/HP and HC/LP groups. The effects and differences were considered significant at $P<0.05$.

5.4 Results

5.4.1 Effects of a high-carbohydrate diet on body weight, reproductive performance, and intermediary metabolism of Nile tilapia broodstock

In this study, we recorded the body weight of the broodstock. There were no significant differences in body weights during the experimental period (Fig. 2A, B). During this period, we collected fertilised eggs twice at day 14 and 28 of the feeding period, and different dietary CHO levels resulted in different reproductive performances, including fecundity and egg weight (Fig. 2C, D, F). At both times of fertilised egg collection, HC/LP-diet females had a lower egg weight and higher fecundity, compared with those of LC/HP-diet females ($P<0.05$). On day 38 of feeding, the ovary and testes were collected from the female and male broodstocks. A higher GSI was observed in females fed the LC/HP diet than that in those fed the HC/LP diet ($P<0.05$) (Fig. 2E). No significant differences were observed in the GSI of the males ($P>0.05$; Fig. 2E).

After day 38 of the feeding period, plasma metabolites, including glucose, cholesterol, triglycerides, total protein, and BUN were measured to reflect the effects of dietary CHO (Table 5.3). In both males and females, glucose and triglyceride levels increased in response to the HC/LP stimuli ($P<0.05$), and the total protein content decreased in the female HC/LP group ($P<0.05$). However, no significant differences in cholesterol or BUN levels were observed between the experimental groups ($P>0.05$). Table 2 shows the effects of dietary CHO on liver and muscle composition. Both males and females showed similar trends in the effect of dietary CHO on chemical composition: 1) HC/LP fish exhibited higher HSI, lipid, glycogen, and triglyceride content; and 2) HC/LP fish had lower protein content compared with that in LC/HP fish ($P<0.05$) (Table 5.3). Muscular contents were similar to those observed in the liver; significant differences were found only in glycogen and triglyceride contents in females, as well as in protein, lipid, and glycogen in males ($P<0.05$; Table 2).

The responses at the molecular level of CHO metabolism in the liver and muscle of Nile tilapia broodstock fed different CHO diets are shown in Table 5.4.

The HC diet induced the upregulation of genes related to glycolysis (both male and female; *pfklr* and *pklr*) and lipogenesis (both male and female, *fasn* and *g6pd*) and downregulation of genes associated with gluconeogenesis (female, *pck1*; male, *g6pca2* and *pck1*) and amino acid catabolism (female, *alat*, *asat,* and *gdh*; male, *alat* and *gdh*) in the liver ($P<0.05$). In the muscle, *glut4* (an enzyme related to glucose transport) was induced in females fed HC/LP ($P<0.05$). In addition, dietary HC/LP induced muscle glycolysis with the upregulation of *hk1*, *pfkma*, *pfkmb,* and *pkma* in females and *pfkma* in males ($P<0.05$) (Table 5.4).

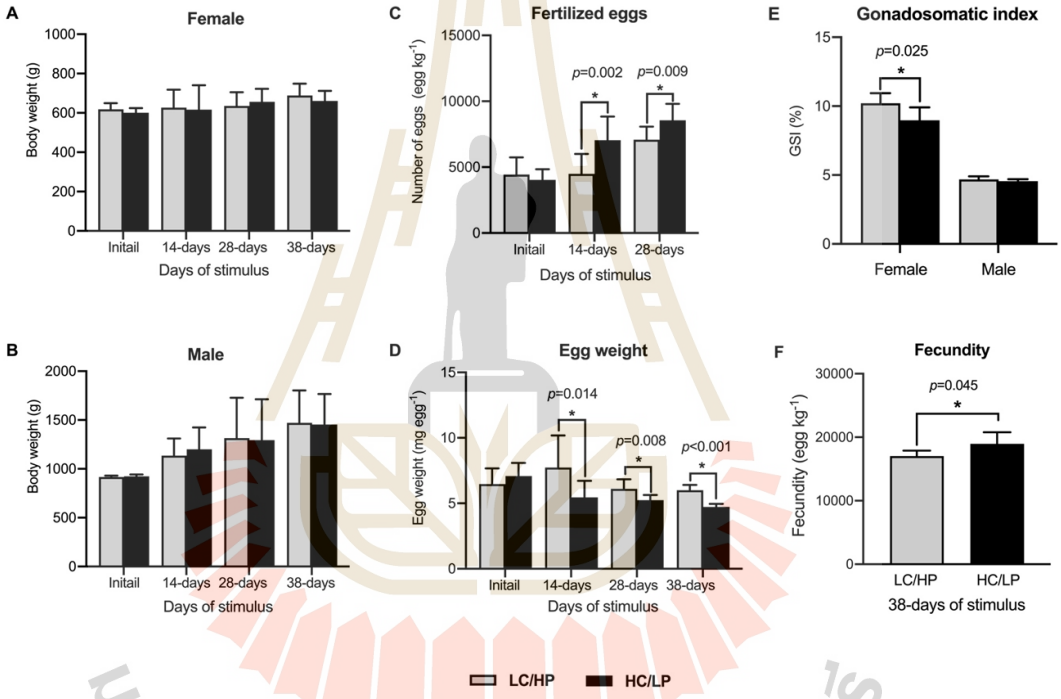

Figure 5.2 Final body weight and reproductive performances of experimental Nile tilapia broodstock. Final body weight of female (A) and male (B) broodstock fed with low-carbohydrate/high-protein (LC/HP) and high-carbohydrate/low-protein (HC/LP) diets. For reproductive performances, number of fertilised eggs (C) and egg weight (D) were recorded at initial (at earthen pond of SUT farm) and at 14- and 28- days of LC/HP or HC/LP feeding. Gonadosomatic index (GSI) (E) and fecundity (F) were determined at 38-days of experimental diet feeding. The asterisk (*) denotes a statistically significant difference between LC/HP and HC/LP dietary stimuli at $P<0.05$

Table 5.4 Plasma metabolites and chemical compositions of broodstock fed experimental diets for 38 days (mean ± SD; female, n = 6; male, n = 3).

	Female			Male		
	LC/HP	HC/LP	*P*-value[1]	LC/HP	HC/LP	*P*-value
Plasma metabolites						
Glucose (mmol L^{-1})	3.68 ± 0.24	4.53 ± 0.35	<0.001	3.65 ± 0.29	4.38 ± 0.21	0.024
Triglyceride (mmol L^{-1})	0.87 ± 0.07	1.14 ± 0.12	<0.001	0.92 ± 0.08	1.13 ± 0.07	0.032
Cholesterol (mmol L^{-1})	1.93 ± 0.19	2.06 ± 0.27	0.359	1.80 ± 0.15	1.90 ± 0.12	0.383
Total Protein (g L^{-1})	36.36 ± 4.26	30.73 ± 3.31	0.029	35.15 ± 4.67	30.61 ± 4.67	0.299
BUN[2] (mmol L^{-1})	1.67 ± 0.37	1.55 ± 0.54	0.661	1.43 ± 0.71	1.19 ± 0.41	0.646
Liver						
HSI[3] (%)	1.14 ± 0.22	3.18 ± 0.31	<0.001	2.02 ± 0.18	2.86 ± 0.24	0.009
Protein (g kg^{-1})	144.99 ± 2.80	84.65 ± 2.32	<0.001	109.98 ± 0.81	76.17 ± 1.21	<0.001
Lipid (g kg^{-1})	21.40 ± 1.69	40.50 ± 1.62	<0.001	31.25 ± 3.05	59.33 ± 0.01	0.004
Glycogen (mg g^{-1})	335.38 ± 55.89	826.79 ± 72.63	<0.001	392.18 ± 20.81	898.39 ± 39.41	<0.001
Triglyceride (mg g^{-1})	20.67 ± 1.77	24.80 ± 1.93	0.003	13.15 ± 1.76	67.95 ± 11.49	0.013
Muscle						
Protein (%)	209.04 ± 3.37	205.97 ± 3.63	0.161	213.95 ± 4.88	193.69 ± 2.95	0.004
Lipid (%)	6.69 ± 2.93	8.48 ± 0.77	0.201	5.05 ± 1.91	9.74 ± 1.69	0.034
Glycogen (mg g^{-1})	23.67 ± 6.26	55.38 ± 23.76	0.010	22.19 ± 7.82	108.45 ± 14.52	0.001
Triglyceride (mg g^{-1})	1.35 ± 0.08	1.95 ± 0.48	0.028	1.46 ± 0.02	2.03 ± 0.32	0.090

[1]An independent t-test was used to analyse the effects of different stimuli (HP/LC and LP/HC diets). HC, high-carbohydrate; HP, high-protein; LC, low-carbohydrate; LP, low-protein.

[2]BUN: blood urea nitrogen

[3]Hepatosomatic index (HSI) = 100 × (liver weight / fish body weight)

Table 5.5 Plasma metabolites and chemical compositions of broodstock fed experimental diets for 38 days (mean ± SD; female, n = 6; male, n = 3).

	Female			Male		
	LC/HP	HC/LP	*P*-value[1]	LC/HP	HC/LP	*P*-value
Plasma metabolites						
Glucose (mmol L^{-1})	3.68 ± 0.24	4.53 ± 0.35	<0.001	3.65 ± 0.29	4.38 ± 0.21	0.024
Triglyceride (mmol L^{-1})	0.87 ± 0.07	1.14 ± 0.12	<0.001	0.92 ± 0.08	1.13 ± 0.07	0.032
Cholesterol (mmol L^{-1})	1.93 ± 0.19	2.06 ± 0.27	0.359	1.80 ± 0.15	1.90 ± 0.12	0.383
Total Protein (g L^{-1})	36.36 ± 4.26	30.73 ± 3.31	0.029	35.15 ± 4.67	30.61 ± 4.67	0.299
BUN[2] (mmol L^{-1})	1.67 ± 0.37	1.55 ± 0.54	0.661	1.43 ± 0.71	1.19 ± 0.41	0.646
Liver						
HSI[3] (%)	1.14 ± 0.22	3.18 ± 0.31	<0.001	2.02 ± 0.18	2.86 ± 0.24	0.009
Protein (g kg^{-1})	144.99 ± 2.80	84.65 ± 2.32	<0.001	109.98 ± 0.81	76.17 ± 1.21	<0.001
Lipid (g kg^{-1})	21.40 ± 1.69	40.50 ± 1.62	<0.001	31.25 ± 3.05	59.33 ± 0.01	0.004
Glycogen (mg g^{-1})	335.38 ± 55.89	826.79 ± 72.63	<0.001	392.18 ± 20.81	898.39 ± 39.41	<0.001
Triglyceride (mg g^{-1})	20.67 ± 1.77	24.80 ± 1.93	0.003	13.15 ± 1.76	67.95 ± 11.49	0.013
Muscle						
Protein (%)	209.04 ± 3.37	205.97 ± 3.63	0.161	213.95 ± 4.88	193.69 ± 2.95	0.004
Lipid (%)	6.69 ± 2.93	8.48 ± 0.77	0.201	5.05 ± 1.91	9.74 ± 1.69	0.034
Glycogen (mg g^{-1})	23.67 ± 6.26	55.38 ± 23.76	0.010	22.19 ± 7.82	108.45 ± 14.52	0.001
Triglyceride (mg g^{-1})	1.35 ± 0.08	1.95 ± 0.48	0.028	1.46 ± 0.02	2.03 ± 0.32	0.090

[1]An independent t-test was used to analyse the effects of different stimuli (HP/LC and LP/HC diets). HC, high-carbohydrate; HP, high-protein; LC, low-carbohydrate; LP, low-protein.

[2]BUN: blood urea nitrogen

[3]Hepatosomatic index (HSI) = 100 × (liver weight/fish body weight)

5.4.2 Effects of a high-carbohydrate diet in broodstock on epigenetic stability through related enzyme modulators at the molecular level

In this study, we evaluated the expression of genes related to DNA methylation writer and eraser in liver (Table 5.6). Our results showed that, in liver, HC/LP diet down-regulated the DNA methylation writer (female; *dnmt1, dnmt3aa* and *dnmt3bb*, male; *dnmt1* and *dnmt3bb*) and up-regulated DNA methylation eraser (female; *tet1, tet2* and *tet3*, male; *tet1* and *tet3*), compared with the LC/HP diet (P <0.05). Similar results were found in muscle, i.e., the DNA methylation writer (female; *dnmt1* and *dnmt3bb*) was downregulation and its eraser (female; *tet1* and *tet2*, male; *tet1*) was upregulation on HC/LP group (P <0.05) (Table 5.7).

Table 5.8 shows the effects of dietary HC on the expression of genes related to hepatic histone methylation and acetylation. In the liver, the mRNA expression of H3K4me3 writers (*setd1a, setd1ba, kmt2a, kmt2ba*, and *kmt2bb*; except female *kmt2a*) and their erasers (*kdm5a, kdm5ba, kdm5bb*, and *kdm5c*) was upregulated in both females and males fed the HC/LP diet ($P < 0.05$). For H3K9me3, the expression of genes associated with its erasers (*kdm4aa, kdm4ab, kdm4b*, and *kdm4c*) was upregulated in both females and males fed the HC/LP diet ($P < 0.05$), whereas no significant difference was observed for the H3K9me3 writer (*suv39h1b*) ($P > 0.05$). In addition, *kat2a, kat6a*, and *gtf3c4* (H3K9ac writers) and *sirt2,* and *sirt5* (H3K9ac erasers) were upregulated in both females and males fed the HC/LP diet ($P < 0.05$). The expression of setd2 (H3K36me3 writer) was upregulated only in males fed the HC/LP diet ($P < 0.05$).

A similar trend was found in muscle. In the HC/LP group, H3K4me3 writers (females: *setd1a* and *kmt2ba*; males: *setd1a, kmt2ba*, and *kmt2bb*) and their erasers (females: *kdm5ba* and *kdm5c*; males: *kdm5a*, *kdm5ba*, *kdm5bb*, and *kdm5c*) were upregulated ($P < 0.05$). The HC/LP diet induced the expression of H3K9me3 erasers (females: *kdm4ab* and *kdm4c*; males: *kdm4ab*, *kdm4b*, and *kdm4c*) compared with the LC/HP group ($P < 0.05$). However, dietary CHO had no significant effect on the H3K9me3 writer (*suv39h1b*), the H3K36me3 writer (*setd2*), or the H3K9ac writers and their erasers, irrespective of sex ($P > 0.05$) (Table 5.9).

Table 5.6 mRNA levels of gene involved in DNA (de)methylation in liver of broodstock Nile tilapia that were fed with experimental diets including a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein (HC/LP) diet (mean ± SD, n = 6 female and n = 3 male).

Genes involved in		**Female**			**Male**		
Epigenetics modification		LC/HP	HC/LP	*P*-value	LC/HP	HC/LP	*P*-value
DNA methylation writers	*dnmt1*	3.01 ± 2.27	0.68 ± 0.35	0.032	3.61 ± 1.01	0.95 ± 0.33	0.012
	dnmt3aa	1.94 ± 0.77	1.14 ± 0.32	0.042	4.38 ± 1.43	2.18 ± 0.42	0.064
	dnmt3ab	2.18 ± 1.35	0.90 ± 0.43	0.052	3.77 ± 1.02	2.93 ± 0.50	0.270
	dnmt3ba	0.80 ± 0.25	0.83 ± 0.40	0.892	1.55 ± 0.73	2.87 ± 1.25	0.190
	dnmt3bb	1.98 ± 0.99	0.71 ± 0.19	0.025	2.75 ± 0.80	1.27 ± 0.14	0.035
DNA methylation eraser	*tet1*	0.89 ± 0.44	2.21 ± 1.15	0.026	2.41 ± 0.63	4.82 ± 1.30	0.044
	tet2	1.13 ± 0.58	2.26 ± 0.71	0.012	2.06 ± 0.45	2.94 ± 0.38	0.061
	tet3	0.83 ± 0.41	2.06 ± 0.96	0.017	1.82 ± 0.57	5.59 ± 0.92	0.004

[1]One-way ANOVA analysis was used to analyze the effects of different stimulus between a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein/ (HC/LP) diet.

Table 5.7 mRNA levels of gene involved in DNA (de)methylation in muscle of broodstock Nile tilapia that were fed with experimental diets including a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein (HC/LP) diet (mean ± SD, n = 6 female and n = 3 male).

Genes involved in		Female			Male		
Epigenetics modification		LC/HP	HC/LP	*P*-value	LC/HP	HC/LP	*P*-value
DNA methylation writers	*dnmt1*	1.38 ± 0.21	0.80 ± 0.25	0.001	0.82 ± 0.15	0.94 ± 0.21	0.444
	dnmt3aa	1.12 ± 0.28	0.99 ± 0.31	0.462	1.26 ± 0.18	1.13 ± 0.17	0.414
	dnmt3ab	1.22 ± 0.18	1.11 ± 0.20	0.353	1.32 ± 0.41	0.88 ± 0.37	0.233
	dnmt3ba	0.67 ± 0.09	0.89 ± 0.26	0.092	0.88 ± 0.25	0.84 ± 0.31	0.858
	dnmt3bb	1.12 ± 0.21	0.83 ± 0.22	0.043	0.81 ± 0.16	0.66 ± 0.21	0.369
DNA methylation eraser	*tet1*	0.98 ± 0.14	1.30 ± 0.21	0.014	0.95 ± 0.07	1.23 ± 0.09	0.014
	tet2	1.02 ± 0.16	1.33 ± 0.12	0.004	0.70 ± 0.07	1.07 ± 0.23	0.054
	tet3	0.97 ± 0.27	1.27 ± 0.25	0.074	0.73 ± 0.08	1.04 ± 0.23	0.098

[1]One-way ANOVA analysis was used to analyze the effects of different stimulus between a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein/ (HC/LP) diet.

Table 5.8 mRNA levels of gene involved in histone modifications in liver of broodstock Nile tilapia that were fed with experimental diets including a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein (HC/LP) diet (mean ± SD, n = 6 female and n = 3 male).

Genes involved in Epigenetics modification		Female			Male		
		LC/HP	HC/LP	*P*-value	LC/HP	HC/LP	*P*-value
H3K4me3 writer	*setd1a*	0.56 ± 0.08	0.80 ± 0.07	**<0.001**	0.49 ± 0.04	2.71 ± 0.09	**<0.001**
	setd1ba	0.65 ± 0.22	1.01 ± 0.19	**0.012**	0.58 ± 0.04	2.67 ± 0.13	**<0.001**
	kmt2a	0.66 ± 0.10	0.70 ± 0.07	0.413	0.71 ± 0.01	2.40 ± 0.26	**0.008**
	kmt2ba	0.53 ± 0.11	0.89 ± 0.08	**<0.001**	0.84 ± 0.07	2.37 ± 0.10	**<0.001**
	kmt2bb	0.71 ± 0.08	0.88 ± 0.16	**0.040**	0.60 ± 0.05	2.41 ± 0.14	**<0.001**
H3K4me3 eraser	*kdm5a*	0.77 ± 0.27	1.35 ± 0.19	**0.001**	0.39 ± 0.22	5.50 ± 1.32	**0.019**
	kdm5ba	0.46 ± 0.10	0.76 ± 0.16	**0.003**	0.49 ± 0.06	2.92 ± 0.25	**<0.001**
	kdm5bb	0.54 ± 0.03	0.91 ± 0.04	**<0.001**	0.44 ± 0.03	2.69 ± 0.14	**<0.001**
	kdm5c	0.54 ± 0.08	0.82 ± 0.05	**<0.001**	0.61 ± 0.03	2.92 ± 0.16	**<0.001**
	riox1	nd	nd		nd	nd	
H3K9me3 specific writer	*suv39h1b*	0.59 ± 0.39	0.78 ± 0.19	0.310	0.84 ± 0.13	2.04 ± 0.92	0.148
H3K9me3 specific eraser	*kdm4aa*	0.51 ± 0.13	0.73 ± 0.05	**0.003**	0.31 ± 0.04	2.93 ± 0.14	**<0.001**
	kdm4ab	0.73 ± 0.03	1.01 ± 0.10	**0.001**	0.21 ± 0.05	3.05 ± 0.20	**0.001**
	kdm4b	0.30 ± 0.10	0.84 ± 0.10	**<0.001**	0.32 ± 0.03	4.39 ± 0.23	**<0.001**
	kdm4c	0.29 ± 0.10	0.84 ± 0.09	**<0.001**	0.16 ± 0	4.30 ± 0.23	**0.001**
H3K36me3 specific writer	*setd2*	0.58 ± 0.12	0.64 ± 0.03	0.221	0.54 ± 0.08	2.11 ± 0.44	**0.022**
H3K9ac specific writer	*kat2a*	0.32 ± 0.12	1.28 ± 0.10	**<0.001**	0.16 ± 0.05	2.86 ± 0.34	**0.004**
	kat2b	nd	nd		nd	nd	
	kat6a	0.54 ± 0.07	0.72 ± 0.07	**0.001**	0.81 ± 0.05	2.68 ± 0.04	**<0.001**
	gtf3c4	0.44 ± 0.10	0.82 ± 0.16	**0.001**	0.43 ± 0.06	2.27 ± 0.11	**<0.001**
H3K9ac specific eraser	*sirt2*	0.47 ± 0.10	0.71 ± 0.09	**0.001**	0.24 ± 0.03	3.10 ± 0.28	**<0.001**
	sirt5	0.51 ± 0.10	0.77 ± 0.12	**0.002**	0.42 ± 0.01	2.88 ± 0.21	**0.002**

[1]One-way ANOVA analysis was used to analyze the effects of different stimulus between a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein/ (HC/LP) diet.

Table 5.9 mRNA levels of gene involved in histone modifications in muscle of broodstock Nile tilapia that were fed with experimental diets including a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein (HC/LP) diet (mean ± SD, n = 6 female and n = 3 male).

Genes involved in Epigenetics modification		Female			Male		
		LC/HP	HC/LP	*P*-value	LC/HP	HC/LP	*P*-value
H3K4me3 writer	*setd1a*	0.61 ± 0.04	0.73 ± 0.03	**<0.001**	0.55 ± 0.02	0.72 ± 0.04	**0.003**
	setd1ba	0.55 ± 0.04	0.59 ± 0.05	0.257	0.51 ± 0.04	0.61 ± 0.05	0.057
	kmt2a	0.62 ± 0.06	0.67 ± 0.03	0.071	0.52 ± 0.04	0.53 ± 0.01	0.716
	kmt2ba	0.57 ± 0.05	0.73 ± 0.07	**0.001**	0.42 ± 0.03	0.52 ± 0.05	**0.032**
	kmt2bb	0.65 ± 0.03	0.68 ± 0.04	0.123	0.42 ± 0.02	0.60 ± 0.04	**0.003**
H3K4me3 eraser	*kdm5a*	0.60 ± 0.08	0.70 ± 0.17	0.262	0.38 ± 0.05	0.56 ± 0.05	**0.010**
	kdm5ba	0.55 ± 0.05	0.66 ± 0.06	**0.010**	0.43 ± 0.04	0.75 ± 0.12	**0.015**
	kdm5bb	0.62 ± 0.03	0.65 ± 0.05	0.251	0.40 ± 0.04	0.58 ± 0.01	**0.001**
	kdm5c	0.57 ± 0.05	0.66 ± 0.08	**0.040**	0.51 ± 0.02	0.61 ± 0.04	**0.015**
	riox1	nd	nd		nd	nd	
H3K9me3 specific writer	*suv39h1b*	0.56 ± 0.15	0.44 ± 0.13	0.146	0.56 ± 0.18	0.84 ± 0.06	0.061
H3K9me3 specific eraser	*kdm4aa*	0.55 ± 0.17	0.45 ± 0.08	0.222	0.68 ± 0.08	0.66 ± 0.10	0.802
	kdm4ab	0.68 ± 0.04	0.78 ± 0.06	**0.006**	0.42 ± 0.02	0.51 ± 0.05	**0.035**
	kdm4b	0.63 ± 0.07	0.58 ± 0.05	0.161	0.60 ± 0.04	0.75 ± 0.05	**0.017**
	kdm4c	0.52 ± 0.03	0.70 ± 0.06	**<0.001**	0.43 ± 0.02	0.53 ± 0.01	**0.001**
H3K36me3 specific writer	*setd2*	0.63 ± 0.03	0.63 ± 0.03	0.763	0.42 ± 0.01	0.68 ± 0.17	0.115
H3K9ac specific writer	*kat2a*	0.50 ± 0.18	0.46 ± 0.08	0.578	0.67 ± 0.08	0.61 ± 0.13	0.533
	kat2b	nd	nd		nd	nd	
	kat6a	0.56 ± 0.21	0.45 ± 0.09	0.295	0.66 ± 0.12	0.67 ± 0.07	0.939
	gtf3c4	0.55 ± 0.18	0.51 ± 0.10	0.628	0.71 ± 0.15	0.75 ± 0.02	0.642
H3K9ac specific eraser	*sirt2*	0.55 ± 0.05	0.52 ± 0.03	0.348	0.71 ± 0.05	0.71 ±0.02	1.000
	sirt5	0.52 ± 0.12	0.53 ± 0.10	0.860	0.72 ± 0.02	0.60 ± 0.09	0.098

[1]One-way ANOVA analysis was used to analyze the effects of different stimulus between a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein/ (HC/LP) diet.

5.4.3 Effects of a high-carbohydrate diet in broodstock on growth performance and intermediary CHO metabolism in Nile tilapia offspring at early developmental stages

The growth performance of the offspring from the broodstock stimulated with the LC/HP and HC/LP diets is shown in Table 5.10. The 7-dph larvae obtained from the HC/LP group were smaller than those obtained from the LC/HP group ($P<0.05$). The 7-daf HC/LP fry demonstrated restored growth rate and appeared to have higher body weight, and the body weight, weight gain, average daily gain, and feed conversion ratio appeared to be similar between the fry of the HC/LP and LC/HP broodstock ($P>0.05$).

The chemical compositions of the entire bodies of the offspring are shown in Table 5.11 Seven days post-hatching, the CF, glycogen, and triglyceride contents were significantly increased in the offspring from broodstock fed the HC/LP diet ($P<0.05$). After the offspring were fed exogenous feed for the first time for 7 days, increases in glycogen and triglyceride content were still observed. However, in the HC/LP broodstock offspring, the CP content decreased ($P<0.05$).

The effects of parental HC on glucose and its related metabolic pathways at the molecular level in 7-dph and 7-daf offspring are shown in Table 5.12. In 7-dph larvae, upregulation of glycolytic genes (*pfkma* and *pfkmb*) and downregulation of genes related to hepatic gluconeogenesis (*g6pca1*) and amino acid catabolism (*alat* and *gdh*) were observed in offspring from the HC/LP fed broodstock ($P<0.05$). No significant differences were observed in the expression of *gck, pfklr*, *pklr*, *fasn*, *g6pd*, *g6pca2, pck1, pck2, asat, glut4, hk1, hk2*, and *pkma* in the whole body of the offspring according to parental diet ($P>0.05$). In 7-daf fry, upregulation of genes associated with glycolysis (*pklr, hk2,* and *pfkmb*) and glucose transport (*glut4*) and downregulation of gluconeogenesis-related genes (*g6pca1* and *g6pca2*) were observed in the offspring of broodstock fed the HC diet ($P<0.05$). However, no significant differences were observed in the expression of *gck, pfklr, pck1, pck2, fasn, g6pd, alat, asat, gdh, hk1, pfkma,* and *pkma* ($P>0.05$).

Table 5.10 Growth performances of offspring from Nile tilapia broodstock fed with low-carbohydrate/high-protein (LC/HP) and high-carbohydrate/low-protein (HC/LP) diets.

Offspring stages	Broodstock stimulus diets	Final weight (g)	Weight gain (g)	ADG[2] (g day^{-1})	SGR[3] (% day^{-1})	FCR[4]
7 days post-hatching	LC/HP	10.39 ± 0.06				
(7dph)[1]	HC/LP	10.10 ± 0.03				
	p-value[5]	<0.001				
7 days after first-feeding	LC/HP	33.29 ± 0.49	23.19 ± 0.56	3.31 ± 0.08	17.03 ± 0.34	0.93 ± 0.02
(7daf)[1]	HC/LP	34.17 ± 0.06	23.99 ± 0.22	3.43 ± 0.03	17.30 ± 0.30	0.90 ± 0.01
	p-value	0.007	0.016	0.016	0.191	0.017
Week 2	LC/HP	131.05 ± 1.10	120.94 ± 1.09	8.64 ± 0.08	18.30 ± 0.11	0.58 ± 0.01
(14 daf)[1]	HC/LP	130.23 ± 0.71	120.05 ± 0.58	8.57 ± 0.04	18.20 ± 0.13	0.59 ± 0.01
	P-value	0.160	0.105	0.108	0.167	0.418

[1] 7dph, 7daf and week 2: FW (final weight), mg; WG (weight gain), mg; ADG, mg/day

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

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

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

[5] An independent t-test was used to analyse the effects of different stimuli (HP/LC and LP/HC diets).

The number of fish samples per replication (n): 7-dph, 200 larvae; 7-daf and 14-daf, 100 fry, n = 6 per HC/LP and LC/HP groups.

Table 5.11 Chemical compositions in whole body of offspring from Nile tilapia broodstock that were fed with experimental diets including a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein (HC/LP) diet (mean ± SD, n = 6).

Parameters	Broodstock stimulus diets	Offspring stage	
		7 days post-hatching	7 days after first-feeding
Crude protein ($g\ kg^{-1}$)	LC/HP	158.15 ± 6.42	190.84 ± 6.99
	HC/LP	167.28 ± 14.29	168.52 ± 5.13
	P-value[1]	0.197	<0.001
Crude fat ($g\ kg^{-1}$)	LC/HP	30.60 ± 6.35	34.78 ± 7.72
	HC/LP	64.45 ± 11.85	37.69 ± 5.50
	P-value	<0.001	0.469
Crude ash ($g\ kg^{-1}$)	LC/HP	15.60 ± 0.92	15.21 ± 1.24
	HC/LP	15.18 ± 0.67	15.78 ± 1.69
	P-value	0.395	0.520
Glycogen ($mg\ g^{-1}$ tissue)	LC/HP	14.65 ± 3.02	15.42 ± 5.17
	HC/LP	33.63 ± 9.20	34.26 ± 6.19
	P-value	0.003	<0.001
Triglyceride ($mg\ g^{-1}$ tissue)	LC/HP	27.71 ± 1.84	21.21 ± 2.24
	HC/LP	34.51 ± 2.32	29.18 ± 4.13
	P-value	<0.001	0.003

[1]An independent t-test was used to analyse the effects of different stimuli (HP/LC and LP/HC diets).

Table 5.12 mRNA levels of genes related to intermediary carbohydrate metabolism in whole body of offspring from Nile tilapia broodstock that were fed experimental diets including a low-carbohydrate/high-protein (LC/HP), and high-carbohydrate/low-protein (HC/LP) diet (mean ± SD, n = 6).

	7 days post-hatching			7 days after first feeding		
Broodstock history	LC/HP	HC/LP	*P*-value[1]	LC/HP	HC/LP	*P*-value
Hepatic glycolysis						
gck	0.93 ± 0.29	1.18 ± 0.36	0.221	0.94 ± 0.20	1.09 ± 0.23	0.270
pfklr	0.93 ± 0.13	1.00 ± 0.10	0.321	0.83 ± 0.22	1.21 ± 0.43	0.082
pklr	0.90 ± 0.22	1.25 ± 0.34	0.057	0.66 ± 0.44	1.37 ± 0.37	0.012
Hepatic gluconeogenesis						
g6pca1	1.05 ± 0.17	0.61 ± 0.27	0.007	1.37 ± 0.27	0.67 ± 0.25	0.001
g6pca2	0.93 ± 0.28	0.73 ± 0.24	0.211	1.29 ± 0.45	0.62 ± 0.19	0.007
pck1	1.06 ± 0.45	1.13 ± 0.66	0.854	1.05 ± 0.31	0.72 ± 0.24	0.064
pck2	1.16 ± 0.39	0.93 ± 0.15	0.209	1.12 ± 0.39	0.79 ± 0.20	0.098
Hepatic lipogenesis						
fasn	0.88 ± 0.10	0.92 ± 0.08	0.453	0.85 ± 0.28	1.22 ± 0.41	0.094
g6pd	1.04 ± 0.16	1.08 ± 0.10	0.606	0.88 ± 0.25	1.08 ± 0.29	0.233
Hepatic amino acid catabolism						
alat	1.16 ± 0.28	0.47 ± 0.12	<0.001	1.23 ± 0.36	0.92 ± 0.30	0.150
asat	0.89 ± 0.08	0.87 ± 0.15	0.778	1.23 ± 0.36	1.01 ± 0.33	0.299
gdh	0.98 ± 0.09	0.77 ± 0.18	0.031	1.09 ± 0.19	0.93 ± 0.15	0.138
Glucose transport and muscle metabolism						
glut4	0.76 ± 0.24	0.93 ± 0.14	0.169	0.64 ± 0.14	1.53 ± 0.28	<0.001
hk1	1.07 ± 0.19	1.14 ± 0.10	0.451	0.93 ± 0.19	1.12 ± 0.40	0.327
hk2	0.96 ± 0.30	1.27 ± 0.21	0.063	0.74 ± 0.08	1.34 ± 0.41	0.016
pfkma	0.74 ± 0.13	0.99 ± 0.19	0.026	0.87 ± 0.28	1.22 ± 0.33	0.075
pfkmb	0.83 ± 0.14	1.03 ± 0.13	0.028	0.77 ± 0.11	1.34 ± 0.32	0.006
pkma	0.89 ± 0.14	1.03 ± 0.10	0.082	1.05 ± 0.21	1.16 ± 0.38	0.528

[1]An independent t-test was used to analyse the effects of different stimuli (HP/LC and LP/HC diets).

5.5 Discussion

Nile tilapia has been extensively studied regarding the adaptation of carbohydrate (CHO) metabolism in relation to growth performance and intermediary metabolism under various nutritional regimes. These include short- and long-term high-carbohydrate (HC) feeding, as well as HC refeeding following fasting in both juvenile and adult stages (Azaza et al., 2015; Boonanuntanasarn et al., 2018a,b; Thongchaitriwat et al., 2024). More recently, nutritional programming of carbohydrate metabolism has also been demonstrated in Nile tilapia, with several early-life intervention strategies—such as glucose injection into the yolk reserve and HC dietary feeding during the fry stage—successfully implemented. These interventions have been shown to enhance the fish's capacity to utilize carbohydrates, particularly in promoting the efficient use of HC diets to achieve a protein-sparing effect that supports growth during later life stages, including the juvenile and adult phases (Kumkhong et al., 2020a,b; Kumkhong et al., 2021; Srisakultiew et al., 2022). Furthermore, previous studies have demonstrated that broodstock diet can exert long-term effects on offspring metabolism (Izquierdo et al., 2001). Similar to findings in mammals, nutritional management of broodstock may serve as a viable strategy to influence both ontogenetic development and long-term metabolic outcomes in progeny (Riddle and Hu, 2021). Therefore, like mammals, the management of broodstock diet could be another nutritional intervention strategy influencing ontogeny metabolism and metabolism later in life (Riddle and Hu, 2021), and epigenetic modification may be a mechanism regulating this metabolic modulation. In the present study, we investigated the effects of a high-carbohydrate diet in both male and female broodstock on reproductive performance, intermediary carbohydrate metabolism, and the regulation of epigenetic modulators at the molecular level. In addition, we demonstrated that offspring derived from high-carbohydrate-fed broodstock exhibited compensatory growth upon receiving an exogenous diet, reflecting parental metabolic programming of carbohydrate metabolism.

5.5.1 Effects of a high-carbohydrate diet on body weight and reproductive performance of Nile tilapia broodstock

Nile tilapia are good users of CHO, and the optimum level of dietary CHO for growth depends on the growth stage. In this study, our results showed that Nile tilapia broodstock, both male and female, fed dietary HC/LP for up to 38 days, did

not exhibit a decrease in growth performance. Similarly, although rainbow trout is a poor user of CHO (carnivorous), a 2-year-old broodstock fed with up to 35% dietary CHO for 1 year showed no negative effects on growth performance (Callet et al., 2020). Broodstock fish (both omnivorous and carnivorous) appear to be able to utilise high levels of dietary CHO, at least in the short term, without any negative effects on growth performance. These findings provide a practical stimuli method for broodstock to further investigate the NP of CHO in broodstocks.

Female broodstock had high fecundity, but low egg weight, when fed the HC/LP diet for up to 28 days, demonstrating a trade-off between egg number and size. It was revealed that dietary lipid at a moderate level increased fecundity in *Oreochromis karongae* broodstock (Nzohabonayo et al., 2017). Additionally, low dietary protein levels in broodstock have been associated with reduced egg size in fish (Masrizal et al., 2015). The HC/LP diet induced an elevation of plasma triglycerides, as well as hepatic and muscular lipids, along with a reduction in plasma protein levels in broodstock, suggesting its potential role in increasing fecundity while concurrently reducing egg weight. A negative relationship between offspring number and size has been demonstrated in cichlids (Kolm et al., 2006). Notably, low GSI was found in female broodstock, but not in males fed the HC/LP diet for 38 days. Coincidentally, rainbow trout females fed a 40% CP/35% CHO diet showed higher relative fecundity with no differences in egg size, but lower GSI compared to those fed a 60% CP/0% CHO diet (Callet et al., 2020, 2022). Our data confirm that nutritional status plays an important role in regulating the reproductive performance (Cerdá et al., 1994; Izquierdo et al., 2001; Chong et al., 2004; Iqbal et al., 2021). Thus, dietary CHO influences reproductive performance in fish broodstock, and its effects may vary according to fish feeding habits.

5.5.2 Effects of a high-carbohydrate diet on intermediary CHO metabolism and its related metabolic pathways of Nile tilapia broodstock

Previous reports on the long- (26 weeks) and short-term (4 days) effects of dietary HC on intermediary metabolism and responsive glucose and related metabolic pathways were demonstrated in Nile tilapia (Boonanuntanasarn et al., 2018a,2018b; Thongchaitriwat et al., 2024). In this study, after we obtained fertilised eggs on days 14 and 28, we decided to perform sampling to determine the effects of

dietary HC/LP in the broodstock (at day 38). Our results showed that, compared to LC/HP broodstock, male and female fish showed obvious intermediary metabolic responses to dietary HC in the plasma, liver (increased glycolysis/gluconeogenesis, lipogenesis and decreased amino acid catabolism at a molecular level), and muscle. The metabolic responses at the molecular level were found to be similar to those reported in previous studies (Boonanuntanasarn et al., 2018a,2018b; Thongchaitriwat et al., 2024). According to the optimum sex ratio for natural breeding in Nile tilapia, the number of male individuals was lower than that of females; nonetheless, the CHO metabolic responses were similar to those observed in females. Overall, these findings show that Nile tilapia broodstock responded as expected to dietary HC. Adult Nile tilapia responded to the HC diet after a short feeding period (4 days). To ensure HC stimulus without negative effects on reproductive performance and/or avoid selection bias, this study used offspring from broodstock fed for 14 days to investigate the parental NP effect. Similarly, a 2-week exposure to an HC diet without adverse effects on broodstock reproductive performance or embryogenesis produced an effective parental NP impact in yellow catfish (Xu et al., 2024). Whether HC feeding in the broodstock could affect CHO and its related metabolism in offspring was discussed in the following sections.

5.5.3 Effects of high-carbohydrate feeding on epigenetic regulators in Nile tilapia broodstock

Metabolic heritability responses to nutrient intakes could be partly linked with epigenetics stability for upregulation and/or downregulation of metabolic enzymes and factors. Among epigenetic changes, DNA methylation and histone modification have been revealed to be the most important factors involving in regulation of metabolic processes of specific nutrients (Tokunaga et al., 2013; You et al., 2020; Xie et al., 2023). DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that maintaining mdC (Dnmt1) during replication and forming mdC (Dnmt3a and Dnmt3b) by transferring a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a dC (Moore et al., 2013). DNA demethylation is controlled by ten-eleven translocation (TETs) protease, which catalyzes 5mC to 5hmC, and then 5hmC is processed to produce 5-fC and 5-cadC, which is eventually converted to cytosine (Kohli and Zhang, 2013). It was revealed that cooperation of

DNMT3A and TET1 regulated promoter epigenetic landscapes and gene expression in mouse embryonic stem cells (Gu et al., 2018). Inhibition of the DNMT1 activity was revealed to involved in a mechanism of hypomethylation that is regulated by hyperglycemia (Shin et al., 2023). In mammals, the diabetic conditions upregulated DNA methylation eraser genes (*tet1, tet2, tet3*) in rat. Also, upregulation of *tet2* and *tet3* was detected in humans with type 2 diabetes mellitus (Yuan et al., 2019). Our results showed that comparing with LC/HP fish, down-regulation of genes involved in DNA methylation writer (female; *dnmt1, dnmt3aa*, *dnmt3bb*, male; *dnmt1, dnmt3bb*) together with up-regulation of genes related to DNA methylation eraser (female; *tet1, tet2, tet3,* male; *tet1, tet3*) were detected in liver of HC/LP fish feeding for 38 days which were corelated to global DNA hypomethylation. Similar trend of up-regulation and down-regulation of genes associated with DNA methylation writer (female; *dnmt1, dnmt3bb*) and eraser (female; *tet1, tet2*, male; *tet1*), respectively, was determined in muscle of experimental Nile tilapia broodstock. In adult Nile tilapia, short-term refeeding with a high-CHO diet exhibited muscular DNA hypomethylation, which might be in accordance with the upregulation of a DNA methylation eraser, *tet* (Thongchaitriwat et al., 2025). However, in juvenile rainbow trout, when compared to fish fed with non-CHO diet, subsequent to fasting, refeeding with HC for 4 days downregulated genes related to DNA methylation writer (*dnmt3aa, dnmt3ab1 , dnmt3ab2, dnmt3ba2*) and methylation eraser (*tet1a*). Because of the complexity of the rainbow trout genome, these examined genes might not be responsible for the observed DNA hypomethylation according to HC diet (Liu et al., 2020). Therefore, with respect to hypomethylation according to hyperglycemia, modulation of expression of genes related to DNA methylation writer and eraser would depend on fish species/habit and stage of fish as well as duration of HC feeding.

Histone methylation and acetylation change chromatin structure which therefore generate epigenetic modified regulating gene expression (Tokunaga et al., 2013; Xie et al., 2023). In type 2 diabetic mice, using western blotting, methylated H3K4me and H3K9me2 increased while acetylated H3K9 and H3K23 decreased in liver (Tu et al., 2015). In rat, increased acetylation of histone 3 lysine residues such as 56 (H3K56), H3K14, H3K9, and H3K27 were found in both *in vivo* embryos of diabetic mothers and *in vitro* embryos incubated with glucose (Yu et al., 2016). In addition,

epigenetic regulators of chromatin through histone modifications are reversible epigenetic changes that are related to the dynamic balance of methylation/acetylation writer and eraser (Hyun et al., 2017), which could be influenced by dietary nutrients (i.e., CHO, protein). Therefore, this study aimed to investigate the effects of high-carbohydrate (HC) feeding on the regulation of histone modification modulators at the molecular level, which play a crucial role in the methylation and acetylation status of histone marks.

After 38 days of high-carbohydrate (high-CHO) feeding in broodstock, upregulation of genes involved in H3K9me3 and H3K36me3 erasure (*kdm4aa, kdm4ab, kdm4b,* and *kdm4c*) was observed in the liver of both female and male broodstock, suggesting hypomethylation of H3K9 and H3K36 in females, as well as hypomethylation of H3K9 in males. However, high-CHO feeding in broodstock also appeared to promote the expression of several genes associated with H3K4me3 and H3K9ac modifications, including both writer genes (*setd1a, setd1ba, kmt2a, kmt2ba, kmt2bb, suv39h1b, setd2, kat2a, kat6a,* and *gtf3c4*) and eraser genes (*kdm5a, kdm5ba, kdm5bb, kdm5c, kdm4aa, kdm4ab, kdm4b, kdm4c, sirt2,* and *sirt5*), suggesting that the high-carbohydrate/low-protein (HC/LP) diet influenced the histone modification landscape via methylation and acetylation modulators.

Similar trends in histone modifications related to the HC diet were observed in muscle, suggesting muscular hypomethylation of H3K9 and H3K36 through upregulation of the kdm4 gene family. Therefore, HC feeding in Nile tilapia broodstock appeared to induce histone methylation and acetylation through epigenetic modulators in both the liver and muscle. In carnivorous Mandarin fish, feeding a carbohydrate-rich diet followed by an anorexic condition could induce hyperglycemia, which increased H3K4me3 levels and upregulated *setd1b* expression in the liver. In addition, elevated H3K4me3 levels could activate the expression of the gluconeogenic gene *pepck* (You et al., 2020). In contrast, in 4-day fasted rainbow trout, refeeding with a high-carbohydrate (high-CHO) diet for four days resulted in a decrease in H3K9ac protein levels in the liver compared with fish refed a no-carbohydrate diet (Marandel et al., 2016). In Nile tilapia, short-term refeeding with a high-CHO diet promoted H3K36 hypomethylation and H3K9 hypoacetylation in the liver and muscle, which might be in accordance with the upregulation of histone methylation (*kdm4*) and acetylation

eraser (*sirt5*). Taken together, hyperglycemic conditions could lead to histone modifications; nonetheless, further studies on metabolism-related histone modifications are required to draw a definitive conclusion.

5.5.4 Effects of high-carbohydrate diet in broodstock on growth performance and intermediary CHO metabolism in Nile tilapia offspring at early developmental stages

The HC- mediated nutritional stimulation in the early life from first feeding for a short (7 days) and long term (28 days) of Nile tilapia fry lowered the growth of fry; however, compensatory growth occurred and resulted in a similar growth pattern during later development (Kumkhong et al., 2020; Srisakultiew et al., 2022). In this study, compared to the broodstock fed the LC/HP diet, a lower body weight of 7-dph fry was observed in the offspring of broodstock fed the HC/LP diet because of the smaller size of eggs. However, the 14-day offspring of HC/LP exhibited compensatory growth (with superior growth [7-daf fry] of fry of the LC/HP diet broodstock). Taken together, although high dietary CHO stimuli in Nile tilapia broodstock led to reduced embryo size, early larvae and fry compensated for their growth.

Direct HC intervention at early life stages significantly affected CHO metabolic responses, which could suggest the induction of glycogenesis and lipogenesis in Nile tilapia. For example, dietary HC stimulation in Nile tilapia fry at the first feeding for 1 or 4 weeks as well as early CHO intervention by glucose injection into the yolk reserve promoted the accumulation of glycogen and triglycerides in the body (Kumkhong et al., 2020a,2020b, 2021; Srisakultiew et al., 2022). In fish, the maternal diet affects embryonic development (Riddle and Hu, 2021). Thus, maternal metabolic responses reflect the chemical composition of the offspring, particularly in larvae before exogenous feeding. Our results showed that the whole-body composition, including increments of glycogen, triglycerides and CF, and low protein of the two tested stages (7-dph larvae and/or 7-daf fry) might be partly due to maternal accumulation effects and/or ontogenic metabolic responses, influenced by both CHO and protein levels in the broodstock diet. Coincidentally, dietary HC in parents (both female and male) could modulate whole-body composition in offspring, including the following: 1) paternal HC history decreased protein and increased glycogen in offspring; and 2) maternal HC history lowered hepatic glucose content in offspring (Callet et al.,

2021). Thus, in fish, CHO and its associated metabolic pathways in offspring (larvae and fry) may be related to the parental HC diet. These findings strongly suggest that the modification of metabolic pathways in the offspring could be partly achieved by the nutrition of parents.

Although the body composition of offspring appeared to be closely related to that of their parents at the molecular level, a few changes were observed in glucose and its related metabolic pathways. Several modulations of genes indicated CHO metabolism responses according to parental HC such as induction of glycolysis and glucose transport as well as suppression of gluconeogenic pathway and amino acid catabolism were observed in 7-dph larvae and 7-daf fry. These CHO responses were similar to the findings obtained after applying the HC feeding stimuli, from the first feeding for short (7 days) and long (28 days) periods, in whole-body fry (Kumkhong et al., 2020a; Srisakultiew et al., 2022). In zebrafish, 4 days post-fertilisation embryos with a history of glucose injection appeared to induce whole-body glycolysis at the molecular level (Rocha et al., 2015). In yellow catfish, parental NP has been shown to induce the expression of glycolytic (*gk, pk*, and *pfk*) and glucose transport (*sglt1*) genes in the offspring; however, there were no effects on gluconeogenesis (*pepck* and *g6pc*) (Xu et al., 2024). Taken together, parental dietary HC feeding could be an effective method for modulating CHO metabolism in offspring, but the degree of metabolic modification might depend on the fish species and experimental condition.

5.6 Conclusion

In conclusion, dietary high-carbohydrate (HC) intake in female broodstock increased fecundity without negatively affecting overall growth, although it was associated with a reduction in ovulated egg size. Despite their smaller initial size, the resulting larvae exhibited compensatory growth, eventually attaining comparable body weight after the initiation of exogenous feeding. The HC diet influenced several aspects of intermediary carbohydrate metabolism in both male and female broodstock, including indications of hyperglycemia, enhanced lipogenesis, and altered amino acid catabolism. At the molecular level, the HC diet upregulated hepatic glycolysis and lipogenesis while downregulating pathways associated with gluconeogenesis and amino acid catabolism. In muscle tissue, it also induced the expression of genes involved in

glycolysis and glucose transport. Dietary HC intake was associated with epigenetic modifications, which modulated the expression of several genes related to both DNA hypomethylation and histone hypomethylation in male and female broodstock. These parental dietary effects were reflected in the offspring, which exhibited similar intermediary metabolic responses under hyperglucidic conditions, indicating a transgenerational influence of dietary carbohydrate on metabolic programming.

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CHAPTER VI
GENERAL CONCLUSION

Nile tilapia (*Oreochromis niloticus*) exhibits pronounced metabolic adaptations to high carbohydrate (CHO) diets, including induced glycolysis and lipogenesis, accompanied by reduced gluconeogenesis and amino acid catabolism. In aquaculture systems, fasting and refeeding events frequently occur due to environmental fluctuations, influencing nutrient metabolism and potentially causing epigenetic responses. This study aims to investigate the effects of short-term refeeding with varying dietary CHO levels on intermediary carbohydrate metabolism and epigenetic modifications in juvenile and adult Nile tilapia. Additionally, it examines whether broodstock exposure to high-CHO diets enhances carbohydrate utilization efficiency in offspring, contributing to improved nutritional strategies for sustainable tilapia production.

In this study found that short-term refeeding after fasting compensated for body weight loss and promoted nutrient recovery in Nile tilapia by increased plasma triglycerides and cholesterol levels, as well as hepatic and muscular triglyceride and glycogen contents. These effects were diet dependent, with the HC/LP (high-CHO/low-protein) diet stimulating glycolysis, lipogenesis, and glycogen deposition, and the LC/HP (low-CHO/high-protein) diet enhancing amino acid catabolism. Overall, short-term HC refeeding exhibited metabolic responses similar to long-term feeding, highlighting the species' strong adaptability to dietary carbohydrates of Nile tilapia, however, the role of epigenetic regulation in this response remains unclear.

This study is the first to demonstrate that global DNA (de)methylation, histone modifications, and epigenetic modulators at the molecular level are influenced by both nutritional status and dietary CHO in Nile tilapia. Short-term refeeding after fasting altered epigenetic regulation, particularly through induced histone hypermethylation and hyperacetylation. Notably, refeeding with a HC/LP diet induced tissue-specific hypomethylation and hypoacetylation patterns in juvenile and adult fish, underscoring

the critical role of dietary CHO–protein balance in shaping epigenetic responses across life stages. Collectively, intermediary metabolism and epigenetic modulation influenced by nutritional status and short-term dietary CHO intake during two key growth stages may suggest that a similar mechanism underlies the effects of a high-carbohydrate diet in Nile tilapia broodstock on intermediary metabolism, reproduction, and offspring performance.

Feeding broodstock Nile tilapia a HC/LP diet altered female reproductive performance, increasing fecundity but reducing egg weight and gonadosomatic index, while not affecting body weight. The HC/LP diet elevated plasma glucose and triglycerides, hepatosomatic index, and hepatic lipid and glycogen contents, with sex-specific muscular responses. HC/LP feeding also modulated glycolysis, lipogenesis, gluconeogenesis, and amino acid catabolism in broodstock, and similar intermediary carbohydrate metabolic adaptations—including enhanced glycolysis and glucose transport and suppressed gluconeogenesis—were observed in their offspring during early development and after first feeding stages. In addition, dietary HC intake was associated with epigenetic modifications, which modulated the expression of several genes related to both DNA hypomethylation and histone hypomethylation in male and female broodstock. These parental dietary effects were reflected in the offspring, which exhibited similar intermediary metabolic responses under hyperglucidic conditions, indicating a transgenerational influence of dietary carbohydrate on metabolic programming.

BIOGRAPHY

Sirijanya Thongchaitriwat was born on April 9, 1997, in Mueang Nakhon Ratchasima, Nakhon Ratchasima Province, Thailand. She completed her secondary education at Boonwattana School, Mueang Nakhon Ratchasima, in 2015. In 2019, she obtained her Bachelor of Science degree in Animal Production Technology (Second-Class Honors) from the Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. In the same year, she was admitted to the Doctor of Philosophy Program in Biotechnology for Aquaculture, School of Animal Technology and Innovation, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Her doctoral research focuses on the nutritional and epigenetic regulation of carbohydrate metabolism in Nile tilapia (*Oreochromis niloticus*).