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**Around the poor use of dietary carbohydrate phenotype
in trout (*Oncorhynchus mykiss*): its epigenetic
consequences and metabolic modulation through a
programming strategy**

Jingwei Liu

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THÈSE

UNIVERSITE DE PAU ET DES PAYS DE L'ADOUR
ÉCOLE DOCTORALE 211-SCIENCES EXACTES ET LEURS APPLICATIONS

Présentée et soutenue le 24 Septembre 2019

par **Jingwei LIU**

pour obtenir le grade de docteur
de l'Université de Pau et des Pays de l'Adour
Spécialité : Sciences agronomiques, biotechnologies agro-alimentaires

**AROUND THE POOR USE OF DIETARY CARBOHYDRATE PHENOTYPE IN TROUT
(*Oncorhynchus mykiss*): ITS EPIGENETIC CONSEQUENCES AND METABOLIC
MODULATION THROUGH A PROGRAMMING STRATEGY**

**PHÉNOTYPE DE FAIBLE UTILISATION DES GLUCIDES ALIMENTAIRES CHEZ LA
TRUITE ARC-EN-CIEL (*Oncorhynchus mykiss*): SES CONSÉQUENCES
ÉPIGÉNÉTIQUES ET SA MODULATION MÉTABOLIQUE VIA UNE STRATÉGIE DE
PROGRAMMATION**

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I dedicate this thesis to my beloved family

You are my pillar of strength

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
°D	degree day
2-OG	2-oxoglutarate
2-OGDD	2-oxoglutarate-dependent dioxygenase
5-caC	5-carboxylcytosine
5-cadC	5-carboxylcytidine
5-fC	5-formylcytosine
5-fdC	5-formylcytidine
5-hmC	5-hydroxymethylcytosine
5-hmdC	5-hydroxymethylcytidine
5-hmU	5-hydroxymethyluracil
5-mC	5-methylcytosine
5-mdC	5-methylcytidine
8-oxodG	8-oxo-7,8-dihydroguanine
ABD-F	4-fluoro-7-sulfamoylbenzofurazan
ADD	ATRX-DNMT3-DNMT3L domain
AID	activation-induced cytidine deaminase
AML	in acute myeloid leukemia
AMPK	5' adenosine monophosphate-activated protein kinase
APOBEC	apolipoprotein B mRNA editing catalytic polypeptide-like
ATP	adenosine triphosphate
BAH	bromo-adjacent homology domain
BER	base excision repair
BHMT	betaine homocysteine methyltransferase
BLAST	Basic Local Alignment Search Tool

C	cytosine
C/EBP β	CCAAT-enhancer-binding protein β
cenH3	histone 3 variant
CGI	CpG island
CGS	CpG island shore
CHD	chromodomain helicase DNA-binding
CHO	carbohydrate
CpA	cytosine-phosphate-guanine
CpC	cytosine-phosphate-cytosine
CpG	cytosine-phosphate-guanine
CpT	cytosine-phosphate-thymine
CTGF	connective tissue growth factor
CTR	control
CXXC	CXXC zinc finger domain
Cys	cysteine
CysGly	cysteinylglycine
dC	cytidine
DHA	docosahexaenoic acid
DM	dry matter
DMAPD	DNA methyltransferase associated protein domain
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DO	dissolved oxygen
DSBH	double-stranded β -helix
EFA	essential fatty acid
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
ERK	extracellular signal-regulated kinases
ESC	embryonic stem cells
FAO	Food and Agriculture Organisation
<i>fbp</i>	fructose 1,6-bisphosphatase
FM	fishmeal
FO	fish oil
<i>g6pc</i>	glucose 6-phosphatase
<i>gck</i>	glucokinase
GCL	glutamate cysteine ligase
GluCys	γ -L-Glutamyl-L-Cysteine
GPX	glutathione peroxidase
GR	glutathione reductase
<i>Gr</i>	glucocorticoid receptor
GS	glutathione synthetase
GSH	reduced glutathione
GSSG	oxidized glutathione
H1	histone 1
H2A	histone 2A
H2A.B	histone 2A variant
H2A.X	histone 2A variant
H2A.Z	histone 2A variant
H2B	histone 2B
H3	histone 3
H3.3	histone 3 variant
H3K36me3	histone H3 lysine 36 tri-methylation
H3K4me3	histone H3 lysine 4 tri-methylation
H3K9ac	histone H3 lysine 9 acetylation
H3K9me3	histone H3 lysine 9 tri-methylation
H4	histone 4
HC	high-carbohydrate
hCys	homocysteine
HDAC	histone deacetylase
HIF	hypoxia-inducible factor

HMT	histone methyltransferase
<i>Hnf4α</i>	hepatocyte nuclear factor 4- α
HPLC	High Performance Liquid Chromatography
HP-LC	high protein/low carbohydrate
HR	homologous recombination
HRE	hypoxia-response element
<i>IGF2</i>	insulin-like growth factor II
INO80	chromatin-remodeling ATPase INO80
ISWI	imitation switch
JHDM	Jumonji C domain containing demethylase
JmjC	Jumonji C
KDM1	lysine demethylase 1
LC3	microtubule-associated protein 1A/1B-light chain 3
LC-PUFA	long-chain polyunsaturated fatty acid
LDHA	lactate dehydrogenase A
lncRNA	long non-coding RNA
LP	low-protein
LP-HC	low protein/high carbohydrate
LUMA	LUMinometric Methylation Assay
macro H2A	histone 2A variant
MAPK	A mitogen-activated protein kinase
MAT	methionine adenytransferase
MBD	methyl-binding domain protein
MBP (MeCP)	methyl-CpG-binding protein
MBT	the midblastula stage
MCD	methionine-coline-deficient
Mda	megadaltons
miRNA	microRNA
MLL	mixed Lineage Leukemia
MMR	mismatch repair
mRNA	messenger RNA
MS	methionine synthase
MSI	microsatellite repeat instability
mtDNA	mitochondrial DNA
MTHFR	methylenetetrahydrofolate reductase
Mya	million years ago
NAD ⁺	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
ncRNA	non-coding RNA
NEFA	non-esterified fatty acid
NFR	nucleosome free region
NRC	National Research Council
OGT	O-linked β -GlcNAc transferase
ORF	open reading frame
OSA	octenylsuccinic anhydride
<i>pck</i>	phosphoenol pyruvate carboxykinases (cytosolic <i>pck1</i> and mitochondrial <i>pck2</i>)
PCNA	nuclear antigen
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PEPCK	phosphoenolpyruvate carboxykinase
<i>pfkl</i>	phosphofructokinase (liver)
<i>pfkm</i>	phosphofructokinase (muscle)
PGC	primordial germ cell
PGC-1 α	peroxisome proliferator-activated receptor γ (PPAR γ) coactivator-1 α
piRNA	P-element-induced wimpy testis (Piwi)-interacting RNA
<i>pklr</i>	pyruvate kinase (liver and red blood cell)
<i>pkm</i>	pyruvate kinase (muscle)
PPAR	peroxisome proliferator-activated receptor
PWWP	Pro-Trp-Trp-Pro domain

qPCR	quantitative polymerase chain reaction
RFTD	Replication foci targeting domain
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rRNA	ribosomal RNA
SaGD	salmonid specific genome duplication
SAH	S-adenosylhomocysteine
SAHH	S-adenosyl homocysteine hydrolase
SAM	S-adenosyl-L-methionine
SDH	succinate dehydrogenase
SFA	saturated fatty acid
SHMT	serine hydroxymethyltransferase
siRNA	small interfering RNAs
<i>slc16a3</i>	solute carrier family 16 (monocarboxylate transporter), member 3
<i>slc2a1/glut1</i>	solute carrier family 2 (facilitated glucose transporter), member 1
<i>slc2a4/glut4</i>	solute carrier family 2 (facilitated glucose transporter), member 4
<i>slc2a2/glut2</i>	solute carrier family 2 (facilitated glucose transporter), member 2
<i>slgt1</i>	sodium-dependent glucose cotransporter
SMUG1	single-strand-selective monofunctional uracil-DNA glycosylase 1
sncRNA	short non-coding RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SSD	small scale duplication
SWI/SNF	switch/sucrose non-fermentable
T2DM	type 2 diabetes mellitus
TCA cycle	tricarboxylic acid
TDG	thymine-DNA glycosylase
TE	transposable element
TET	ten-eleven translocation methylcytosine dioxygenase
TF	transcription factor
TFA	trifluoroacetic acid
TGD (or Ts3R)	teleost-specific genome duplication
THF	tetrahydrofolate
TNF- α	tumour necrosis factor- α
Trdmt 1	tRNA aspartic acid methyltransferase 1
tRNA	transfer RNA
TSS	transcription start site
UHRF	ubiquitin like with PHD and ring finger domains
VGD1	the first round vertebrate whole genome duplication
VGD2	the second round vertebrate whole genome duplication
WGD	whole genome duplication
ZFIN	Zebrafish Information Network



GENERAL INTRODUCTION

GENERAL INTRODUCTION

I. Fast development of aquaculture industry

Fish, crustaceans and molluscs are important food sources for human. With the explosion of world population in 21th century, the demand for aquatic products is increasing simultaneously. Between 1961 and 2016, the average annual increase in global food fish consumption (3.2%) outpaced population growth (1.6%) (Fig. 0-1) (FAO, 2018). According to the report of Food and Agriculture Organisation (FAO) in 2018, fish provides about 17 percent of animal protein consumed by the global population in 2015, and this proportion is expected to be expanding continuously in the future (FAO, 2018). In addition to animal protein, fish contain unique long-chain polyunsaturated fatty acids (LC-PUFAs) and highly bioavailable essential micronutrients such as vitamins and minerals (Fisheries, 2014). These compounds, which are often not readily available elsewhere in diets, have beneficial effects on humans. Therefore, fish is well acknowledged as a major nutrient-dense animal-source food, overshadowing that of most terrestrial animal-source food (Béné et al., 2016).

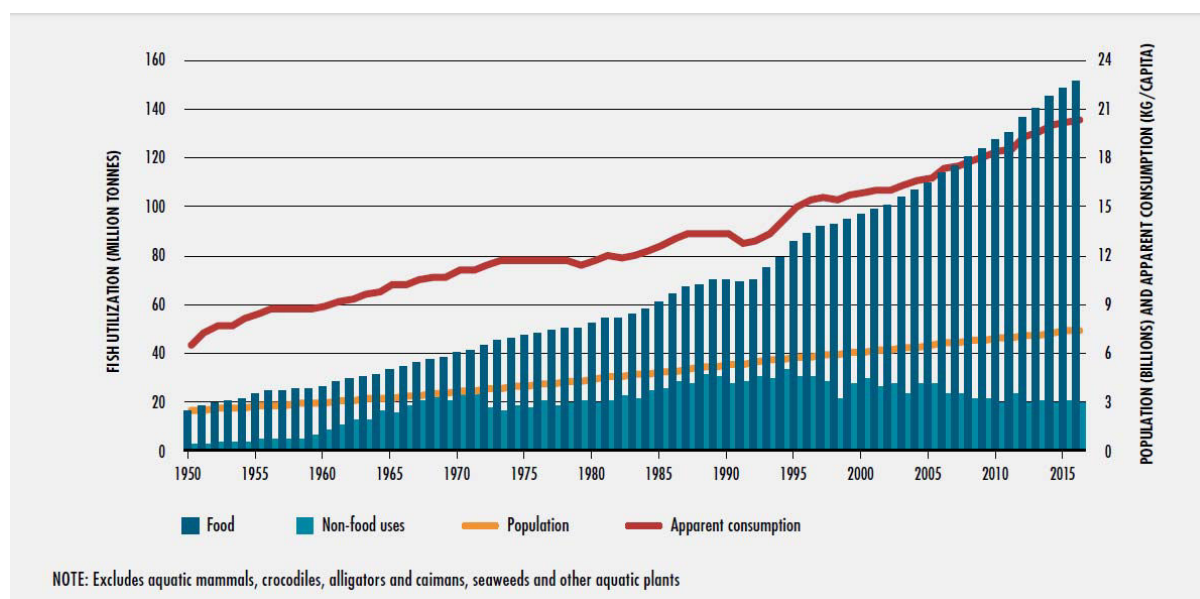


Figure 0-1 World fish utilisation and apparent consumption (FAO, 2018)

In order to keep pace with the growing demand for fish consumption and avoid overfishing, aquaculture is contributing significant quantities to the world's supply of fish for human consumption (Fig. 0-2), although the capture fishery production has remained static

over the past four decades. Aquaculture is considered the fastest growing food-producing sector in the world. Fish obtained from aquaculture grew from 4.7 million metric tons in 1980 to 13.1 million metric tons in 1990, reaching 80 million metric tons (valued at USD 232 billion) in 2016. This production accounts for 47 percent of the 171 million metric tons total global fish production (FAO, 2018).

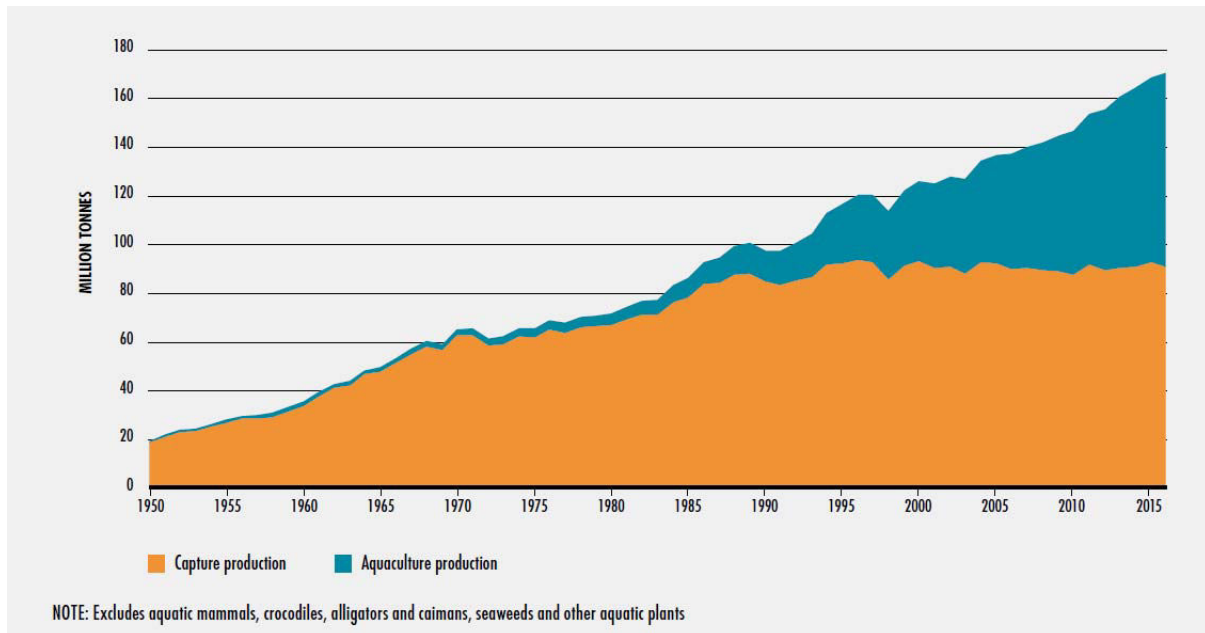


Figure 0-2 World capture fisheries and aquaculture production (FAO, 2018)

II. Fish nutrition, aquafeeds and the bottleneck in aquaculture

Fish nutrition is one of the most crucial elements for aquaculture. The cost of aquafeeds typically accounts for about 50 percent of the production cost for aquaculture, representing the largest operating cost item of most fish and crustacean farming operations (Tacon and Metian, 2008). Any reduction in feed cost either through developing dietary formula, husbandry improvement or other direct/indirect means is hence a priority for the development of the aquaculture industry (Craig et al., 2017).

The nutritional requirements of fish vary in their feeding habits. While omnivorous and herbivorous fish (*i.e.* most fresh water species) have relatively low protein requirements (ranging from 25% to 35% of the diet), carnivorous fish (*i.e.* most diadromous and marine species) have high protein requirements (ranging from 40% to 55% of the diet) (NRC, 2011;

Wilson, 2003). Apart from this, the essential fatty acid (EFA) requirements between freshwater/diadromous fish and marine species are also different. The freshwater/diadromous species generally require C-18 polyunsaturated fatty acids (PUFA; namely 18:3n-3 and/or 18:2n-6) whereas marine fish have a strict requirement for LC-PUFA [namely 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA)] (Tocher, 2010).

Fishmeal (FM), characterised by high protein content with balanced amino acid profile, high protein digestibility and palatability and no anti-nutritional factors, is considered the most adequate protein source for fish (Hardy, 2010). Also, fish oil (FO), a rich source for LC-PUFA, is indispensable to cover the essential fatty acid requirements in diets for marine fish species (Oliva-Teles et al., 2015). Consequently, FM and FO represent the dominant ingredients in compound feeds for marine/carnivorous fish (Naylor et al., 2000).

However, the production of FM and FO relies on wild capture fisheries of small pelagic species such as anchoveta (*Engraulis ringens*). The stock abundance of these species is dominated by the El Niño phenomenon, which leads to the fluctuation of their catches and therefore the instability of FM and FO production (FAO, 2018). Moreover, as the global production of capture fisheries is now limited due to the sustainable management of wild marine source preservation, the availability of FM and FO is also limited. The finite supply and the growing demand of FM and FO for the quick expansion of aquaculture industry resulting in the concomitant increase in their prices. The relatively inadequate availability and the rising costs of FM and FO hence becomes a bottleneck for the growth of carnivorous fish production (Hardy, 2010; Oliva-Teles et al., 2015; Tacon and Metian, 2008). Efforts have been made to increase the FM and FO production from fish processing by-products, which were often wasted previously. Nonetheless, reducing the reliance of aquafeeds on FM and FO is of the utmost importance for the sustainable development of the aquaculture industry, and in particular the culture of carnivorous fish like marine species and salmonids (Naylor et al., 2000).

III. Dietary carbohydrates: promising substitutes for FM and FO as well as challenging risks for carnivorous fish

Researches to develop terrestrial alternatives for FM and FO become a hotspot for both scientific and the industrial fields in aquaculture (Gatlin et al., 2007; Klinger and Naylor, 2012). As one of the most promising candidates, carbohydrates (starches and sugars), draw

much attention since they are conventional plant-based ingredients and competitive in market price. Carbohydrates could be an excellent source of energy and carbon for fish feeds. However, similar to other vertebrates, fish can survive and grow with carbohydrate-free diets because of their ability to efficiently synthesise glucose from non-carbohydrate precursors such as lactate, pyruvate and amino acids (Kamalam et al., 2016; NRC, 2011). Nonetheless, dietary carbohydrates are conventionally included in aquafeeds to reduce feed cost and for their binding activity during feed manufacturing (Craig et al., 2017). Moreover, the optimal inclusion level of dietary carbohydrates, can save the precious dietary proteins and lipids for growth purpose only instead of being utilised as an energy source, which is termed “sparing effect”. Meanwhile, the optimal inclusion of dietary carbohydrates can also help to reduce the nitrogen waste in water thus protecting the environment (Kamalam et al., 2016; Médale et al., 1995; Shiao and Peng, 1993).

However, the ability of dietary carbohydrate utilisation varies among fish species. It is demonstrated that this ability is closely linked to their trophic level (Polakof et al., 2012). For instance, fish feeding at a low trophic level are capable to utilise up to 50% of dietary carbohydrates, *i.e.* omnivorous species Nile tilapia (*Oreochromis niloticus*) (Boonanuntanasarn et al., 2018). By contrast, the optimal dietary carbohydrate level is reduced to 10-20% for species at the higher trophic level, namely the carnivorous fish (Enes et al., 2011; Polakof et al., 2012; Wilson, 1994) (**Table 1**).

Table 1 Optimum/recommended dietary digestible carbohydrates level for various fishes

Feeding habits	Type of fish	% digestible carbohydrates
Omnivorous	Nile Tilapia	~ 40
	Common carp	30-40
	Grass carp	37-56
	Channel catfish	25-30
Carnivorous	Atlantic salmon	≤ 20
	Rainbow trout	≤ 20
	Asian seabass	≤ 20
	Yellowtail	≤ 10

Table modified from Wilson *et al.*, (1994).

The breeding of carnivorous fish species represents an important section in global aquaculture marketing and their consumption is increasing (FAO, 2018). Salmonids are high-

value commercial species that are popular in Europe due to the ideal flesh quality and nutritious value. Trade in salmonids has increased at an average of 10 percent per year in value terms since 1976, and since 2013 it is the largest single fish commodity by value (FAO, 2018). However, as carnivorous species, salmonids require a high level of FM and FO in diets and have low capacity to use dietary carbohydrates (Naylor et al., 2000; Polakof et al., 2012). For instance, rainbow trout (*Oncorhynchus mykiss*), one of the most important commercial salmonids species, is considered a “poor user” of dietary carbohydrates. Persistent postprandial hyperglycaemia and reduced growth performance are evoked when feeding on a diet rich in dietary carbohydrates (> 20%) in this species (Panserat et al., 2000a; Polakof et al., 2012). The defect of low dietary carbohydrate utilisation in carnivorous species including salmonids remains a great challenge for the further replacement of FM by this promising ingredient, and hence hinders the further expansion of aquaculture. Consequently, a comprehensive understanding of the ‘poor dietary carbohydrate utilisation phenotype’ of carnivorous fish, as well as finding solutions to improve their capacities to use dietary carbohydrates, would contribute to the sustainable development of the aquaculture industry.

IV. Epigenetics, a potential mechanism in phenotypic adaptation

Numerous researches have been conducting on one hand to investigate the causes for the low capacity to use dietary carbohydrates in carnivorous fish, and on the other hand to try to improve their adaptation for carbohydrates-rich diets. Epigenetics is arising as a new field to study the interactions between environmental factors (nutritional and non-nutritional ones) and phenotypic plasticity. Notably, evidence has been found that epigenetic mechanisms, particularly DNA methylation, may be associated with the poor dietary carbohydrate utilisation phenotype in carnivorous fish like rainbow trout (Craig and Moon, 2013; Marandel et al., 2016a). Indeed, previous studies showed that dietary high carbohydrates can trigger the hepatic global DNA hypomethylation in rainbow trout, a phenotype that has been frequently reported in animals with impaired glucose homeostasis (Dhliwayo et al., 2014; Williams et al., 2008). However, the detail pathways through which epigenetic mechanisms exert influences remain unclear.

Besides, epigenetics has recently attracted considerable attention for its potential roles in many areas of agricultural production (Álvarez-Venegas and De-la-Peña, 2016; Goddard and Whitelaw, 2014; González-Recio et al., 2015; Ong-Abdullah et al., 2015) as well as in

aquaculture (Geurden et al., 2007; Vagner et al., 2009). Within aquaculture, two key areas where epigenetics could make a major contribution have been identified: i) selection of epigenetic marks that may improve brood-stock holding or conditioning and ii) environmental manipulation, where the intention is to induce an ‘epigenetic memory’ either within or between generations to produce a desired phenotype (namely programming) (Eirin-Lopez and Putnam, 2018; Gavary and Roberts, 2017). Therefore, epigenetics may provide an excellent chance to tailor the ‘poor carbohydrate utilisation phenotype’ in carnivorous fish and thus contributing to the potential improvement of FM substitution by dietary carbohydrates.

V. General objectives of the present thesis project

Based on the above context, the present thesis aimed at: **i)** investigating the epigenetic mechanisms underlying the ‘poor dietary carbohydrate utilisation phenotype’ in one of the best studied and of great agronomical interest carnivorous fish species, rainbow trout, and **ii)** testifying if the dietary carbohydrate utilisation of trout juveniles could be improved through programming strategy during early life stage.



CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Overview of epigenetics

The term “epigenetics” was coined by Conrad Hal Waddington in the early 1940s, which was originally defined as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” (Waddington, 2011). It derived from the word “epigenesis”, a theory of development proposed by Aristotelian in the 7th century by which the adult form develops from the embryo through gradual steps, in contrast to being fully pre-formed in the zygote (preformationism theory) (Gould, 1977; Waddington, 1956).

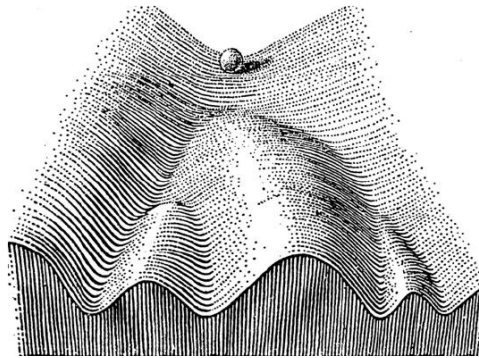


Figure 1-1 Waddington’s classic Epigenetic Landscape. The "epigenetic landscape" proposed by Conrad Waddington shows a ball rolling downhill with different valleys, as a visual metaphor for a cell (represented by the ball) can lead to different phenotypic outcomes or cell fates through a certain range of possibilities (represented by different valleys). Figure reprinted from Waddington, 1957 (Waddington, 2014).

The Greek prefix “*epi-*” means “above” or “upon”, therefore “epigenetics” literally means beyond or above “genetics”. At the time when Waddington invented his term, genetics was still a very young scientific field, with the physical structure of the gene and how it functioned barely understood. Waddington’s views of epigenetics focused on how identical genotype gives rise to different phenotypes during development and more specifically the process of cellular differentiation. He depicted this concept in his famous “epigenetics landscape” drawing in 1957 (**Fig. 1-1**), but the underlying mechanisms remained mysterious (Waddington, 2014). The specific epigenetic mechanisms that regulate genetic programming were not discovered until decades later when two papers independently documented the important role of cytosine methylation (Holliday and Pugh, 1975; Riggs, 1975).

Throughout the past three-quarters century, the definition of this word has been reformed several times along with the rapid advances of molecular biology and our increasingly deeper understanding of the gene regulatory mechanisms (Berger et al., 2009; Bird, 2007; Haig, 2004; Holliday, 1994; Nanney, 1958). Currently, one of the most widely accepted definition is “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” (Russo et al., 1996). This definition will be therefore used as a reference throughout the present work. Nonetheless, the conceptual delimitation of what may or may not be considered as epigenetic is still under debate in some occasions.

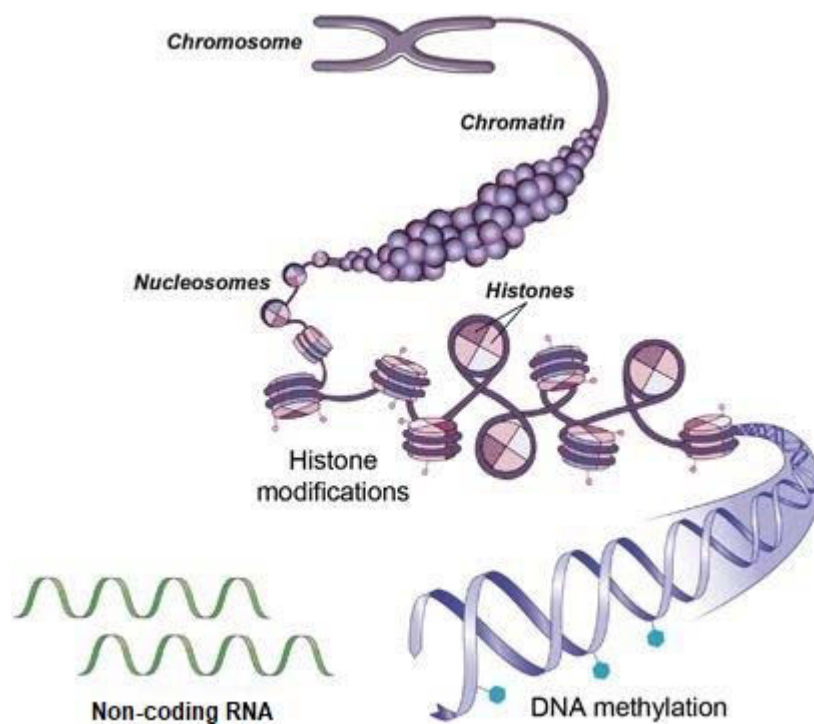


Figure 1-2 Epigenetic mechanisms involved in gene expression regulation. Eukaryotic DNA is wrapped around core histone proteins (histone octamers) and packaged into compact chromatin structures termed nucleosomes. Figure modified from (Weber-Stadlbauer, 2017).

Up to date, the main discovered epigenetic mechanisms involved in gene expression regulation include 1) DNA methylation, 2) histone modifications, 3) nucleosome remodelling, and 4) non-coding RNAs (**Fig. 1-2**). Somehow, there are still disputes about whether or not non-coding RNAs could be considered as epigenetics (Goldberg et al., 2007), probably because they work as an ‘indirect’ epigenetic modulator through the alterations in DNA methylation and histone modifications. Compared to DNA methylation and histone modifications, researches concerning the latter two mechanisms are relatively fall behind.

These mechanisms work independently but also cooperatively to control chromatin architecture, accessibility of genetic loci to transcriptional machinery and gene expression levels.

1.1.1 DNA methylation

As one of the most extensively studied and well characterised epigenetic mechanisms, DNA methylation is corresponding to the addition of a methyl group onto DNA molecule. This modification predominantly occurs at the 5th-carbon position of cytosine (C) positioned next to a guanine (G), namely CpG dinucleotides in vertebrates (Bird, 2002). But DNA methylation is also known to occur at non-CpG sites like CpA, CpT, and CpC (Jang et al., 2017). The symmetrical presence of CpG methylation marks on both DNA strands allows the post-replicative maintenance of methylation patterns, rendering DNA methylation a key feature of epigenetic regulation (Lyko, 2018).

The discovery of DNA methylation may date back to the time as early as the DNA was identified as the genetic material. However, it was only until the 1980s that several studies found that DNA methylation can play an important role in gene regulation (Holliday and Pugh, 1975). Generally, DNA methylation is considered as a repressive mark that characterises heterochromatin in vertebrates. When DNA methylation occurs at the promoter region of a gene, this modification has usually been implicated in transcriptional repression (Naveh-Many and Cedar, 1981).

As the present thesis focuses mainly on this epigenetic mark, the detail information about DNA methylation, *i.e.* the key mediators involved in regulation of DNA methylation, the functional roles of this mark, *etc.*, will be described in depth in next section.

1.1.2 Histone modifications

Histones are a family of alkaline proteins that are associated with DNA in the nucleus and help to condense it. Five types of histones have been identified: H1, H2A, H2B, H3 and H4. Based on their functions, histones can be divided into core histones (H2A, H2B, H3 and H4) and linker histone (H1). 145–147 bp DNA wraps around the core histone octamer (composed of two copies of each of the histone protein H2A, H2B, H3, and H4) to form the nucleosome, the basic subunit of chromosome (Luger et al., 1997). By contrast, the linker histone H1 is not a component of nucleosome, but links nucleosomes into higher order of chromosome structures (Thoma et al., 1979).

Histone variants and posttranslational modifications are the most studied histone-associated epigenetic mechanisms. Histone variants are non-allelic isoforms of the canonical histones, which differ by only a small number of amino acids from their major canonical histone counterparts (Henikoff and Smith, 2015; Talbert et al., 2012). So far, the discovered eukaryotic histone variants include H2A.B, H2A.X, H2A.Z, macroH2A, H3.3 and cenH3 (Henikoff and Smith, 2015). Whereas canonical histones are generally produced during S-phase in a DNA replication-dependent manner, histone variants can be synthesised independent of replication throughout the cell cycle (Henikoff and Shilatifard, 2011). Replacement of the canonical histones by their corresponding variants can lead to the alteration of the fundamental structure of the nucleosome and its stability (Talbert and Henikoff, 2010). Studies have demonstrated critical roles of variant histones in chromosome segregation, transcriptional regulation, DNA repair and sperm packaging (Henikoff and Smith, 2015).

Table 2 Some transcriptional activating and repressing histone modifications

Activating modifications	Repressing modifications
<u>Acetylation</u>	
H2A: K5, K9, K13	
H2B: K5, K12, K15, K20	
H3: K9, K14, K18, K23, K56	
H4: K5, K8, K13, K16	
<u>Methylation</u>	<u>Methylation</u>
H3: K4, K36, K79	H3: K9, K27
H3: R17, R23	H4: K20
H4: R3	
<u>Phosphorylation</u>	
H3: T3	
H3: S10, S28	
H3: Y41	
H2A.X: S139	
<u>Ubiquitination</u>	<u>Ubiquitination</u>
H2B: K120	H2A: K119
H2B: K123	
	<u>Sumoylation</u>
	H2A: K126
	H2B: K6, K7
	H4: K5, K8, K12, K16, K20

(Choudhuri, 2011)

Histone proteins can be also subjected to various reversible covalent posttranslational modifications on their flexible N-terminus (the so-called “tail”) that protrudes from the

nucleosome (Bannister and Kouzarides, 2011). Posttranslational modifications of histones include but not limited to methylation, acetylation, phosphorylation, ubiquitination and sumoylation (Nathan et al., 2003). These modifications could be involved in the alteration of gene expression not only through the alteration of the chromatin structure (from compact heterochromatin to loose euchromatin), but also through recruiting specific proteins and chromatin-remodelling complexes (Bannister and Kouzarides, 2011; Kouzarides, 2007). The correlation between specific posttranslational histone modifications and their effects on gene transcription has led to the histone code hypothesis (Strahl and Allis, 2000). **Table 2** lists some of the main studied histone modification markers (Choudhuri, 2011).

1.1.3 Nucleosome remodelling

Genome-wide nucleosome mapping data for various eukaryotic organisms reveal a non-random distribution of nucleosomes. Nucleosomes are normally depleted at many enhancer, promoter, and terminator regions. By contrast, they typically occupy preferred positions within genes and non-genic regions. So-called “nucleosome free regions” (NFRs) are localized just upstream of the transcription start site (TSS) (Radman-Livaja and Rando, 2010; Struhl and Segal, 2013). This pattern allows the free access of transcription factors and coactivators to their target sites on DNA, therefore is crucial for transcription initiation. The ATP-dependent chromatin remodelling complexes (also known as chromatin remodelers), *i.e.* ISWI (imitation switch), SWI/SNF (switch/sucrose non-fermentable), CHD (Chromodomain Helicase DNA-binding), and INO80 are large multi-subunit complexes (> 1 MDa) that highly conserved across the eukaryotic kingdom (Chen et al., 2017; Liu et al., 2012). They are capable in altering DNA-histone contacts through ATP hydrolysis and thereby leading to the changes in structure, composition and positioning of nucleosomes. These effects include i) repositioning or sliding nucleosomes (Hamiche et al., 1999; Whitehouse et al., 1999), ii) ejecting the histone octamer to expose DNA (Boeger et al., 2004; Lorch et al., 1999), iii) exchanging and incorporating histone variants (Mizuguchi et al., 2004), and iv) ejecting or exchanging dimers, such as H2A-H2B dimers to destabilise nucleosome and unwrap DNA (Bruno et al., 2003).

1.1.4 Non-coding RNA

Non-coding RNAs (ncRNAs) are RNAs that transcribed from DNA but not translated into proteins. The accomplishment of mammalian transcriptome landscapes analyses in recent years strikingly discovered that the vast majority of the genome is pervasively transcribed

(62-75%). However, the primary transcripts from protein-coding genes account for only 2.94% of the genome (Consortium, 2012; Djebali et al., 2012). Some of the ncRNAs have well-known functions: the canonical ncRNAs such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are key elements for protein synthesis, while small nucleolar and small nuclear RNAs (snoRNAs and snRNAs) are found in specific cellular locations, which can function through modifying other RNAs (e.g., rRNAs and tRNAs) and processing of pre-mRNA (Matera et al., 2007). Nonetheless, the functions of many of the rest ncRNAs remain less understood.

Except for ncRNAs with specific functions, other ncRNAs are broadly classified into short ncRNAs (sncRNAs, < 200 nucleotides, nt) and long ncRNAs (lncRNAs, >200 nt) based on the length of their mature products (Kapranov et al., 2007). The sncRNAs can be further divided into small interfering RNAs (siRNAs), microRNAs (miRNAs), P-element-induced wimpy testis (Piwi)-interacting RNAs (piRNAs) (Diamantopoulos et al., 2018).

Ever since the discovery of the first miRNA *lin-4* in 1993, miRNAs have attracted certain interests of researchers and are likely the most extensively studied sncRNAs (Lee et al., 1993). Mature miRNAs are short (approximately 22 nt), single-stranded endogenous RNA molecules whose function is to silence gene expression (Yates et al., 2013). miRNA-mediated gene regulation can be achieved at both transcriptional and post-transcriptional levels. At post-transcriptional level, miRNA can bind 3'untranslated region (3'UTR) of various messenger RNAs (mRNAs) leading to the degradation of the targeted mRNA(s), repression of translation, or destabilisation of the mRNA by shortening of the poly (A) tail (Lam et al., 2015; MacFarlane and R Murphy, 2010; Wilczynska and Bushell, 2015). Latterly, evidence showed that miRNAs can be also imported into nucleus, bind chromosomal DNA sequences, and modulate gene expression through recruiting chromatin-modifying proteins thereby altering chromatin structure (Li, 2012).

siRNAs, a class of double-stranded sncRNAs (about 22 nt in length, either transcribed from cellular genes or infecting pathogens, or artificially introduced into the cells), share many similarities with miRNAs in their roles during the process of posttranscriptional gene regulation and RNA interference (RNAi). The major difference between siRNAs and miRNAs is that the siRNAs are highly specific with only one mRNA target, whereas the miRNAs have multiple targets (Lam et al., 2015).

By contrast, piRNAs are a distinct class of sncRNAs (about 21 to 36 nt in length) specially expressed in the germline of many species where they repress transposons to maintain genome integrity (Aravin et al., 2006; Girard et al., 2006; Hiraoka and Siomi, 2016; Houwing et al., 2007). In contrast to miRNAs, genes encoding piRNAs are concentrated in specific clusters with very low sequence conservation, even between closely related species (Girard et al., 2006; Ozata et al., 2019).

In terms of lncRNAs, the 200 nt cutoff seems arbitrary, which roughly corresponds to the retention threshold of protocols for the purification of long RNAs (Ulitsky, 2016). However, there is no widely accepted definition of lncRNA that is based on biological argumentation currently. According to their genomic proximity relative to protein-coding genes, lncRNAs can be classified into one or more of these broad categories: 1) sense lncRNAs, which overlaps with the sense strand of a protein-coding gene; 2) antisense lncRNAs, when overlapping the opposite strand of a protein-coding gene; 3) bidirectional lncRNAs, which are transcribed from the opposite strand, in the opposite direction and within 1 kb of the promoter of coding genes; 4) intronic lncRNAs, which are derived entirely from within an intron of another transcript (although this may be either a true independent transcript or a product of pre-mRNA processing); 5) intergenic lncRNAs, when it lies within the genomic interval between two genes (Ponting et al., 2009).

Although lncRNAs share many common features with protein coding mRNAs in their biogenesis and forms (*i.e.* RNA polymerase II transcribed, 5'-capped, spliced, and 3'-polyadenylated), there are many unique features of lncRNAs that distinguish them from mRNAs [*i.e.* lack of open reading frames (ORFs), special 3'-terminal processing, *etc.*] (Chen et al., 2017; Quinn and Chang, 2015). Besides, the general expression levels of lncRNAs appear to be lower but more tissue-specific and/or developmental stage-specific than mRNAs (Mercer et al., 2008; Palazzo and Lee, 2015; Ramsköld et al., 2009). Recent studies showed that, although there is poor sequence conservation of lncRNAs among species, their specific secondary structures, mechanisms of action, and localisation appear to be highly conserved (Lanzafame et al., 2018; Zampetaki et al., 2018). The uncovered functions of lncRNAs include: 1) imprinting, best illustrated by *Xist* induced transcriptional silencing of one X chromosome in mammalian females for dosage compensation; 2) scaffold/guide for epigenetic and transcription factors; 3) enhancer activation; and 4) molecular sponges (lncRNA with multiple antisense sequences of miRNAs may act as an endogenous miRNA

sponge to attenuate miRNA-mediated gene silencing, as exemplified by the circular RNA) (Chen et al., 2017; Uchida and Dimmeler, 2015).

In summary, DNA methylation, histone modifications, nucleosome remodelling, and non-coding RNAs all together constitute multiple layers of epigenetic mechanisms, which work independently but also cooperatively with connections and interplays in controlling chromatin structure and gene expression (Geiman and Robertson, 2002). The present study focuses on DNA methylation only, which will be discussed in detail in the next following subchapters.

1.2 DNA methylation

1.2.1 DNA methylation machinery and pathways

1.2.1.1 The writing of DNA methylation

The methyl group is transferred from S-adenosyl-L-methionine (SAM) to cytosine through the mediation of a group of enzymes, namely DNA methyltransferases (DNMT). DNMTs are large multi-domain proteins containing a catalytic C-terminal domain and an N-terminal part with regulatory functions (Bestor, 2000). The catalytic motifs of cytosine-5 DNMTs are highly conserved and can be used to identify these enzymes in DNA sequences (Fig. 1-3) (Pósfai et al., 1989).

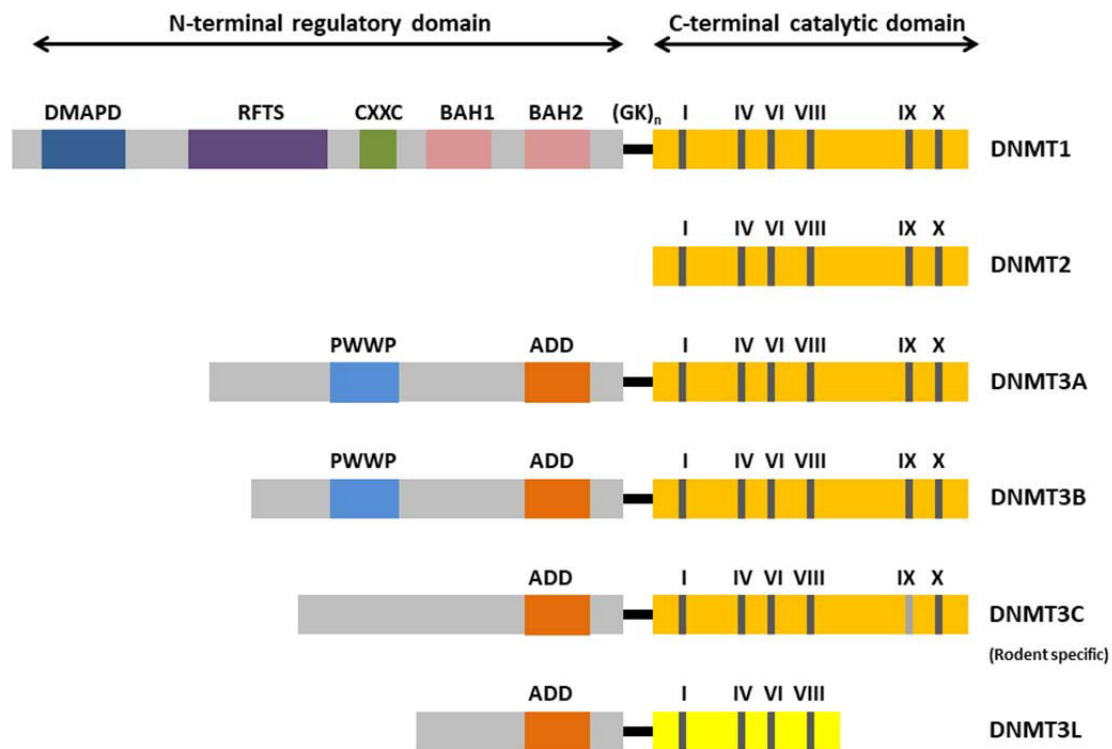


Figure 1-3 Domain structure of DNMT enzymes in mammals. Abbreviations used: DMAPD: DNA methyltransferase associated protein domain, RFTD: Replication foci targeting domain, CXXC: CXXC zinc finger domain, BAH1/2: bromo-adjacent homology domains 1/2, PWWP: Pro-Trp-Trp-Pro domain, ADD: ATRX-DNMT3-DNMT3L domain. Figure modified from (Barau et al., 2016; Jeltsch and Jurkowska, 2016).

The mammalian DNMT family consists of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3C and DNMT3L. As the first described DNMT, DNMT1 preferentially recognises hemi-methylated DNA through its functional partner ubiquitin-like with PHD and ring finger domain 1 (UHRF1), thereby copying the methylation pattern from the native DNA strand onto the neo-synthesised strand (Hermann et al., 2004; Hirasawa et al., 2008). Thus, DNMT1 acts primarily as a maintenance methyltransferase during DNA replication. From a structural point of view, the N-terminal regulatory region of DNMT1 contains DAMPD (DNA methyltransferase associated protein domain), RFTS (replication foci targeting domain), CXXC (CXXC zinc finger domain) and BAH1/2 (bromo-adjacent homology domains 1 and 2), which allow it to recognise the methylation target site, bind DNA molecules and interact with other proteins (Maresca et al., 2015). The C-terminal region encloses 10 conserved amino acid motifs characteristic for DNA-(cytosine-C5)-Methyltransferases, which is folded into a widely conserved structure and is responsible for the catalysis of DNA methylation (Jeltsch and Jurkowska, 2016) (**Fig. 1-3**).

DNMT2 harbours the full set of sequence motifs in its C-terminal domain but lacks the N-terminal domain, making this enzyme has not been shown to have any methyltransferase activity for DNA. Latter researches demonstrated that DNMT2 could methylate cytosine 38 in the anticodon loop of tRNA^{Asp} through a DNA methyl transferase-like catalytic mechanism, thus was proposed to be renamed as Trdmt1 (TRNA Aspartic Acid Methyltransferase 1) (Goll et al., 2006).

The DNMT3 family of enzymes are implicated in *de novo* methylation, with DNMT3L serves as the stimulatory factor of DNMT3A/DNMT3B but lacks catalytic activity on its own (Okano et al., 1998; Suetake et al., 2004). DNMT3A and DNMT3B have similar domain arrangements and exhibit 3-fold higher preference for unmethylated DNA than hemi-methylated DNA, thus acting as *de novo* DNA methyltransferases (Yokochi and Robertson, 2002). When looking at their structures in detail, both DNMT3A and DNMT3B have the regulatory PWWP (Pro-Trp-Trp-Pro) and the ADD (ATRX-DNMT3-DNMT3L) domains in N-terminal part. The PWWP domain is proved to mediate the DNA binding, whereas the ADD domain is responsible for protein-protein interactions (Maresca et al., 2015). The C-terminal domains of DNMT3A and DNMT3B share 81% sequence identity and contain all the DNMTs catalytic motifs. By contrast, DNMT3L lacks the PWWP domain together with the DNMTs motifs IX and X and all the important catalytic residues in its C-terminal part (Jeltsch and Jurkowska, 2016). Interestingly, existing only in eutherian mammals and in some

marsupials, DNMT3L has been shown to be involved in genomic imprinting (Yokomine et al., 2006).

DNMT3C was previously annotated as a pseudogene. Very recently, it was identified in the male germline of mice. It is supposed that DNMT3C evolved from a specific duplication of DNMT3B in rodent genomes. DNMT3C contains methyltransferase motifs in C-terminal position and an N-terminal ADD (ATRX-DNMT3-DNMT3L) domain, but lacks the PWWP domain (Barau et al., 2016).

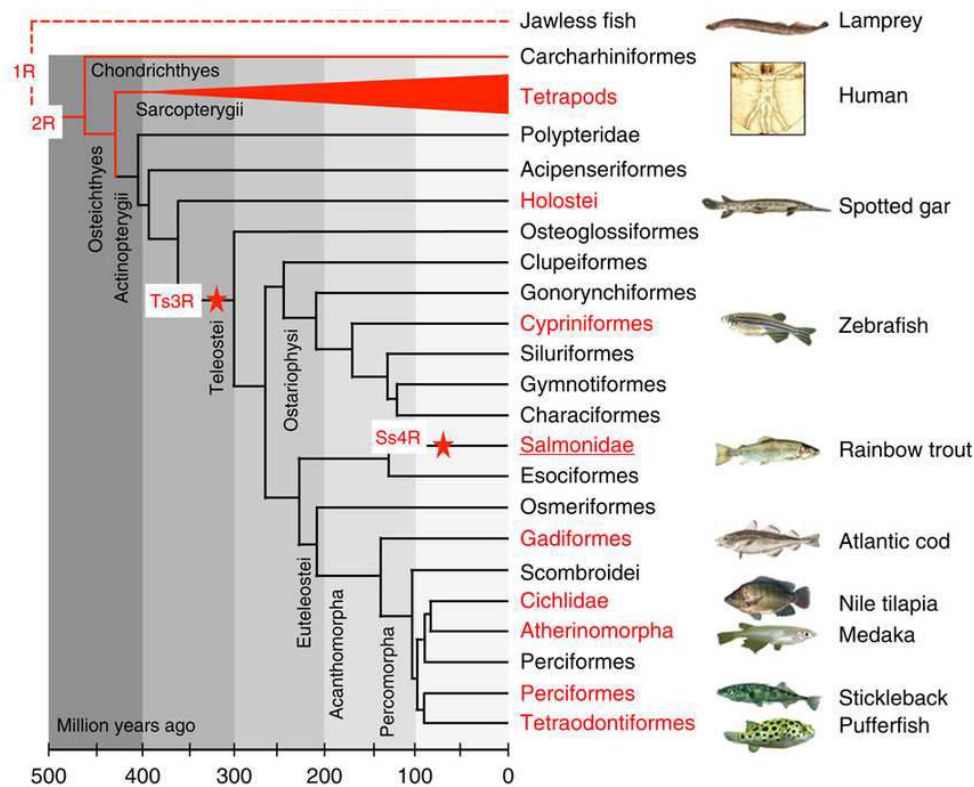


Figure 1-4 Whole genome duplication events during the evolution of vertebrates. The red stars show the position of the teleost-specific (Ts3R) and the salmonid-specific (Ss4R) whole-genome duplications (Berthelot et al., 2014).

In teleost species, studies showed that orthologues of DNMT1, DNMT3A and DNMT3B are encoded in their genomes, suggesting that pathways involved in maintenance and *de novo* methylation are generally conserved in vertebrate species. (Fellous et al., 2018; Firmino et al., 2017; Goll and Halpern, 2011; Skjærven et al., 2014). No mammalian DNMT3L orthologue was identified in teleost genomes, which might be possibly linked to the lack of genomic imprinting (Yokomine et al., 2006). However, due to the additional round of a whole genome duplication (WGD) event (**Fig. 1-4**) that occurred before the radiation of the teleost lineage [TGD (or Ts3R), teleost-specific genome duplication, 320 Mya (million years ago)], an

increase in the number of copies of *dnmt* genes has been found in teleost genomes (Amores et al., 1998; Ravi and Venkatesh, 2018). For instance, the *de novo* methyltransferase *dnmt3* was shown to be more divergent in teleost compared with mammals: 4 to 9 genes were identified as orthologous to mammalian *dnmt3a* and *dnmt3b* genes in different teleost species like zebrafish (*Danio rerio*), sole (*Solea senegalensis*), medaka (*Oryzias latipes*) and Atlantic salmon (*Salmo salar*) (Best et al., 2018; Campos et al., 2012; Dasmahapatra and Khan, 2015; Firmino et al., 2017). Nonetheless, the evolutionary history and the orthologous relationship of the *dnmt3* genes remain elusive. Indeed, studies did by Campos *et al.* reported that, among the six identified *dnmt3* genes in zebrafish genome, there are 2 DNMT3A orthologues and 4 DNMT3B orthologues (Campos et al., 2012). The authors hypothesised that the 2 *dnmt3a* genes derived from the TDG event, whereas the 4 *dnmt3b* genes arose from two successive lineage-specific duplication events. **However, with the recent discovery of *Dnmt3c* gene in mice, this hypothesis could be challenged. Besides, the consequences of the extra *dnmt* copies identified in teleost genomes remain uncovered.**

1.2.1.2 The erasing of DNA methylation

Compared with most histone modifications, DNA methylation is a relatively stable epigenetic mark. Nevertheless, loss of DNA methylation has been observed in different biological contexts. Contrary to the enzymatically controlled, straightforward methylation writing mechanism, a DNA demethylase capable to directly convert 5-methylcytosine (5-mC) to cytosine (C) has not yet been identified. This direct conversion is also unlikely because of the difficulties of braking non-activated carbon-carbon bonds. Instead, several alternative and indirect demethylation pathways have been highlighted (Shown in **Fig. 1-5**) (Wu and Zhang, 2014).

1.2.1.2.1 Passive, replication-dependent dilution

Once established by *de novo* DNA methylation, the methylation pattern can be preserved during the DNA replication process through the function of maintenance DNA methyltransferase DNMT1. Therefore, the lack or inhibition of DNMT1 function can lead to the gradual loss of 5-mC during successive rounds of replication, which is termed passive DNA demethylation (**Fig. 1-5**) (Law and Jacobsen, 2010; Messerschmidt et al., 2014). Due to its intrinsic dependence on DNA replication, the passive demethylation mechanism cannot account for the rapid loss of DNA methylation in slowly or non-dividing cells. In addition,

this mechanism does not allow locus-specific demethylation, but only proceed at a global level (Messerschmidt et al., 2014).

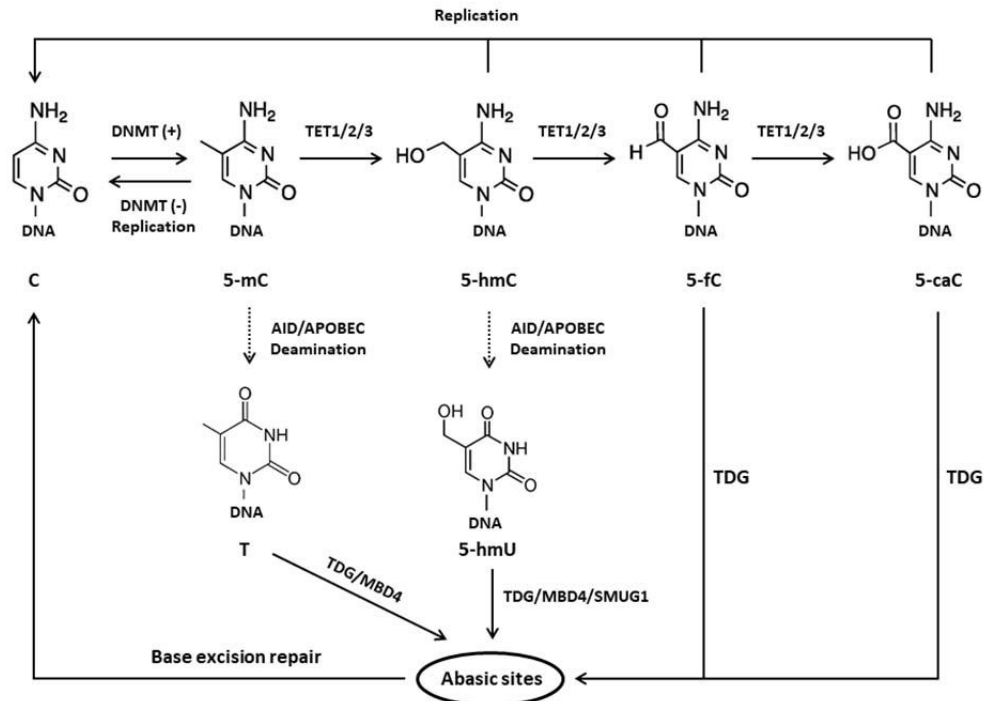


Figure 1-5 Passive and active DNA demethylation pathways. DNA methylation (5-mC) is established and maintained by DNMT. Passive DNA demethylation is caused by a reduction in activity or absence of DNMTs thereby leading to replication-dependent dilution. Active demethylation via oxidation pathway: 5-mC can be stepwise oxidized by TET (Ten-Eleven translocation) family of dioxygenases to form 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC). 5-fC and 5-caC can be excised by glycosylases such as TDG, followed by base excision repair (BER) to generate C. Active demethylation via deamination pathway: AID/APOBEC family members can deaminate 5-mC or 5-hmC to form thymine or 5-hydroxymethyluracil (5hmU). These intermediates are replaced by cytosine during BER mediated by glycosylases like TDG, SMUG1 and MBD4. Abbreviation: AID activation-induced deaminase, APOBEC apolipoprotein B mRNA-editing enzyme complex, BER base excision repair, DNMT DNA methyltransferase, MBD4 methyl-binding domain protein 4, SMUG1 single-strand specific monofunctional uracil-DNA glycosylase, TET1/2/3 Ten-Eleven Translocation enzyme, TDG thymine DNA glycosylase. Figure modified from Sadakierska-Chudy et al., 2015 as well as Li and Zhang, 2014 (Li and Zhang, 2014; Sadakierska-Chudy et al., 2015).

1.2.1.2.2 Active demethylation pathway

The discovery of TET (Ten-Eleven translocation methylcytosine dioxygenase) enzymes was a breakthrough in epigenetics. TET1 was first identified as an MLL fusion partner in acute myeloid leukemia (AML) patients (Ono et al., 2002). However, in 2009, two cutting-

edge papers discovered that human TET1 is capable of converting 5-mC to 5-hydroxymethylcytosine (5-hmC). This striking finding for the first time confirmed the hypothesis of active DNA demethylation mechanisms (Tahiliani et al., 2009). During the last decade, a flurry of studies have identified some of the key players in active demethylation process (Bhutani et al., 2011), which act through first modifying the methylated cytosine (by hydroxylation, deamination, oxidation or a combination of these modifications) and subsequently replacement of the modified nucleotide by DNA repair (**Fig. 1-5**) (Bhutani et al., 2011; Zhu, 2009).

Three enzyme families are involved in active demethylation pathways: TET family, which modifies methylated cytosine through iterative oxidation of 5-mC to generate 5-hmC, 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC) (Ito et al., 2010; Tahiliani et al., 2009); the AID/APOBEC family, which deaminates the 5-mC or 5-hmC to Thymine (T) or 5-hydroxymethyluracil (5-hmU), respectively (Nabel et al., 2011; Schutsky et al., 2017); and base excision repair (BER) glycosylases family, which mediate DNA repair, *i.e.* thymine DNA glycosylase (TDG), methyl-binding domain protein 4 (MBD4) (Bhutani et al., 2011; Cortellino et al., 2011).

These enzymes constitute the TET-TDG mediated oxidative demethylation pathway and the AID/APOBEC mediated deamination pathway. While the former pathway has been widely accepted and confirmed, the facticity of AID/APOBEC remains controversial. Notably, it has been shown that the enzymatic activity of AID/APOBEC is inversely proportional to the size of the substituent at the C5 position of cytosine. Thus, AID/APOBEC enzymes have 10-fold reduced activity on 5-mC compared to cytosine, and moreover, 5-hmC is not a substrate of AID/APOBEC enzymes (Rangam et al., 2012; S Nabel et al., 2012). These results challenged the plausibility of proposed pathways that have invoked 5-hmC deamination in DNA demethylation. In the thesis, I will thus focus on TET-TDG mediated oxidative demethylation pathway.

The TET enzymes are large proteins (about 180 to 230 kDa) which belong to members of the Fe (II)/ 2-oxoglutarate (2-OG) dependent oxygenase family of enzymes. Mammalian TET proteins consist of three family members, namely TET1, TET2 and TET3 (Lorsbach et al., 2003).

All TET proteins contain a core catalytic region in the C terminal which comprises a cysteine (Cys)-rich domain and a conserved double-stranded β -helix (DSBH) domain that

including the binding sites for the cofactors Fe (II) and 2-oxoglutarate (2-OG) within it (**Fig. 1-6**) (Rasmussen and Helin, 2016). With the presence of molecular oxygen, Fe (II) and 2-OG, TET enzymes catalyse the oxidation of 5-mC to other intermediate forms of oxidase cytosine, generating CO₂, succinate, and a high-valent Fe (IV)-oxo intermediate (Kohli and Zhang, 2013). A large low complexity insert is found within the DSBH domain, with its length varies greatly among three TET members, and the sequence is less conserved (Iyer et al., 2009). Although its detail function remains to be defined, a recent study hypothesised that this low complexity insert may have regulatory roles via post-translational modifications (Bauer et al., 2015). The Cys-rich domain in TET enzymes can modulate the chromatin targeting of TET proteins (An et al., 2017).

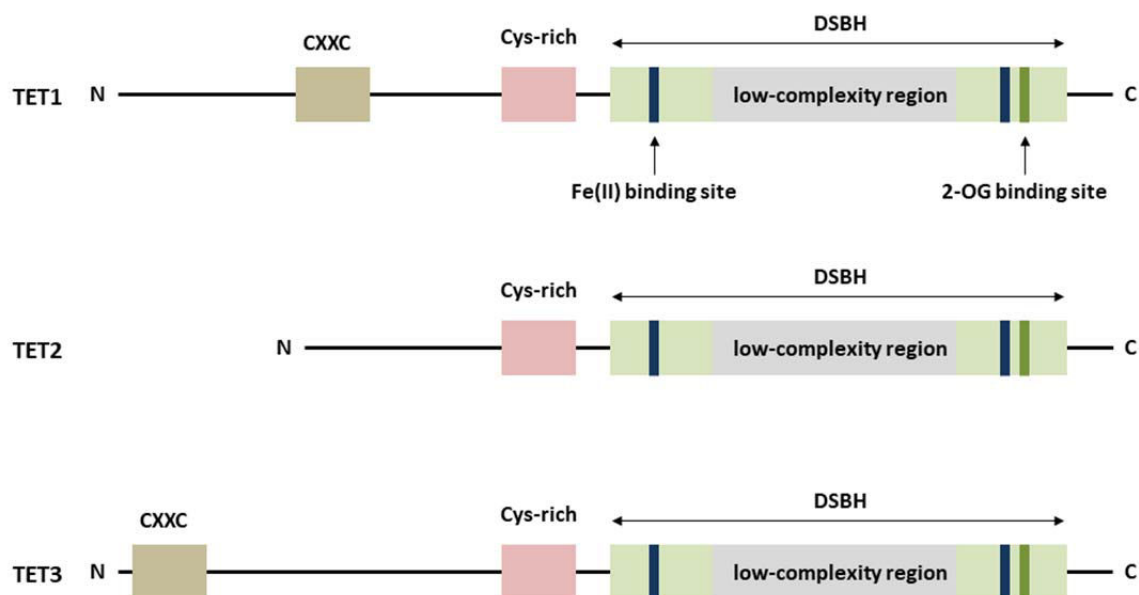


Figure 1-6 Domain structure of the TET family proteins. All the TET family members possess a conserved core catalytic domain in the C terminal, comprising the cysteine (Cys)-rich domain and the double-stranded β -helix (DSBH) domain. Key catalytic motifs, like Fe (II) binding site and 2-OG binding site are found in the DSBH domain together with a large low-complexity insert within it, whose function remains unclear (Rasmussen and Helin, 2016).

At the N terminal region, TET1 and TET3 have a CXXC domain that composed of two Cys₄-type zinc finger motifs. This domain can bind directly to DNA and facilitate recruitment to genomic target sites (Long et al., 2013; Pastor et al., 2013; Zhang et al., 2010). Interestingly, a chromosomal inversion apparently split the ancestral *TET2* gene during

evolution, breaking the segment encoding the CXXC domain from the part encoding catalytic domain. Thus, the ancestral CXXC domain of TET2 is now encoded separately by a neighboring gene, CXXC4.

Concerning the next step after the successive oxidation of 5-mC by TET proteins in active demethylation pathway, TDG is likely the most promising candidates for BER compared to other DNA glycosylase enzymes, because it has been shown to interact with various transcription factors, chromatin-modifying enzymes and DNMTs (Dalton and Bellacosa, 2012). In particular, unlike other DNA glycosylases, TDG is essential for embryonic development (Cortázar et al., 2011; Kohli and Zhang, 2013). Originally, TDG is well-known for its function as a DNA repair enzyme that can remove a normal base, T, from a genomic T·G mismatch (Cortázar et al., 2007). However, evidence showed that C bases with 5-position substituents may destabilise the N-glycosidic bond through electronic effects, making them eligible targets for base excise by TDG (Bennett et al., 2006). These observations opened up the possibility that TDG could directly excise TET oxidation products. Indeed, although no significant base excision activity has been observed with C, 5-mC and 5-hmC, TDG has robust base excision activity on 5-fC and 5-caC properly base paired to G in duplex DNA *in vitro* (Maiti and Drohat, 2011).

In terms of teleost, studies related to the active DNA demethylation mechanism are just emerging, and most of the results are obtained in zebrafish. It has been proved that, similar to mammals, all the enzyme families involved in TET-TDG pathway are present in teleost (Kamstra et al., 2015a). There are three *tet* genes in the genome of zebrafish, each one presents as a single well-conserved orthologue for mammalian *TET1*, *TET2* and *TET3* (Almeida et al., 2012). By contrast, two paralogues of *tdg*, annotated as *tdg1* and *tdg2*, were identified in zebrafish (Jessop et al., 2018), which were originally hypothesised to be derived from a lineage-specific duplication event, but no comprehensive studies with the information from other species were done to confirm it. Above all, the presence of all the DNA demethylation machinery in teleost species suggests that this mechanism is conserved during vertebrate evolution. However, researches concerning the active DNA demethylation are still a very young field of investigation, and information related to teleost is much less. **Questions remain to be answered about how the active DNA demethylation functions and what roles it may play during various biological processes.**

1.2.2 DNA methylation dynamics during gametogenesis and embryogenesis

There are significant variances in the prevalence and patterns of genomic DNA methylation among taxa. At the low extreme is the nematode worm *Caenorhabditis elegans*, whose genome lacks detectable 5-mC (Greer et al., 2015). In vertebrates, the methylated cytosine level is found to be highest and widely dispersed throughout the entire genome. By contrast, plants and invertebrates show 'mosaic' methylation patterns because only specific genomic elements are targeted (Suzuki and Bird, 2008). In addition, the DNA methylation patterns are actually more dynamic than previously thought, as they can also vary within a single individual across life cycle or different physiological conditions. It has been well documented in mammals that whole genome DNA methylation patterns undergo dramatic remodelling during two major processes in an organism's life: gametogenesis and early embryonic development (Hackett and Surani, 2013; Messerschmidt et al., 2014). These dynamic events are supposed to reset parental methylation patterns thereby reprogramming methylome signatures in embryos and offspring.

In mammals, a global DNA demethylation event occurs during early embryogenesis quickly after fertilisation, which is of great importance for establishing pluripotency. After fertilisation, the hypermethylated sperm genome (around 80-90% CpG methylation) undergoes a rapid loss of global 5-mC content before the onset of DNA replication (Mayer et al., 2000). Latter researches proved this process is mainly attributed to the TET3-mediated active demethylation mechanism (Wossidlo et al., 2011). Conversely, the hypomethylated maternal genome (around 40%) remains static from the early pronuclear to the two-cell stage, but is shown to be gradually demethylated in a passive demethylation manner over the subsequent cleavage divisions (Wossidlo et al., 2011). As a result, both parental genomes have acquired low levels of methylation by the early blastocyst stage (Sanz et al., 2010). This widespread demethylation is followed by the gradually increasing of 5-mC content through *de novo* of DNA methylation thus reprogramming the embryonic DNA methylome to reach a hypermethylation level at epiblast stage (about 70% of CpG sites) (Jessop et al., 2018; Santos et al., 2002) (**Fig. 1-7**).

The subsequent differentiation of epiblast distinguishes embryonic soma and the primordial germ cells (PGCs), which show different fates in methylation pattern. The somatic fated cells continue to increase their 5-mC content and acquire distinct methylomes according to their lineages, whereas the PGCs confront a phase of comprehensive erasure of 5-mC,

which resets the PGCs into a ‘blank’ state (Hackett and Surani, 2013). This second global demethylation process is complete before the sexual specification of PGCs. From this time point onward, the re-methylation process initiates to re-establish the parental imprints in the gametes and eventually leading to sperm and oocyte-specific methylation patterns (Messerschmidt et al., 2014) (**Fig. 1-7**).

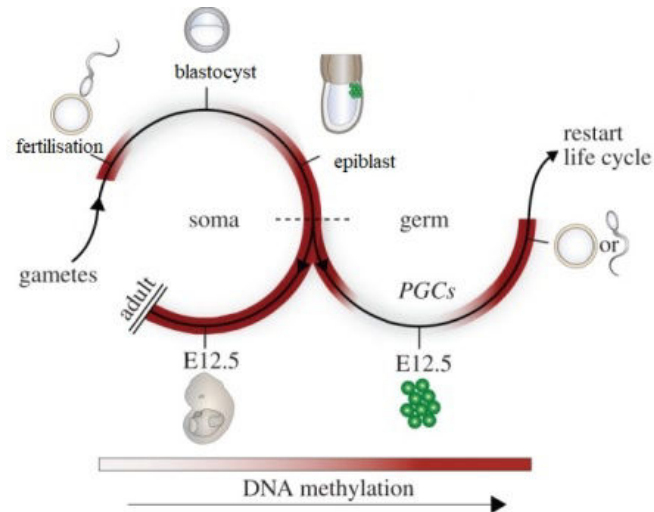


Figure 1-7 Global DNA methylation dynamics during the life cycle in mammals. After fertilization, genome-wide DNA demethylation occurs in the zygote. The low-point of global methylation occurs at the early blastocyst stage, after which global re-methylation begins and reaches near completion by epiblast stage. At the epiblast stage, cells either continue to develop towards a somatic fate (left) or are specified as primordial germ cells (PGCs-right). Somatic fated cells acquire distinct methylomes according to their lineage but maintain high global levels of DNA methylation. PGCs initiate a phase of comprehensive DNA demethylation, which is complete before sexual specification, and which enables subsequent establishment of a unique gamete-specific methylome during gametogenesis. Mature gametes can then fuse to form the zygote and initiate a new life cycle (Hackett and Surani, 2013).

Up to date, there is a lack of information about the DNA methylation dynamics during gametogenesis in teleost. One of the biggest obstacles for exploring DNA methylation dynamics in teleost is possible because of the presence of large amount of yolk in the oocytes of many species such as salmonids. This abundance of yolk makes it very difficult to extract genomic DNA in developing ovaries and embryos of teleost with appropriate quantity and quality. However, few studies concerning the DNA methylation dynamics during embryogenesis were conducted in teleost during the past decades, most of which were done in zebrafish. Results obtained remain controversial about whether the widespread DNA demethylation process is conserved in teleost embryonic development or not. One study did in zebrafish demonstrated a lower level of DNA methylation in 1-2 cell stage embryos

compared to those in both oocytes and sperm, which further increased until the late blastula (4hpf) (Mhanni and McGowan, 2004). Contrarily, studies conducted in goldfish and medaka failed to observe the variations in global DNA methylation levels during embryonic development (Labbé et al., 2017; Walter et al., 2002).

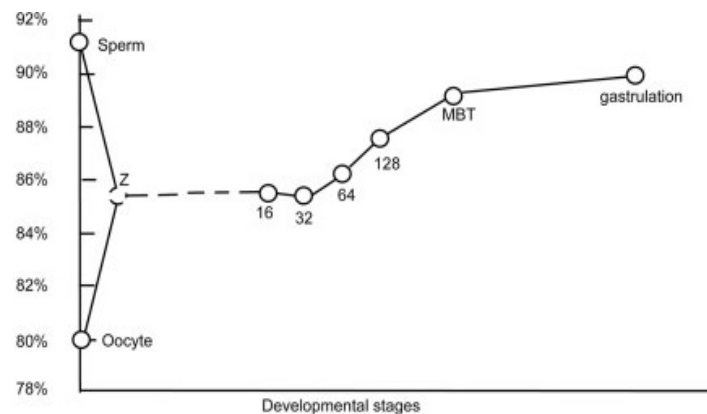


Figure 1-8 Global DNA methylation level during zebrafish development. The average methylation level is calculated as the percentage of methylated CpG in all CpG content. “Z” means the mean of methylation level between oocyte and sperm. MBT: the midblastula stage (1k cell) (Jiang et al., 2013).

Two recent works did in zebrafish with more advanced whole-genome shotgun bisulfite sequencing method obtained similar results, which demonstrated that the global methylation level at the 16-cell stage is equivalent to the average methylation level of sperm (91%) and oocyte (80%). From 16-cell on, this percentage continues to rise until gastrulation stage, when the methylation levels reaching those observed in sperm (Jiang et al., 2013; Potok et al., 2013). However, no data about the DNA methylation level in earlier developmental stages were reported, thus it is hard to conclude whether or not there is a global DNA methylation erasure existence (**Fig. 1-8**). Interestingly, these two studies also found that during zebrafish development, the maternal genome is gradually programmed to a pattern similar to that in sperm. This remodelling process includes both passive demethylation of oocyte-specific hypermethylated regions and *de novo* methylation of oocyte hypomethylated regions (Bogdanović and Gómez-Skarmeta, 2013).

However, debates arise questioning if the global demethylation prior to gametogenesis is truly required in teleost species as in mammals. In contrast to mammals, whether or not the genomic imprinting mechanism exists in teleost species remain controversial. PGCs in teleost

are pre-determined germplasm which inherited from oocyte at the beginning of embryogenesis and could further develop into germ cells (Raz, 2003). Thus there is no need for the fish embryos to generate PGCs and reset for naïve pluripotency, which is linked to genome demethylation (Jessop et al., 2018; Labbé et al., 2017).

1.2.3 DNA methylation regulation by nutrients, oxidative stress and hypoxia

Changes of epigenetic marks like the global DNA demethylation and re-methylation during certain life stages are natural processes, which occur not only under tightly controlled and pre-programmed mechanisms but also can be influenced by both internal and external factors including metabolism, aging, nutrition, pollutants, *etc.*. These factors may reshape the epigenome and alter gene expression, thereby giving rise to phenotypic plasticity. In the following sub-chapter, general mechanisms and recent insights related to the DNA methylation regulatory by nutrients, oxidative stress and hypoxia will be discussed. These three environmental factors are key players which mediated epigenetic regulations. In addition, they are of great importance for the survival, growth, development and health of aquatic species.

1.2.3.1 DNA methylation regulation by nutrients

Numerous studies have focused on the link between diets and DNA methylation to elucidate how variations in dietary components may induce long-term consequences for health and the pathogenesis of diseases (Chmurzynska, 2010; Lim and Song, 2012; Liu et al., 2017). Most data exist concerning the effects of dietary regulations of DNA methylation are associated with perinatal nutrition *i.e.* protein restriction, methionine and/or folate deficiency, and high-carbohydrate and/or high-fat consumptions derived metabolic disorders like diabetes, obesity and cardiovascular diseases later in life (Anderson et al., 2012; ElGendy et al., 2018; Ng et al., 2010; Niculescu and Zeisel, 2002; Vansant, 2016; Zhang, 2018). Here I will briefly discuss the metabolic pathways closely linked with DNA methylation, and how dietary nutrients may affect the DNA methylome.

1.2.3.1.1 Metabolic pathways involved in DNA methylation

As mentioned in previous sub-chapters, the reaction of DNA methylation is mediated through the function of DNMT, with S-adenosylmethionine (SAM) serves as the primary methyl donor and S-adenosylhomocysteine (SAH) as a byproduct during methyltransferase reactions (Anderson et al., 2012). The biosynthesis of SAM is involved in the cyclical

cellular process named one-carbon metabolism, where many dietary nutrients like methionine, folate, choline, betaine and other B vitamins function as substrates or cofactors (Friso et al., 2017)(Fig. 1-9). In this sense, any dietary nutrients that can alter the methyl availability may potentially influence DNA methylation.

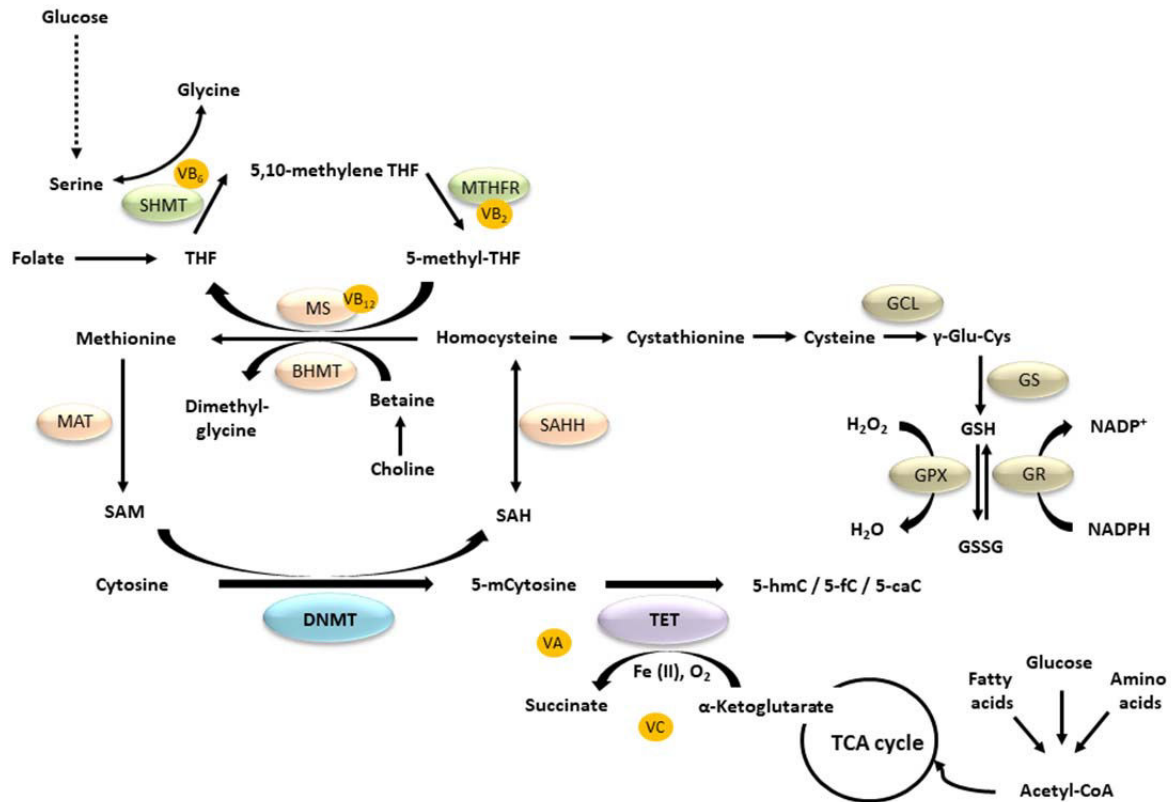


Figure 1-9 Metabolic pathways and DNA methylation/demethylation regulations. BHMT: betaine homocysteine methyltransferase; DNMT: DNA methyltransferase; GCL: glutamate cysteine ligase; GPX: glutathione peroxidase; GR: glutathione reductase; GS: glutathione synthetase; GSH: reduced glutathione; GSSG: oxidized glutathione; MAT: methionine adenylyltransferase; MS: methionine synthase; MTHFR: methylenetetrahydrofolate reductase; SAH: S-adenosylhomocysteine; SAHH: S-adenosyl homocysteine hydrolase; SAM: S-adenosylmethionine; SHMT: serine hydroxymethyltransferase; TET: ten-eleven translocation methylcytosine dioxygenase; THF: tetrahydrofolate. Modified from (Masih et al., 2014).

Briefly, SAM can be generated from methionine and ATP by methionine adenylyltransferase (MAT) (Finkelstein, 1990). After the methyl transfer reaction, SAM is converted to SAH, which can be further hydrolysed into homocysteine by S-adenosyl homocysteine hydrolase (SAHH) (Finkelstein, 1990). This reaction is reversible when homocysteine is not efficiently removed, because the thermodynamics of SAHH favours SAH synthesis instead of hydrolysis (De La Haba and Cantoni, 1959), which in return

resulted in an accumulation of SAH and inhibition of DNA methylation. The clearance of homocysteine can be achieved through two processes: one is remethylation to methionine, and the other is entering the transsulfuration pathway to form glutathione (GSH), which plays an important role in antioxidant defense of oxidative stress (**Fig. 1-9**) (Schulz et al., 2000; Selhub, 1999). The regeneration of methionine from homocysteine can be catalysed by methionine synthase (MS) with Vitamin B₁₂ as cofactor and 5-methyl-tetrahydrofolate (5-methyl-THF) donating a methyl group to become THF, which comprises an important step of folic cycle. In addition, THF can be also produced from dietary folate, and subsequently recycled to form 5-methyl-THF through a 5, 10-methylene THF intermediate. The former reaction is catalysed by serine hydroxymethyltransferase (SHMT) with Vitamin B₆ as a cofactor, while the latter is driven by methylenetetrahydrofolate reductase (MTHFR) with Vitamin B₂ as cofactor. An alternate pathway for homocysteine conversion to methionine is through the function of betaine homocysteine methyltransferase (BHMT), where betaine, from the diet or derived from choline, serves as the methyl donor (**Fig. 1-9**) (Craig, 2004; Kadayifci et al., 2018; Ulrey et al., 2005).

On the other hand, there is still a lack of knowledge in understanding the dietary modulations of active DNA demethylation process. TET enzymes belong to the family of Fe (II)- and 2-oxoglutarate-dependent dioxygenases, therefore the dietary factors that can modulate 2-oxoglutarate and Fe (II) availabilities are supposed to have an impact on TET function. Besides, TET activities can be also inhibited by intermediates of the TCA cycle such as fumarate and succinate, which are downstream products of 2-oxoglutarate (Xiao et al., 2012). Recent studies have found that Vitamin C can enhance the function of TET thereby leading to DNA hypomethylation. One explanation for this effect showed that Vitamin C can interact with the TET enzymes in their catalytic domains, which possibly promotes their folding and/or recycling of the cofactor Fe (II) (Blaschke et al., 2013; Yin et al., 2013). It has been hypothesised that Vitamin C may serve as a cofactor for TETs (Minor et al., 2013). Another study reported that Vitamin A can also stimulate TET and induce the DNA demethylation process, but detail mechanisms underlying this effect remain to be understood (Hore et al., 2016). Zhang and collaborators' work found that the O-linked β -GlcNAc transferase (OGT) was a major TET3-interacting protein and can regulate TET3 subcellular localisation and enzymatic activity. The O-GlcNAcylation of TET3 was regulated by glucose metabolism (Zhang et al., 2014).

Above all, it is generally believed that the possible ways through which dietary factors may affect the DNA methylation patterns include: i) altering the availability of substrates or cofactors that are necessary for methylation process; ii) modulating the function of DNMT and/or TET; iii) adjusting the activities of enzymes regulating the one-carbon cycle (Zhang, 2015).

1.2.3.1.2 Methyl-donor nutrients (dietary protein, vitamins, etc.) and DNA methylation

So far, numerous researches have investigated the effects of supplementation/deficiency in dietary methyl-donors or corresponding cofactors on the DNA methylation level in mammals. These studies have been thoroughly reviewed by some authors recently (Crider et al., 2012; Friso et al., 2017; Kadayifci et al., 2018; Zeisel, 2017; Zhang, 2015; Zhang, 2018). Theoretically, diets deficient in methyl-donor nutrients (*i.e.* methionine, folate, *etc.*) could hinder the synthesis of SAM, which might further lead to the insufficient DNA methylation process and thus DNA hypomethylation. In turn, the increased availability of methyl-donor nutrients through dietary supplementation is supposed to enlarge the SAM pool therefore enhancing DNA methylation. However, there is not a simple correlation between methyl-donor concentration and DNA methylation, and the regulation of DNA methylation is much complex than originally presumed. In particular, the methylation reaction converts SAM to SAH, which is a strong inhibitor for almost all the methylation reactions (Yi et al., 2000). Indeed, increasing evidence demonstrated that the level of SAH is more closely correlated with global DNA methylation level compared to SAM (Mattocks et al., 2017; Mirbahai et al., 2013). Also, in most cases researchers consider not only the SAM and SAH concentrations separately but also the ratio between them—SAM/SAH, the so-called ‘methylation index’ (Waterland, 2006).

Take one of the best investigated fields, dietary methionine deficiency as an example. A previous study in rats found that maternal protein deficiency resulted in hepatic DNA hypermethylation in fetal liver, which could be due to the alteration of methionine and homocysteine metabolism (Rees et al., 2000). Another study in mice showed that 1-week feeding of methionine-choline-deficient (MCD) diet decreased the SAM content and the SAM/SAH ratio in the liver, whereas the total 5-mC level exhibited a slightly decreased trend in MCD group, but no statistically significant difference was achieved. In addition, upregulation of *Tet2* and *Tet3* gene expression, as well as *Dnmt1* and *Dnmt3a* was observed in rats fed the MCD diet, indicating that the sophisticated DNA methylation regulation existed

even when dietary methyl donors are restricted (Takumi et al., 2015). It has been shown that a methyl-deficient diet feeding for 9 weeks resulted in DNA damage and pronounced global loss of DNA methylation in the rat liver, these abnormalities can be completely restored after refeeding a methyl-sufficient diet. However, when the dietary methyl-deficient exposure extended to 18 weeks, the refeeding of methyl-sufficient diet failed to restore the altered DNA methylation status (Pogribny et al., 2009).

Besides, increasing evidence showed that the nutritional regulation of methylation reaction can be tissue-specific, and the effects may also depend on ages and sex. For instance, a hepatic global DNA hypomethylation and brain hypermethylation occurred together in methyl deficient rats (Pogribny et al., 2008; Pogribny et al., 2006), and a short-term methionine restriction increased hepatic global DNA methylation in adult (1-year-old) but not young (8-week-old) mice (Mattocks et al., 2017).

1.2.3.1.3 Dietary lipids and DNA methylation

The type and amount of dietary fat significantly affect the metabolism and the development of obesity and other diseases. Increasing evidence has demonstrated that DNA methylation may play important roles in the regulation of these processes.

It has been showed that a high-fat diet could influence global DNA methylation level in various animal models. One study in vervet monkeys proved that the global DNA methylation level in white blood cells was twofold lower in monkeys fed a high-fat diet compared to monkeys fed a standard diet (Pheiffer et al., 2014). Similarly, high dietary fat induced global DNA hypomethylation has also been reported in mice (Ciccarone et al., 2019; Yokoyama et al., 2018). By contrast, another study in mice showed that a high-fat diet increased muscular global DNA methylation, whereas dietary supplementation of fish oil decreased global DNA methylation in adipose tissue (Amaral et al., 2014). Dietary docosahexaenoic acid supplementation in pregnant women showed slightly higher (1%) global DNA methylation in cord blood leukocytes, but statistical difference was not achieved (Lee et al., 2013). Some recent studies explored the effects of high-fat diet on genome-wide DNA methylation profile and gene expression patterns, where tremendous differentially methylated regions induced by high-fat diet were identified, including both hyper and hypomethylation of genes involved in several important metabolic pathways (Keleher et al., 2018; Zhang et al., 2015; Zhou et al., 2016).

Dietary fat profile can also affect the DNA methylome in different manners (Voisin et al., 2015). Overfeeding of diets rich in saturated fatty acids (SFA) or polyunsaturated fatty acids (PUFA) resulted in distinct epigenetic changes in human adipose tissue. The global degree of DNA methylation levels increased with the intervention of both PUFA and SFA. However, 1797 genes were differentially induced by PUFA, whereas 125 genes were differentially induced by SFA among the 4875 CpG sites differentially methylated regions identified (Perfilyev et al., 2017).

It is still a mystery at present how dietary lipids modify DNA methylation. Evidence has shown that the expression levels of DNMTs (DNMT1, 3A and 3B) and the methyl-CpG-binding domain protein 2 (MBD2) were affected by dietary lipids (Lu et al., 2018). In addition, it is hypothesised that the global changes in DNA methylation/hydroxymethylation levels induced by high-fat diet could be associated with upregulation of TET (Ciccarone et al., 2019). The β -oxidation of fatty acids can produce acetyl-CoA, which enters the TCA cycle and thereby potentially affecting the concentration of the TET cofactor, 2-oxoglutarate. Also, the peroxidation of lipids is known to generate reactive oxygen species (ROS), which might alter the oxidative status and further affect the DNA methylation reaction.

1.2.3.1.4 Dietary carbohydrates and DNA methylation

Currently, there are relatively fewer studies concerning the effects of dietary carbohydrates on DNA methylation regulation compared to dietary proteins and lipids. Instead, mounting researches are done to investigate the epigenetic modulations and consequences involved in diabetes, which has long been considered as a disorder of carbohydrates metabolism due to its hallmark feature of hyperglycaemia.

Researches did by Srinivasan and collaborators showed that feeding the neonatal rat with a high-carbohydrate (HC) milk from 4-day to 28-day old altered the size and number of pancreatic islets compared to mother-fed rats. (Petrik et al., 2001; Srinivasan et al., 2001). Although epigenetic markers were not measured in these studies, the HC diet-induced alterations in key genes expressions (*i.e.* pancreatic duodenal homeobox factor-1, which modulates pancreatic ontogeny as well as preproinsulin gene expression in islets) may likely under epigenetic control. In addition, previous studies found that rat pups fed on high-carbohydrate (HC) milk immediately developed hyperinsulinemia, which persisted into adulthood despite weaning onto lab diet on day 24 (Hiremagalur et al., 1993). A subsequent study showed that the exposure to HC diet during the suckling period of rats induced long-

term increase of the Neuropeptide Y (*Npy*) mRNA level in hypothalamus. This endurable alteration in *Npy* expression could be explained by the changes observed in promoter CpG methylation as well as the H3K9 acetylation (Mahmood et al., 2013).

Because dietary carbohydrates and dietary lipids are closely linked in intermediary metabolism, some studies investigated their regulatory effects together (high-fat and high-carbohydrate diet) (Lomba et al., 2010b; Zheng et al., 2015b). One study showed that mice fed a Western diet (42% kcal fat, 0.2% total cholesterol and 34% sucrose) for 12 weeks resulted in lower global methylation in the frontal cortex compared to those fed a control diet (19.2% kcal fat, 0% added total cholesterol and 12% sucrose) (Yokoyama et al., 2018). Actually, most researches concerning the effects of high-fat diets were done by increasing the dietary lipids content and decreasing dietary carbohydrates (high carbohydrate/low-fat diet vs high fat/low carbohydrates diet) (Lomba et al., 2010a; Schwenk et al., 2013; Zhou et al., 2016). Therefore, it is hard to discriminate their respective effects through *in vivo* feeding trial.

One study tested the effects of dietary fructose on the pathogenesis of metabolic syndrome in rats through oral solution method. They found that rats received 20% fructose solution for 14 weeks developed non-alcoholic fatty liver disease. Increased hepatic mitochondrial DNA (mtDNA) content, decreased global 5-mC and 5-hmC levels of mtDNA were observed in fructose-fed rats compared to control group (water solution) (Yamazaki et al., 2016). Another study did in mice using intraperitoneal injection method to investigate the glucose delivery on DNA methylation regulation. Results showed that although the global 5-mC level in liver remained stable after glucose injection, the global hepatic 5-hmC and 5-fC contents were significantly increased (Yang et al., 2016).

Nowadays, more and more studies were performed *in vitro* to explore the role of glucose on DNA methylation regulation directly. Lai and collaborators reported that reducing glucose level in the culture media of HepG2 cells resulted in down-regulation of the mRNA levels of all three TET enzymes (Lai et al., 2018). By contrast, another study showed that high glucose conditions can decrease the TET2 protein level and thereby reducing 5-hmC content in several human cell lines. They also demonstrated that the low levels of 5-hmC seen in high glucose conditions is achieved through the inhibition of AMPK-mediated TET2 phosphorylation and increased TET2 degradation (Strzyz, 2018; Wu et al., 2018). Several other studies using different cell lines also reported that high glucose can alter the DNA

methylation of specific genes thereby regulating their expression (Ishikawa et al., 2015; Palacios-Ortega et al., 2016). Study did by Zhang *et al.* reported that high glucose can promote the cytoplasmic translocation of Dnmt3a, thereby decrease binding of Dnmt3a on connective tissue growth factor (CTGF) promoter and increase its expression in human glomerular mesangial cells. They further discovered that this high glucose-induced translocation of Dnmt3a could be mediated through the ERK/MAPK signalling pathway (Zhang et al., 2016).

1.2.3.2 DNA methylation regulation by oxidative stress

Reactive oxygen species (ROS) refer to a group of chemical species that are formed upon incomplete reduction of oxygen, including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), *etc.* ROS can either be produced endogenously through aerobic mitochondria respiration, or through exogenous oxidising agents like ionising radiation and heavy metals (Niu et al., 2015). Although ROS are originally considered as toxic product with damaging effects on DNA, protein, and lipid, it is now well acknowledged that ROS also play important roles in several biological processes such as cellular signalling and bacteria defencing (D'Autréaux and Toledano, 2007). Under normal condition, antioxidants (superoxide dismutase, catalase and reduced glutathione) can efficiently remove ROS to prevent tissue damage. However, overproduction of ROS or deficient in antioxidant defences can lead to oxidative stress, which has been linked to several pathological processes (Betteridge, 2000). As oxidative stress and aberrant DNA methylation can be often observed simultaneously in many diseases like cancer and diabetes, studies about their relationships and how they may affect disease development have always been research hotspots.

Previous studies have demonstrated that ROS could serve as an import factor that affects epigenetic mechanisms including DNA methylation (Ziech et al., 2011). The oxidative stress-induced alterations in DNA methylation can be achieved through different mechanisms including: i) DNA oxidation damage; ii) SAM depletion; iii) inhibition of TETs activity through iron oxidation and TCA intermediates (**Fig. 1-10**).

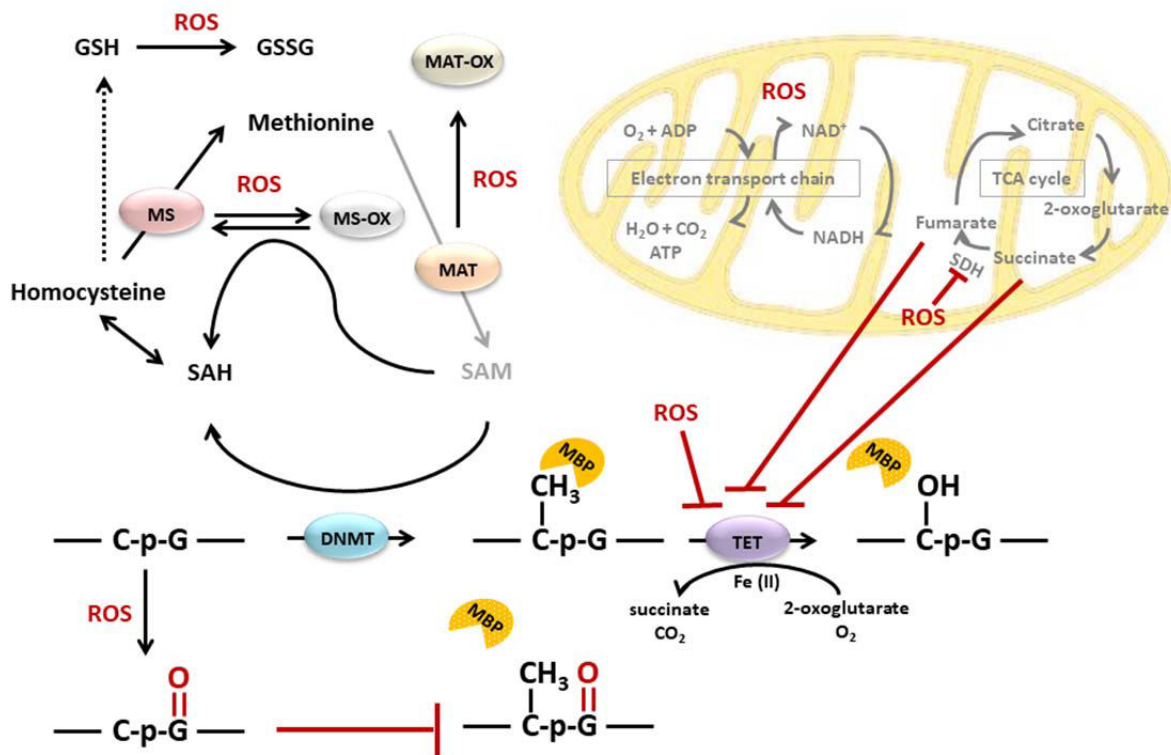


Figure 1-10 Oxidative stress influences chromatin modifiers. DNMT: DNA methyltransferase; GSH: Glutathione; MAT: Methionine adenosyltransferase; MS: Methionine synthase; NAD⁺: Nicotinamide adenine dinucleotide; ROS: Reactive oxygen species; SAM: S-adenosylmethionine; SDH: Succinate dehydrogenase; TET: Ten-Eleven-Translocation DNA demethylase. Figure modified from Kreuz and Fischle (Kreuz and Fischle, 2016).

1.2.3.2.1 DNA oxidative damage and DNA methylation

In CpG dinucleotides, the cytosine is the preferred base for DNA methylation, whereas the guanine is the most susceptible nucleoside for oxidative damage. Under oxidative stress, guanine becomes easily oxidised to form 8-Oxo-7,8-dihydroguanine (8-oxodG), which has been regarded as the most reliable marker of DNA damage (Ziech et al., 2011). 8-oxodG can be bound and excised by OGG1 and then repaired by the BER pathway.

Previous studies have shown that the presence of 8-oxodG can directly inhibit the adjacent cytosine methylation (Turk et al., 1995; Weitzman et al., 1994). Likewise, the repair of 8-oxodG was also diminished when the 8-oxodG was preceded by 5-methylcytosine (Zawia et al., 2009). Besides, evidence was found that oxidation of guanine in CpG dinucleotides can

also weaken the binding affinity of methyl-CpG-binding proteins (*i.e.* MeCP2), leading to the hypomethylation status. Similarly, when 5-methylcytosine is oxidised to 5-hydroxymethylcytosine, its affinity to methyl-binding protein is also greatly reduced (Valinluck et al., 2004). In addition, 8-oxodG also has great influence on gene expression regulation through the interaction with transcription factors (Ba et al., 2014; Dattilo et al., 2016).

1.2.3.2.2 Oxidative stress induced SAM depletion and DNA methylation

Because DNMT requires SAM as methyl donor, depletion of SAM by oxidative stress may interrupt DNA methylation process (Kloypan et al., 2015). This oxidative stress-induced reduction of SAM pool could be triggered through different manners. Studies showed that oxidation of MAT resulted in inactivation of its function and reduced SAM synthesis from methionine (Avila et al., 1998). Besides, the other enzyme which catalyses the conversion of 5-methyl-THF and homocysteine to methionine and THF, namely MS, is easily oxidised and inactivated by oxidative stress. MS can be reactivated by MS reductase utilising SAM, establishing a futile cycle without any net SAM production (Jarrett et al., 1998). Finally, it has been demonstrated that oxidative stress can promote the homocysteine entering the transsulfuration pathway for the generation of GSH. Thus, less homocysteine is directed toward the generation of methionine, leading to the depletion of SAM (Cyr and Domann, 2011; Kreuz and Fischle, 2016; Niedzwiecki et al., 2013) (**Fig. 1-10**).

1.2.3.2.3 Redox regulation and TETs

As stated above, TETs mediate DNA demethylation process requires an important cofactor Fe (II). During the catalytic process, Fe (II) can be oxidised to Fe (III) and then reduced back to Fe (II) through the participation of ascorbate (Dickson et al., 2013). However, Fe (II) itself is susceptible to direct oxidation under oxidative stress conditions, thus leading to the inactivation of TETs and DNA hypermethylation (Niu et al., 2015). Besides, pro-oxidant changes in the redox state, notably marked by a reduction in GSH/GSSG ratio, could recruit ascorbate as free radical scavenger, thus preventing its ability to regenerate Fe (II) from Fe (III) (Lamadema et al., 2018). Consistently with this, one study found that antioxidant supplementation largely attenuated the oxidative stress-induced DNA hypomethylation and as well as DNA damage in human sperm (Tunc and Tremellen, 2009).

Additionally, previous studies have proved that the accumulation of TCA intermediates succinate and fumarate could inhibit the activity of TETs by competing for the 2-oxoglutarate-binding site (Bargiela et al., 2018). The alteration in concentrations of succinate and fumarate has been observed in oxidative stress associated mitochondria dysfunction (Frizzell et al., 2012; Pan et al., 2007). Evidence was found that the alteration in SDH (succinate dehydrogenase, catalyse succinate to fumarate, also a component of the Complex II in electron transport chain) might play important role in oxidative stress-induced epigenetic modifications. ROS can abolish the SDH function (Moser et al., 2009), thus leading to excess of succinate concentration and reduced TETs activity. SDH mutation in gastrointestinal stromal tumor resulted in succinate accumulation, TET inhibition and global DNA hypermethylation (Killian et al., 2013; Salminen et al., 2014).

1.2.3.3 DNA methylation regulation by hypoxia

For all the aerobic organisms, oxygen is an indispensable environmental factor that plays vital roles in biological processes. Insufficient oxygen availability, or hypoxia, due either to changes in ambient oxygen level or because of deficient oxygen homeostasis, can profoundly affect the metabolism, development and the pathologies of many diseases including cancer (Dunwoodie, 2009; Palmer and Clegg, 2014; Wilson and Hay, 2011). Under certain conditions, the influences of hypoxia can persist permanently throughout lifetime or may even transfer across generations (Gluckman et al., 2008; Ho and Burggren, 2012; Reeves et al., 2003; Wang et al., 2016). The detailed mechanisms concerning how hypoxia provokes durable alterations in cellular functions remain to be elucidated. But many studies have proven that these ‘metabolic memories’ induced by hypoxia may at least partly be linked to epigenetic mechanisms (Perez-Perri et al., 2011). It has been well acknowledged that hypoxia is a powerful regulator of histone modifications through the modulation of Jumonji histone demethylases (Hancock et al., 2015; Pollard et al., 2008). Increasing evidence pointed out that hypoxia can also affect both DNA methylation and demethylation processes (Lamadema et al., 2018; Shahrzad et al., 2007).

1.2.3.3.1 Hypoxia and DNA methylation: HIF-dependent pathway

The cellular responses to hypoxia have been well characterised by prevalent alterations in the expression of a vast number of genes. Many of these genes are controlled by hypoxia-inducible factors (HIFs), the master regulator for oxygen sensing and transcription factor for hypoxic-inducible genes’ expression. HIFs are heterodimers consisting of an oxygen-

sensitive α -subunit and a constitutively expressed β –subunit (Bruick, 2003; Semenza, 2003). Under hypoxic conditions, the HIF- α -subunit and β –subunit can form an active HIF. The HIF can thereby bind to the hypoxia-response element (HRE) at the promoter of a plethora of target genes, leading to their transcriptional activation (Appelhoff et al., 2004; Palmer and Clegg, 2014)

Previous studies have found that both DNMTs and TETs can be regulated by HIF (Lai et al., 2018; Zhang et al., 2019). Study did by Watson *et al.* (2013) showed that hypoxia induced pro-fibrotic changes in human cardiac fibroblast cells together with a concomitant global DNA hypomethylation and increased expression of DNMT1 and DNMT3B. The authors found that DNMT1 and DNMT3B gene promoter regions contain a consensus sequence for a HIF-1 α -binding HRE, and proved the hypoxia-induced upregulation of DNMT enzymes is mediated by HIF-1 α (Watson et al., 2013). Similarly, hypoxia-induced overexpression of TETs has been observed both in mice and in zebrafish (Wang et al., 2017). Lin and collaborators reported that hypoxia upregulated the expression of all three members of TET enzymes as well as the 5-hmC level in HepG2 cells. Knockdown of HIF-1 α attenuated the hypoxia-induced expression of TET enzymes (Lin et al., 2017). These results indicated that hypoxia could control the expression of TET enzymes and regulate DNA methylation in a HIF-dependent manner.

Besides, one study in mice found that hypoxia induced genomic DNA hypomethylation and reduced the steady-state of SAM content both *in vivo* and *in vitro*. They also observed a surprisingly upregulated expression of methionine adenosyltransferase 2A (MAT2A), a key enzyme that is responsible for converting methionine to SAM in the one-carbon cycle. This overexpression is mediated through HIF-1 α activation pathway, although it remains hard to explain how this upregulation in MAT2A leads to a decrease in SAM (Liu et al., 2011).

Interestingly, it has been demonstrated that the consensus HIF binding site sequence 5'-RCGTG-3' contains a CpG dinucleotide, suggesting that HIF dependent gene regulation could be intrinsically affected by DNA methylation under hypoxia (Nanduri et al., 2017; Wenger et al., 2005). Moreover, evidence has also been found that TET may in return regulate HIF (Kao et al., 2016). For instance, one study demonstrated that Tet1 could stabilise HIF- α and enhance HIF- α transcription activity (Wang et al., 2017).

1.2.3.3.2 Hypoxia and DNA methylation: links to 2-oxoglutarate-dependent dioxygenases (2-OGDDs)

One of the most important strategies for cellular adaptation to hypoxic condition is the shift from mitochondrial oxidative phosphorylation to an anaerobic glycolytic pathway. A critical step in this shift is HIF-mediated activation of pyruvate dehydrogenase kinase-1 (PDK-1). This enzyme inactivates pyruvate dehydrogenase (PDH) which is the mitochondrial enzyme responsible for converting pyruvate to acetyl-CoA. In addition, lactate dehydrogenase A (LDHA), the key enzyme that converts pyruvate to lactate, is also activated by HIF. Like this, a reduction of delivery acetyl-CoA into the TCA cycle may thereby reduce the production of nicotinamide adenine dinucleotide (NADH) and inhibit electron transport chain (Palmer and Clegg, 2014) (**Fig. 1-11**). It can be thus hypothesised that hypoxic conditions may result in changes in levels of TCA metabolites, which are known to affect the activity of 2-OGDDs.

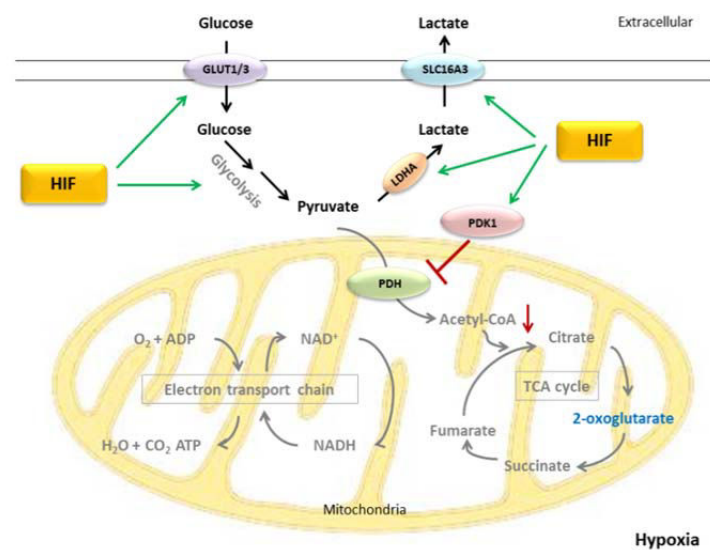


Figure 1-11 HIF-dependent regulation on glycolysis and TCA cycle. Hypoxic condition triggers a shift from mitochondria oxidative phosphorylation to anaerobic glycolysis pathway. Green arrows represent activating effects by HIF, red ones represent inhibiting effects. LDHA: lactate dehydrogenase A; GLUT: glucose transporter; PDH: pyruvate dehydrogenase; PDK1: PDK-1; SLC16A3: solute carrier family 16 member 3. Figure modified from Lin et al.(Lin et al., 2014).

The most important 2-OGDDs involved in hypoxic responses including PHD1-3 (prolyl hydroxylases of HIF α subunits) as well as TET enzymes. As 2-OGDDs, the activity of TETs inherently depends on the level of co-substrates: oxygen, Fe^{2+} , 2-oxoglutarate as co-substrates. Thus hypoxia inducing the shortage of oxygen substrate can result in reducing

TET catalytic activity which further contributes to DNA hypermethylation. This reduction in TET activity could occur independently of hypoxia-associated alterations in TET expression (Thienpont et al., 2016). On the other hand, the hypoxia-induced alteration in TCA metabolites like 2-oxoglutarate, fumarate and succinate may also affect the DNA methylation process through the interference of TETs activities (Lamadema et al., 2018).

1.2.4 Global DNA hyper and hypomethylation and the related consequences

1.2.4.1 DNA methylation variations and the consequences on the genome stability

The DNA methylation status and DNA methylation-related enzymes can affect genome integrity. The possible mechanisms involved in DNA methylation-mediated alterations in genome stability include: i) regulating transcriptional activity; ii) maintaining the stability of repeat elements; iii) preventing the mutations and chromosomal rearrangement; iv) participating in DNA repair mechanisms that prevent and/or correct genetic errors (Zhou and Robertson, 2016).

1.2.4.1.1 DNA methylation and transcriptional regulation

DNA methylation at promoters and gene bodies regulates transcriptional activity in different ways. The predominant DNA methylation occurs at CpG sites in mammals. 80% of these CpG dinucleotides are methylated. Instead, CpG islands, which contain longer stretch of CpG rich region and typically located at gene promoters, often remain unmethylated (Bird and Wolffe, 1999). Hypermethylation of CpG islands in promoters generally linked to gene silencing, whereas hypomethylation permits active transcription (De Smet et al., 1999). About 70% mammalian genes possess CpG islands in promoter, including housekeeping genes, developmental genes, tumour suppressors and cell-cycle genes. Aberrant hypermethylation at promoters of these genes is often observed in cancer or other diseases (Das and Singal, 2004). In contrast to promoter methylation, gene body methylation is often associated with active transcription (Jones, 1999; Yang et al., 2014b). Besides, it has been observed that exons tend to be more methylated than introns, and that the transcriptional start sites (TSS) proximal region and transcription termination sites are devoid of methylation (Lister et al., 2009). These findings indicate a role of DNA methylation in transcriptional elongation and termination, and perhaps alternative splicing (Jeltsch, 2010).

The DNA methylation-mediated gene transcription can be achieved through i) directly prevention of transcription factor binding, or ii) indirectly through attracting of methyl-CpG binding domain proteins that recruit ‘repressor complexes’ thus leading to histone modifications and alterations in chromatin structure (Bird and Wolffe, 1999; Jones et al., 1998). The proposed mechanisms of DNA methylation mediated gene repression are illustrated in **Fig. 1-12**.

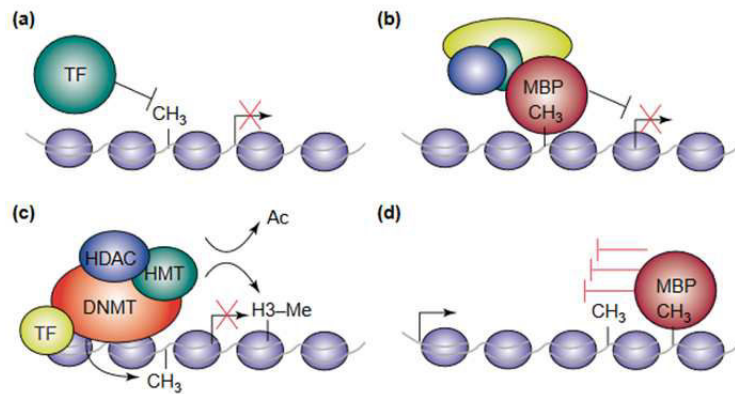


Figure 1-12 Mechanisms of DNA-methylation-mediated repression. (a) DNA methylation in the cognate DNA-binding sequences of some transcription factors (TF) can result in inhibition of DNA binding. By blocking activators from binding targets sites, DNA methylation directly inhibits transcriptional activation. (b) Methyl-CpG-binding proteins (MBPs) directly recognize methylated DNA and recruit co-repressor molecules to silence transcription and to modify surrounding chromatin. (c) In addition to their DNA methyltransferase activities, DNMT enzymes are also physically linked to histone deacetylase (HDAC) and histone methyltransferase (HMT) activities. In this case, the addition of methyl groups to DNA is coupled to transcriptional repression and chromatin modification. (d) DNA methylation within the body of genes can also have a dampening effect on transcriptional elongation. MBPs might be involved in inhibiting elongation, either directly or by their effects on the surrounding chromatin structure (Klose and Bird, 2006).

DNA methylation can also occur in the context of non-CpG sites, the gene-regulatory functions of this form of DNA methylation are less clear. However, increasing evidence was obtained in recent studies which showed that methylation at non-CpG sites also has effects on gene expression regulation (Patil et al., 2014). These findings highlight the need to uncover the originally negligent functions of non-CpG methylation.

1.2.4.1.2 DNA methylation and repeat sequences

The vast majority of vertebrate genomes are occupied by non-coding regions (in human, 98.5%), which are important contributors to genome/chromosome stability. Repeat elements comprise nearly half of these non-coding sequences. Interspersed repeats [*i.e.* transposable elements (TEs)] and tandem repeats (*i.e.* microsatellites, minisatellites, and satellites) are the

two main groups of repeat elements (Ahmed and Liang, 2012). Evidence showed that both groups can be affected by DNA methylation, thus leading to the alterations in genome stability and disease onset.

TEs are discrete mobile DNA segments that capable of moving and integrating randomly within the genome, which is associated with genome instability. DNA methylation of TEs can preserve their stability trough silencing gene transcription. Besides, numerous TEs are key components of heterochromatin enriched at regions flanking centromeres and telomeres (Schueler and Sullivan, 2006). DNA methylation levels on these TEs are functionally related to the formation and stability of constitutive heterochromatin (Zhou and Robertson, 2016).

Microsatellite loci are repetitive sequences typically consisting of one to four nucleotide repeats, and they are particularly susceptible to mutations through length changes. DNA methylation appears to protect against microsatellite repeat instability (MSI) through both the DNA methylation induced transcriptional repression of genes involved in MSI, and the DNMT1 protein involved mismatch repair (MMR) (Putiri and Robertson, 2011).

1.2.4.1.3 DNA methylation and chromosomal recombination

DNA hypomethylation is generally associated with an increased frequency of gene rearrangements and chromosomal translocations as a consequence of increased homologous recombination (HR) (Zhou and Robertson, 2016). HR often occurs between DNA regions sharing high sequence identity. It occurs regularly during meiosis and also occasionally in somatic cells. Transcription increases the frequency of HR by exposing single stranded DNA and facilitates the homologous sequences approaching each other, whereas DNA methylation mediated transcriptional silencing inhibits HR (Domínguez-Bendala and McWhir, 2004). It is generally believed that HR is the basis for many mutational events. Thus, significantly increases mutation rates observed in extensive DNA hypomethylation condition could be potentially linked to the increasing rate of mitotic chromosomal recombination (Chen et al., 1998).

1.2.4.1.4 DNA methylation and DNA-damage repair

DNA replication and chromosomal rearrangements are the most likely processes to yield mutations. DNA damage repair serves as an important mechanism that reduces the mutation rate and maintains the whole genome stability. Previous studies point toward a role for

DNMT1 in DNA-damage repair, which appears to be independent of its maintenance methylation activity. DNMT1 is targeted to newly replicated DNA *via* the interaction with proliferating cell nuclear antigen (PCNA) and ubiquitin-like with PHD and ring finger domains 1 protein (UHRF1) (Hervouet et al., 2018). PCNA, also known as the polymerase processivity factor or sliding clamp, is required for DNA replication as well as DNA repair (Kelman, 1997). Evidence demonstrated that accumulation of DNMT1 and its binding partners PCNA and MBD4, as well as the repair-associated factor MLH1 were found at DNA damage sites (Mortusewicz et al., 2005; Ruzov et al., 2009). PCNA is involved in multiple DNA repair processes including both MMR and nucleotide excision repair (Nichols and Sancar, 1992; Umar et al., 1996), indicating that DNMT1 may function to facilitate this function. This speculation was further supported by the findings which showed that DNMT1 deficiency impairs MMR function (Loughery et al., 2011).

1.2.4.2 DNA methylation variations and the consequences on metabolic syndromes: focus on hyperglycaemia and diabetes

The metabolic syndrome refers to the constellation of several metabolic disorders including central obesity, hypertension, hyperglycaemia, insulin resistance, and dyslipidemia (*i.e.*, high triglycerides, low high-density lipoprotein cholesterol, and small low-density lipoprotein particles). Metabolic syndrome has been postulated to increase an individual's risk of developing chronic diseases such as Type II diabetes, cardiovascular disease, and several types of cancer (Carson and Lawson, 2018; Garvey and Lara-Castro, 2006).

Increasing evidence has demonstrated that genetic factor is not the only determinant for metabolic syndrome. Instead, metabolic syndrome is also closely related to lifestyle and environmental factors like nutrition. Epigenetics provides a unique lens for investigating how these factors interact to influence health outcomes, since it can be modulated by both environmental factors and metabolic status. Remodelling in epigenetic landscapes induced by environmental factors has been proved to affect the development of metabolic syndrome (Kunes et al., 2015). Here, I will mainly focus on global DNA methylation changes involved in hyperglycaemia and diabetes.

Aberrant DNA methylation, both hypermethylation and hypomethylation, has been observed in several metabolic disorders and diseases. For instance, global DNA hypomethylation accompanied by region-specific hypermethylation (especially for promoters of tumor suppressor genes) is frequently found in tumor cells (Das and Singal, 2004). Thus,

both the gene-specific and global DNA methylation might be proposed as a biomarker for metabolic disease risk (Pheiffer et al., 2014).

Hyperglycaemia is the key feature of diabetes. It is characterised by high level of fasting glucose as well as high level of postprandial glucose in blood (Baron, 1998). Numerous studies have been done to investigate if global DNA methylation correlates with diabetic hyperglycaemia, which might provide the possibilities of using epigenetic regulation for the early detection and prevention of diabetes.

One population-based study in human tested the association of metabolic syndrome and its individual components (fasting glucose, high-density lipoprotein cholesterol, triglycerides, blood pressure, and waist circumference) with global leukocyte DNA methylation level. Results showed that individuals with the metabolic syndrome had low DNA methylation level compared to participants without the syndrome. This DNA hypomethylation is independently associated with hyperglycemia and low high-density lipoprotein cholesterol (Luttmer et al., 2013). Accordingly, a study in vervet monkey also reported that decreased global DNA methylation level in blood cells was found in diabetic animals compared to normal ones. They also observed an inverse correlation between global DNA methylation and blood glucose concentration, although the association was not statistically significant (Pheiffer et al., 2014). Diabetic and/or hyperglycaemic experiment involving global DNA hypomethylation have been reported in several studies with various animal models including mammals and teleost (Dhliwayo et al., 2014; Marandel et al., 2016a; Olsen et al., 2012) (Lan et al., 2016; Williams et al., 2008). Using zebrafish as model, Olsen and collaborators demonstrated that hyperglycemia-induced global DNA hypomethylation and aberrant gene expression could be durable and even transmissible to daughter cell tissue, which was never exposed to hyperglycaemia before (Olsen et al., 2012).

Interestingly, evidence showed that not only global DNA hypomethylation but also global DNA hypermethylation could be involved in diabetes. Study did by Williams *et al.* found genomic DNA hypermethylation in the liver of Zucker (type 2) diabetic fatty rat. This result was completely different from their previous findings, which showed hepatic DNA hypomethylation in type 1 diabetic rats (Williams et al., 2008; Williams and Schalinske, 2012). Another study in humans observed that mothers with gestational diabetes mellitus displayed a significantly increase global placental DNA methylation (Reichetzeder et al., 2016). In addition, one previous study investigated the effect of diabetes status and

hyperglycaemia on global DNA methylation (Pinzon-Cortes et al., 2017). They found that the global DNA methylation level was significantly lower in the pancreas of Type 2 diabetes mellitus (T2DM) patients compared to the healthy control. However, global DNA hypermethylation was observed in the blood of T2DM patients. These results indicated that tissue-specific alterations in global DNA methylation could be independently associated with T2DM. The authors also hypothesised that the increased global DNA methylation level in blood could be linked with the hyperglycaemia induced oxidative stress. Furthermore, study did by Maghbooli *et al.* demonstrated that there was a positive significant correlation between diabetes duration and global DNA methylation levels in peripheral blood leukocytes, indicating that chronic hyperglycaemic exposure could play an independent role in global DNA methylation levels in T2DM patients (Maghbooli et al., 2015).

1.2.5 Non-CpG methylation and its functional highlights

In mammalian genomes, the cytosine within CpG dinucleotides is the primary site for DNA methylation. However, methylation of cytosine in CpA, CpT and CpC dinucleotides (known as non-CpG methylation) has also been reported for many years, yet remains less understood. Non-CpG methylation was originally described in plants genomes (Zhang et al., 2006). In mammals, non-CpG methylation was initially detected in embryonic stem cells (Ramsahoye et al., 2000). Latter studies found non-CpG methylation to be predominant in pluripotent cell types, decrease upon differentiation and near complete absence in various somatic cell types (Ziller et al., 2011). However, recent studies showed that non-CpG methylation is also enriched in brain tissue and oocytes (Jang et al., 2017; Lister et al., 2013; Tomizawa et al., 2011; Yu et al., 2017). These evidence proved that non-CpG methylation is actually more widespread than previously thought and may play important roles in gene regulation.

Among the different types of non-CpG methylation, methylation is most frequently found at CpA sites. In the human embryonic cell line HUES48, mean global levels of CpG, CpA, CpT and CpC methylation were 67.85%, 6.68%, 1.48% and 0.63% of all CpG, CpA CpT and CpC dinucleotides tested respectively (Ziller et al., 2011). This trend in the frequency of methylation at each dinucleotide (CpG > CpA > CpT > CpC) has been widely described in other studies (Jang et al., 2017; Patil et al., 2014).

In mammals, non-CpG methylation is catalysed by the *de novo* methyltransferases DNMT3A, DNMT3B and DNMT3L, whereas the maintenance methyltransferase DNMT1 is

not associated with non-CpG methylation patterns (Arand et al., 2012). This finding coupled with the fact that non-CpG methylation is mostly asymmetrical, indicating that non-CpG methylation would need to be re-established *de novo* after each cell division in order to be maintained (Patil et al., 2014).

Recent studies have proved that non-CpG methylation also has a functional role in transcriptional regulation. Similar to CpG methylation, non-CpG methylation can induce alterations in gene expression, although the detail mechanisms remain elusive (Han et al., 2011; Huang et al., 2018b; Sharma et al., 2016). Transcriptional repression of non-CpG methylation may be mediated by the interaction between DNA binding proteins and DNA regulatory elements (Patil et al., 2014). For instance, previous studies showed that the recombinant methyl-CpG binding protein 2 (MeCP2) can bind to methylated cytosines within both CpG and non-CpG dinucleotides (Guo et al., 2014). Interestingly, the authors found that the MeCP2 binding affinity for methylated non-CpG sites was lower in the absence of CpG methylated sites. Another study did by Inoue and Qishi (2005) reported that non-CpG methylation decreased gene transcription through reducing transcription factor binding (Inoue and Oishi, 2005).

A genome-wide promoter methylation analysis did by Barres *et al.* (2009) identified differential non-CpG methylation within the *PGC-1 α* [peroxisome proliferator-activated receptor γ (PPAR γ) coactivator-1 α] promoter in skeletal muscle from T2DM patients (Barres et al., 2009). The methylation levels were negatively correlated with *PGC-1 α* mRNA level. In addition, the authors described that an acute increase of non-CpG methylation at the *PGC-1 α* promoter in human muscle cells could be induced by exposure to tumour necrosis factor- α (TNF- α) or free fatty acids. Meanwhile, the palmitate-induced non-CpG methylation at the *PGC-1 α* promoter could be prevented by silencing of DNMT3B (Barres et al., 2009). This study not only provided evidence that non-CpG methylation is present in somatic cells and involved in regulating gene transcription, but also indicated that non-CpG methylation could be influenced by environmental changes (Pinney, 2014). Another study showed that acute exercise induced transient reduction in non-CpG methylation at the *PGC-1 α* promoter and increase in its mRNA level in human skeletal muscle (Barres et al., 2012). The authors hypothesised that non-CpG methylation may play a specialised role as compared to CpG methylation in mediating transient or rapid methylation.

1.2.6 Potential roles of 5-hmC, 5-fC and 5-caC

Emerging evidence has shown that the oxidised forms of 5-mC, *i.e.* 5-hmC, 5-fC, 5-caC, act not only as intermediates during 5-mC demethylation, but also constitute stable epigenetic marks of the genome and may possess unique regulatory functions.

1.2.6.1 5-hmC

It has been widely reported that the level of 5-hmC varies among different cells, with relatively higher abundance in pluripotent or multipotent cells such as ESCs and neural stem cells (Ito et al., 2011; Song et al., 2011). Besides, an inverse linear correlation between the proliferation rate of cells and the global level of 5-hmC has been found in both rat and porcine tissues (Gackowski et al., 2015). These results highlighted the important role of 5-hmC in regulating cellular pluripotent state. In addition, previous studies in mammals as well as in teleost species have found that the 5-hmC pattern is dynamically regulated during embryonic development (Kamstra et al., 2015b; Ruzov et al., 2011; Wang and Bhandari, 2019). While a global loss of 5-mC occurs during early embryonic developmental stages, the global 5-hmC level increases, indicating the existence of an active demethylation process at these periods (Ruzov et al., 2011). Although the functional roles of 5-hmC are still elusive, it has been increasingly recognised as an important epigenetic player that can regulate gene expression.

The genomic distribution of 5-hmC is diversified at enhancer, promoter, TSS, gene body, 3' UTR or intragenic region (Shi et al., 2017). One study in mice classified the ESC DNA methylome into un-, low-, and fully methylated regions (UMRs, LMRs, and FMRs, respectively). 5-hmC is enriched in LMRs of ESCs, which have a 10-50% level of methylation and frequently located in distal regulatory regions of the genome such as enhancers (Yu et al., 2012a). By contrast, in neuros, 5-hmC seems to be enriched at the gene bodies of neuronal function-related genes where 5-mC is strongly depleted, and the 5-hmC/5-mC ratio correlates well with gene expression levels (Mellén et al., 2012). It is conceivable that 5-hmC takes roles as *cis* element, *i.e.* to promote or repress gene expression by binding to TFs (Shi et al., 2017). Besides, previous studies have shown that 5-hmC can be also recognised by specific binding proteins such as MeCP2 and MBD3, which further regulating gene expression (Mellén et al., 2012; Yildirim et al., 2011). It has been found that 5-hmC is more abundant in constitutive exons than in alternatively spliced exons (Khare et al., 2012) in brain, indicating that 5-hmC may possibly have functions in alternative splicing.

1.2.6.2 5-fC/5-caC

5-fC and 5caC are further oxidation products of 5-hmC. They are consistently low in abundance across all mammalian tissues and cells examined (about 10 to 1000-fold less than 5-hmC) (Ito et al., 2011). 5-fC and 5-caC are currently considered committed demethylation intermediates (Song et al., 2012), which account for the portion of 5-hmC sites that are destined to demethylation (Song and He, 2013). Indeed, studies in murine ESCs found that 90% of 5-fC-marked regions reside in 5-hmC-enriched regions; however, they only mark about 30% of the hydroxymethylome (Song et al., 2013). Previous studies showed that 5-fC is enriched at distal regulatory elements such as poised enhancers (namely inactive enhancers but can be quickly activated once particular cellular signal or stimuli are given), LMRs and promoters of 'poised' genes (Song et al., 2013). Besides, a small fraction of 5-fC was also detected in the previously defined UMRs (Song et al., 2013), where high abundances of TET proteins have also been observed (Stadler et al., 2011). 5-fC and 5-caC can be efficiently removed by TDG, and previous studies have shown that *Tdg* depletion can result in an accumulation of 5-fC and 5-caC at enhancers, multiple TF-binding sites, LMRs and promoters of genes with low to intermediate expression (Shen et al., 2013; Song et al., 2013).

Despite the low abundances of 5-fC and 5-caC, they may exert potential functional roles in gene regulation through recruiting various protein partners. The formyl group of 5-fC and carboxyl group of 5-caC offer special chemical anchors for protein recognition. Previous studies have identified many more potential binding proteins or readers for 5-fC/5caC than for 5-hmC in mouse ESCs (Iurlaro et al., 2013; Spruijt et al., 2013). These results demonstrated that 5-fC/5-caC may recruit unique proteins thus playing specific regulatory roles during DNA demethylation (Song and He, 2013). Besides, previous studies showed that the *Tdg* depletion induced 5-fC/5-caC accumulation at distal regulatory regions seems to coordinate with the binding of transcriptional coactivator p300 (Song et al., 2013), which has a crucial function in chromatin remodelling and gene expression regulation. Moreover, it has been proved that 5-fC and 5-caC can induce a transient pausing of RNA polymerase II elongation on gene bodies, suggesting their potential regulating roles in gene transcription (Kellinger et al., 2012; Wang et al., 2015).

1.3 Epigenetics and metabolic programming

1.3.1 Concept of metabolic programming

It has been widely acknowledged that environmental disturbances (either nutritional or non-nutritional ones) encountered during ‘windows of plasticity’ may induce long-term consequences in metabolism and alter phenotypes in living organisms. This process is termed ‘metabolic programming’ (Lucas, 1991). Numerous mammalian studies were found investigating how environmental stimuli (malnutrition, smoke, alcohol, *etc.*) during perinatal stage may induce the risks for the onset of metabolic disorders and chronic diseases (*i.e.* obesity and diabetes) in adult stages (Bergen, 2006; Hellemans et al., 2010; Langley-Evans, 2015; Martínez et al., 2012). Metabolic programming raises increasing importance in the therapeutic intervention of metabolic syndromes and diseases development in mammals (Gallou-Kabani and Junien, 2005; Patel and Srinivasan, 2002; Vickers, 2014).

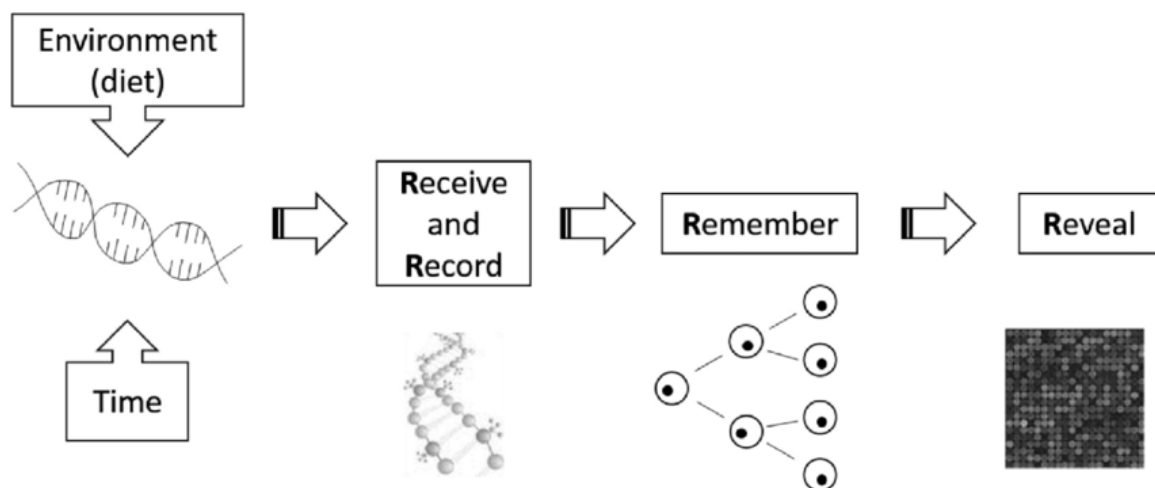


Figure 1-13 The ‘four Rs’ of epigenomics. A conceptual model of the key processes through which altered epigenomic markings as a result of environmental exposures are ‘Received’, ‘Recorded’, ‘Remembered’ and ‘Revealed’. Figure captured from (Mathers, 2008).

Epigenetics is considered as one of the most likely mechanisms mediating the long-lasting programming effects, since epigenome is particularly sensitive to environmental signals at some critical time points and epigenetics by definition refer to heritable alterations in gene expression. The possible mechanisms through which epigenetics participates the metabolic programming process have been well illustrated by Mathers in his ‘four Rs’ theory (Mathers, 2008). In this theory, it is hypothesised that the environmental stimuli experienced during critical periods can be received and recorded as epigenetic modifications; these modifications can be remembered throughout mitosis and/or meiosis, leading to alterations in gene

expression and metabolism and finally revealed as a specific phenotype later in life (**Fig. 1-13**).

1.3.2 Programmed phenotypes and suspected epigenetic mechanisms: selected examples from invertebrates up to mammals

1.3.2.1 Nematode

The model organism nematode (*Caenorhabditis elegans*) has been widely used for biological studies in development, adaptive behaviours (*i.e.* foraging), neuroscience, and aging (Leung et al., 2008). *C. elegans* has also been recognised as an important experimental model for environmental stimuli induced programmed phenotypes. The well-known example is the early environmental stress caused *C. elegans* larvae developmental trajectory alteration. Under conditions of plentiful food and low population density, *C. elegans* goes through four larval stages (L1 to L4) to reach reproductive mature adulthood. However, harsh environmental conditions (starvation, high population density, high temperature *etc.*) in the late L1 stage trigger L1s to enter diapause and arrest development as dauer larvae (Golden and Riddle, 1982; Golden and Riddle, 1984). Dauer larvae are stress resistance and can live for several months. Once dauers encounter a favourable condition, they can exit the dauer stage and resume development by molting into L4 larvae and, subsequently, into reproductive adults (Cassada and Russell, 1975) (**Fig. 1-14**).

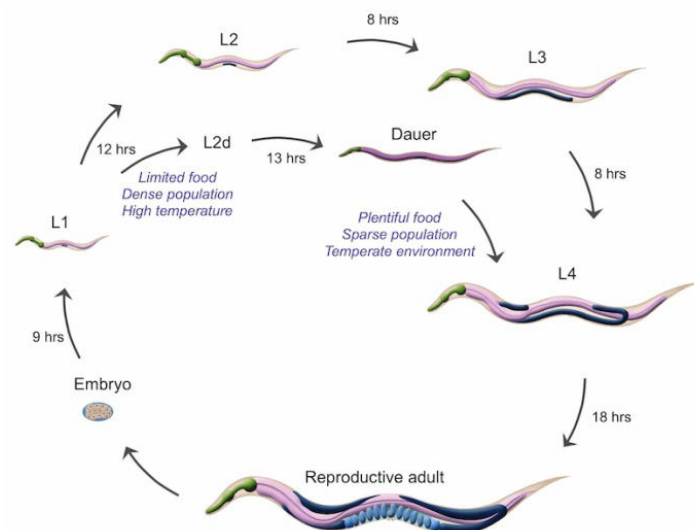


Figure 1-14 Life cycle of *C. elegans* with dauer branch. Figure captured from WORMATLAS (Wolkow and Hall, 2015).

Numerous studies have proved that the early-life stress including dauer arrest can lead to alterations in metabolic gene expression and thereby metabolism (Dalley and Golomb, 1992; Kimura et al., 1997; Meléndez et al., 2003; Wang and Kim, 2003). Some alterations can be ‘memorised’ for long-term, thus resulting in phenotypic variation (Hall et al., 2010). Studies also showed that wild *C. elegans* that are normally highly exploratory, whereas early-life starvation leads to adaptive ‘cautious foraging’ behaviour in which exploration is reduced (Pradhan et al., 2018). In addition, diet restriction can increase of lifespan in diverse species including *C. elegans* (Lithgow and Walker, 2002; Meléndez et al., 2003; Vellai et al., 2003), this lifespan extension effects could be observed even after several generations (Rechavi et al., 2014). Evidence proved that this persistent memory of past conditions could be associated with the starvation-induced transgenerational inheritance of small RNAs (Rechavi et al., 2014).

1.3.2.2 Mammals

1.3.2.2.1 *Quasi-experiment of Dutch famine*

Bulk evidence obtained from clinical, experimental and epidemiological studies in mammals suggested inadequate perinatal conditions (*i.e.* undernutrition, overnutrition) can permanently change the structure and function of specific organs or homeostatic pathways, thereby programming the individuals’ health and well-being in their adulthood. One of the best known researches in this area is the ‘Dutch famine’ (also known as Dutch Hunger Winter) study, which represents a natural experiment that provides evidence for the effects of famine on long-term health outcomes. This famine took place in the western part of the Netherlands during World War II. It was caused by the Nazi food embargo and lasted from November 1944 to May 1945. Individuals prenatally experienced the Dutch famine have been repeatedly found to have metabolic disorders later in life, including higher body mass index, elevated plasma lipids, increased risks for obesity, T2D and cardiovascular diseases (Roseboom et al., 2011; Schulz, 2010; Vaiserman, 2011). Results also demonstrated that the likelihood of a particular syndrome development in adulthood depended on the periods of pregnancy when the famine occurred. Exposure to the famine during early gestation tended to have greater risks of severe complications in adulthood compared to those during the mid- and late gestation stages (Ekamper et al., 2014).

It has been reported that the alterations in DNA methylation pattern induced by this famine exposure during perinatal periods may play important roles in the long-term programming effects on metabolism later in life. Results from the studies of Dutch famine strikingly found that, compared to their unexposed same-sex siblings, individuals who had been exposed to this famine during periconceptional (or early gestational) stage showed hypomethylation of insulin-like growth factor II (*IGF2*) gene 6 decades later (Heijmans et al., 2008). *IGF2* is a key factor that regulates the growth and development and is maternally imprinted (*i.e.*, the paternal allele is expressed). Imprinting is maintained through the *IGF2* differentially methylated region, and its hypomethylation results in bi-allelic expression of *IGF2*, which is associated with increased risks of cancer (Chao and D'Amore, 2008; Fenner, 2017).

1.3.2.2 Maternal protein restriction model

Another widely studied experimental model works with the maternal low protein diet. Numerous studies have demonstrated that protein restriction during pregnancy is associated with low birth weights and the later susceptibility to certain chronic diseases and metabolic disorders such as hypertension (de Brito Alves et al., 2016; Goyal et al., 2015; Langley-Evans et al., 1999; Sellayah and Cagampang, 2018; Woods et al., 2001), obesity (Bech et al., 2014; Guan et al., 2005; Han et al., 2012), insulin resistance (Berends and Dearden, 2018; Chen et al., 2009; Fernandez-Twinn et al., 2005; Ozanne et al., 2003) and diabetes (Berends and Dearden, 2018; Dahri et al., 1991; Petry et al., 2001; Su et al., 2016).

Accumulating evidence demonstrated that the programming effects of maternal malnutrition on progeny health are mostly mediated through alterations of DNA methylation level of the promoter regions of specific genes. These alterations vary among different tissues, and also vary between different genders.

Liver is considered the vital organ for metabolism and thus represents an important target when investigating metabolic programming. The maternal protein restriction during gestation caused promoter DNA hypomethylation of glucocorticoid receptor (*Gr*) and the peroxisome proliferator-activated receptor α (*Ppar- α*), as well as the concomitant up-regulated of their mRNA levels in the liver of offspring (Lillycrop et al., 2005). GR and PPARs are of great importance for embryogenesis (Cole et al., 1993; Michalik et al., 2002). In adults, GR activity is a main regulator of blood pressure (Yang and Zhang, 2004), whereas PPAR activities are central to carbohydrate and lipid metabolism (Djouadi et al., 1998; Wang, 2010). Similar

results have been obtained in many other studies (Lillycrop et al., 2008; Lillycrop et al., 2007; Sato et al., 2017; Sinclair et al., 2007; Zheng et al., 2015a). Interestingly, supplementation of folic acid in maternal protein-restricted diet can prevent these programming effects on GR and PPAR in rat offspring (Lillycrop et al., 2005). Evidence can be also found for the programming effects of maternal protein restriction on epigenetic regulation of gluconeogenic genes in the liver of progeny. Maternal protein restriction can induce hepatic high expression of PEPCCK in the offspring (Desai et al., 1995), and this alteration can be observed even in multiple generations (Burdge et al., 2007). Study in piglet found that maternal low protein diet during pregnancy resulted in increased mRNA and protein level of Glucose-6-phosphatase (G6PC), as well as the promoter hypomethylation in the liver of newborn offspring (Sun et al., 2012). These results may partly explain the inadequate prenatal nutrition mediated impaired glucose metabolism in adulthood.

Apart from liver, there are also some programming studies focusing other tissues like skeletal muscle and adipose tissue. For instance, it was reported that protein restriction for pregnant rats resulted in increased mean CpG island methylation and reduced mRNA level of peroxisome proliferator-activated receptor γ (PPAR γ) coactivator-1 α (*Pgc-1 α*) in the skeletal muscle of 18-month old female descendent (Zeng et al., 2013). Adult mice born to mothers fed a low-protein diet during pregnancy showed lower body weight and higher food intake compared to those born to mothers fed a control diet (Jousse et al., 2011). These alterations could be linked to the maternal nutritional history induced changes in promoter CpG methylation and the inhibited mRNA level of leptin gene in adipose tissue.

Epigenetic mechanisms other than DNA methylation have also been reported to be involved in the long-term metabolic programming effects induced by maternal protein restriction study. For example, the histone modifications of C/EBP β (H3ac and H4ac), a family of transcription factors that regulates the expression of genes involved in energy homeostasis and muscle development, have also been shown to be influenced by the maternal low-protein diet in the skeletal muscle of rat female offspring (Zheng et al., 2011). The increased promoter H3ac and H4ac levels could be linked to the elevated C/EBP β mRNA and protein levels (Zheng et al., 2011). Besides, study in mice using microarray methods showed that maternal low protein diet feeding resulted in downregulated mmu-miR-615, mmu-miR-124, mmu-miR-376b, and upregulated mmu-let-7e were significantly while, mmu-miR-708 and mmu-miR-879 in the liver of 21-day-old pups (Zheng et al., 2017). Bioinformatic analysis showed target genes were mapped to inflammatory-related pathways (including

MAPK, TGF-beta and Toll-like receptor). In addition, one study in rats showed that, maternal low protein diet throughout gestation and lactation lead to decreased expression of hepatocyte nuclear factor 4- α (*Hnf4 α*), a key pancreatic transcription factor for β -cell differentiation and glucose homeostasis, and the subsequent development of glucose intolerance in adult offspring (Sandovici et al., 2011). Such persistent reduction was demonstrated to be associated with the suboptimal early nutrition induced epigenetic silencing at the enhancer region, which weakens the P2 promoter-enhancer interaction (Sandovici et al., 2011).

1.3.2.2.3 Prenatal hypoxia

Environmental factors other than nutrition during early life stage can also profoundly affect the metabolism of adulthood. Of note, hypoxia plays an indispensable role in fetal development. It participates in several vital embryonic processes such as placentation, angiogenesis, and hematopoiesis (Fajersztajn and Veras, 2017). Intrauterine hypoxia, either alone or as a consequence of other adverse physiological conditions (*i.e.* malnutrition, pollution exposure, obesity, stress), has been increasingly studied as an experimental model for programming effects (de Wijs-Meijler et al., 2018; Ducsay et al., 2018; Vargas et al., 2017; Zeman and Okuliarova, 2017).

Amongst, the studies of fetal hypoxia and adult cardiovascular dysfunction are of particular interest. For example, studies in guinea pig demonstrated that prenatal hypoxia reduced mitochondrial maximal respiration and respiratory reserve capacity and complex IV activity rates in hearts of 90-day male pups, indicating that prenatal hypoxia exposure induce adverse programming effects on cardiac mitochondrial and ventricular function (Thompson et al., 2018). Another example comes from the study in sheep, which showed that long-term high altitude hypoxia exposure during pregnancy increased the fetal heart susceptibility to ischemia/reperfusion injury and cardiac dysfunction (Zhang et al., 2019). Results also showed that fetuses with fetal hypoxia experience had higher protein levels of hypoxia-inducible factor (HIF)-1 α and DNA methyltransferases type 3b (DNMT3b) in hearts compared to those with normoxic history. In addition, high altitude hypoxia exposure during pregnancy resulted in increased autophagy biomarker, LC3B-II protein levels and LC3B-II/LC3B-I ratio in the fetal hearts (Zhang et al., 2019). Further analysis showed that long-term high altitude hypoxia exposure during pregnancy induced a significant increase of miR-210 in sheep fetal hearts, with a 4.3 fold increase in the males and a 2.9 fold increase in females (Zhang et al., 2019). A genome-wide analysis to study the impact of antenatal hypoxia on DNA methylome and

transcriptome profiling in fetal and adult offspring hearts was performed in rats (Huang et al., 2018a). Results highlighted that CpG islands (CGIs) and CpG island shores (CGSs) within 10kb upstream of the transcription start site (TSS) were hypomethylated, compared to CGIs and CGSs within gene bodies. In addition, the authors also found that antenatal hypoxia induces opposite changes in methylation patterns in fetal and adult hearts, with hypermethylation in the fetus and hypomethylation in the adult. Further pathway analysis showed that enrichment of inflammation-related pathways was significantly greater in the adult male heart than those in the female heart (Huang et al., 2018a).

Apart from heart, studies have shown that fetal hypoxia has been associated directly or indirectly with the long-term programming of brain and kidney function in adulthood (Fajersztajn and Veras, 2017). Most recently, studies have found that chronic gestational hypoxia can accelerate ovarian aging and decrease ovarian reserve in adult rat progeny, indicating that fetal hypoxia has important implications for fertility for future generations (Aiken et al., 2019a; Aiken et al., 2019b).

1.3.3 Metabolic programming for fish nutrition in aquaculture

Nowadays, metabolic programming has been also considered as a novel breeding strategy. Through this strategy, a moderate environmental intervention during early life is postulated to modify the adaptive plasticity of livestock to better cope with their future environment (Moghadam et al., 2015). The concept of metabolic programming was first applied in aquaculture by Geurden *et al.* in 2007, demonstrating that a short hyperglucidic stimulus during the early life induced a long-term increase in activities of carbohydrate digestive-enzymes in juvenile rainbow trout (Geurden et al., 2007). This pioneer study confirmed the possibility of utilising metabolic programming strategy in aquatic species, thus opening a new research field for aquaculture practice.

Since then, several studies related to metabolic programming were done in different fish species, aiming at tailoring specific metabolic pathways or physiological functions in juvenile/adult stages (Fang et al., 2014; Gong et al., 2015; Izquierdo et al., 2015; Rocha et al., 2016a; Vagner et al., 2009; Vagner et al., 2007). These adaptations may help the organisms to improve their performance under certain conditions, *i.e.* a better use of substitutes for fish meal and fish oil, and hence contributing to maintain the sustainable development of the aquaculture industry (Panserat et al., 2013; Panserat et al., 2018).

As stated above, one of the most important factors involved in metabolic programming concept is the ‘windows of metabolic plasticity’. Similar to mammals, embryogenesis and early developmental stages represent periods of high sensitivity and metabolic plasticity in fish (Rocha, 2015). However, since most of the teleost are oviparous species in contrast to viviparous mammals, their embryonic development is quite different. The mammalian embryogenesis is accomplished in uterus of the mother, whereas most teleost species directly ovulate into water. The mammalian fetus is linked to maternal nutritional experiences through the umbilical cord and placenta, whereas fish embryos rely exclusively on their endogenous yolk reserves before first feeding. Therefore, carefully selecting the optimal stimuli to apply at the appropriate ‘windows of metabolic plasticity’ should be of great importance for metabolic programming study in aquatic species. In terms of metabolic programming strategy, the advantage of oviparous development aquatic species is that the environmental stimuli considered could be both nutritional and non-nutritional ones. The earlier studies mainly focus on nutritional stimuli. In fact, most of the existing practices were conducted based on two main strategies: i) through direct nutritional interventions at first feeding stage; ii) through manipulations on parental nutrition thus modulating the nutrient transfer to the yolk reserves of embryos.

Using metabolic programming strategy to modify the glucose metabolism of teleost, especially the carnivorous species, and thereby improving their carbohydrates utilisation ability is one of the major targets in this research field. For instance, the first attempt that brought metabolic programming concept into teleost studies was done in rainbow trout, aiming at improving its dietary carbohydrate utilisation (Geurden et al., 2007). In this study, a hyperglucidic stimulus was applied to trout alevins at the first-feeding stage, which induced long-term alterations in carbohydrate digestive enzymatic activities in juvenile trout. But no persistent molecular adaptations were found for the genes involved in glucose transport or metabolism. In a subsequent study, Geurden *et al.* (2014) demonstrated that a 5-day high carbohydrate dietary stimulus at first feeding induced a long-term influence on muscle glucose metabolism and on the profile of intestinal microbiota in trout (Geurden et al., 2014). These results proved the possibility of using metabolic programming strategy to modify dietary carbohydrate utilisation in trout.

Later, the efficiency of using metabolic programming strategy to tailoring glucose metabolism was further confirmed in other species, *i.e.* zebrafish (Fang et al., 2014), seabream (Rocha et al., 2016b), Siberian sturgeon (Gong et al., 2015; Liang et al., 2017) and

most recently in sea bass (Zambonino-Infante et al., 2019). Results from these studies showed that an early high carbohydrate dietary stimulus can induce long-term programming effects on glucose metabolism through affecting the glucose metabolism-related gene expression, activities of key enzymes in glucose metabolic pathways, and carbohydrate digestion. In the latest research, Zambonino-Infante *et al.* (2019) reported that a high-carbohydrate nutritional stimulus at early life can increase hypoxia tolerance in juvenile stage, although after the 2-month challenge test with high-carbohydrate diet this effect disappeared (Zambonino-Infante et al., 2019). The variations in programming effects revealed the complexity when dealing with this issue. Further studies remain to be done in order to pilot the programming outcomes. What's more, none of these studies investigated epigenetic mechanisms underlying the metabolic programming effects.

The present thesis mainly focuses on recent works related to metabolic programming strategy of improving the dietary carbohydrates utilisation in aquatic species. Nonetheless, several other studies can be found which aimed at using metabolic programming strategy to promote plant-based diets utilisation in teleost (Balasubramanian et al., 2016; Clarkson et al., 2017; Lazzarotto et al., 2016; Lazzarotto et al., 2015; Michl et al., 2017; Perera and Yufera, 2016; Vera et al., 2017). or increase their adaptation to low-HUFA (highly unsaturated fatty acids) diets (Castro et al., 2016; Izquierdo et al., 2015; Turkmen et al., 2017a; Turkmen et al., 2017b; Vagner et al., 2009; Vagner et al., 2007).

1.4 Rainbow trout as a model to study epigenetics and programming:- understanding the low dietary carbohydrate utilisation and 'glucose-intolerant' phenotype

Rainbow trout (*Oncorhynchus mykiss*), a carnivorous salmonid species, is one of the best studied experimental models in aquaculture. As was previously mentioned in the above chapters, trout is considered a poor user of dietary carbohydrates. The prolonged postprandial hyperglycaemia together with a growth reduction when fed with a high carbohydrate diet (>20%) in the long term becomes a big challenge for reducing the fishmeal with high proportion of carbohydrates in this species, which further impeding the sustainability of aquaculture. Many researches were done aimed at understanding the reasons for this limitation as well as seeking solutions to overcome this obstacle. Nonetheless, trout provides a special chance to investigate the epigenetic mechanisms that underpin the low dietary carbohydrate utilisation and glucose-intolerant phenotype. Moreover, using trout as a model to test the feasibility of metabolic programming strategy in tailoring this poor ability to use carbohydrate-rich diet may further contribute to the knowledge transfer to other carnivorous species.

1.4.1 Embryonic development of rainbow trout

As the early life stages show different sensibilities to environmental stimuli, the establishment of persistent metabolic memory could be restricted to specific developmental stages, which is termed 'windows of plasticity'. A brief description of the embryonic development of rainbow trout will be introduced here. The chronology of development and classification of developmental stages are according to the Vernier Table (**Table 3**) (Vernier, 1969), which was performed at 10 °C, the optimal temperature for salmonid embryonic growth efficiency (Marr, 1966). The embryonic development of trout can be divided into four periods with 37 stages based on easily identifiable features (Vernier, 1969). Because the time required to reach any developmental stage or to hatch is dependent on a specific amount of accumulated thermal units, the developmental stage can be characterised by degree days (°D, calculated as the sum of mean daily water temperature).

- **Cleavage period** (stages 1-10): average 40 °D in duration it begins at fertilisation and ends when the terminal node appears.
- **Gastrulation period** (stages 11-17): extends to the time of blastopore closure at around 90 °D.

- **Caudal bud period** (stages 18-29): extends from 100 °D to the end of hatching at about 350 °D.
- **Alevin period** (stages 30-37): development follows until complete absorption of the yolk sac.

Table 3 The chronology of development and the characteristics of each stage in trout

Periods	Stages	Characteristics of the corresponding stages	Age in °D
Cleavage			
	1	Zygote, no cleavage	
	2	2 blastomeres	10
	3	4 blastomeres	11.7
	4	8 blastomeres	13.3
	5	16 blastomeres	15
	6	32 blastomeres	17.5
	7	Early morula	20
	8	Late morula	25
	9	Onset of blastodisc expansion	30
	10	Blastula	35
Gastrulation			
	11	Appearance of the terminal caudal node	40
	12	Onset of the formation of the embryonic axis	50
	13	Embryo clearly visible. Epiboly 1/3 completed	60
	14	0-5 somites. Epiboly 1/2 completed	70
	15	Optic vesicles. Epiboly 2/3 completed	75
	16	Otic placodes. Epiboly 3/4 completed	80
	17	Primitive crystalline lens. Otic vesicles formed. Blastopore closed.	90
Caudal bud			
	18	Caudal bud not differentiated. Formation of the mesencephalon. Primitive intestinal tract visible.	100
	19	Caudal bud with less than 10 somites. Olfactory placodes. Metencephalon and myelencephalon clearly distinct.	110
	20	Caudal bud with 10-20 somites. Heart beats.	120
	21	Blood circulation begins. All blood from the posterior region returns to the heart via the yolk sac. Pectoral fin buds. External edge of the choroid pigmented.	140
	22	Posterior cardinal veins formed. Return of caudal blood supply to heart via either the yolk sac or the cardinal veins. Entire choroid pigmented.	160
	23	Return of caudal blood supply to heart via only the cardinal veins. Intestinal artery supplies the subintestinal vein. Differentiated hepatic portal vein coexists with the afferent vitelline vein.	170
	24	Primordial caudal fin formed of a mass of mesenchyme. Hepatic portal vein exists alone. Six aortic arches established.	180
	25	Caudal flexion of the vertebral column near the primordial caudal fin. Primordial anal fin formed of a mass of mesenchyme.	200
	26a	Primordial dorsal fin formed of mesenchyme. Posterior intestine and urinary duct completely separated.	220
	26b	First myotomic buds appear in the middle of the primordial anal fin	240
	27	First myotomic buds appear in the middle of the primordial dorsal fin. Myotomic buds of the anal fin reach the mesenchyme. Primitive capillary bed forms near the caudal fin.	250
	28	Myotomic buds appear in the primordial pelvic fins. An indentation on the dorsal border of the embryonic fin fold marks the boundary between the tail and the growing caudal fin.	270
	29	Alternation of radials and myotomic buds in the middle of the primordial dorsal and anal fins. Appearance of first dermal rays in the caudal fin.	310
Alevin			
	30	Hatching.	350 (340 to 370)
	37	Yolk sac absorbed.	850

Table modified from Vernier, 1969 (Vernier, 1969).

1.4.2 Dietary carbohydrate utilisation and glucose metabolic pathways in trout

1.4.2.1 Dietary carbohydrate utilisation in teleost

As was reviewed by Kamalam *et al.* (2016), the capability to use dietary carbohydrates in teleost is determined by different factors including biological factors (*i.e.* feeding habit, genotype, physiological condition, exercise), abiotic factors (*i.e.* temperature, salinity, photoperiod, stress) and nutritional factors (*i.e.* the botanical origin of the starch and its molecular complexity, diet processing, carbohydrates inclusion level, other nutrients such as protein and lipids) (Kamalam *et al.*, 2016).

Similar to other vertebrates, after food intake, the digestive process in teleost begins with a chain of elaborate mechanical and enzymatic processes for breaking down complex molecules into simple absorbable units in gastrointestinal tract. The main final product after carbohydrate digestion is glucose, which will be subsequently absorbed along the gastrointestinal tract and transported across the epithelium to the bloodstream and thereby circulating to other organs for cellular metabolism (Rust, 2003) (**Fig. 1-15**).

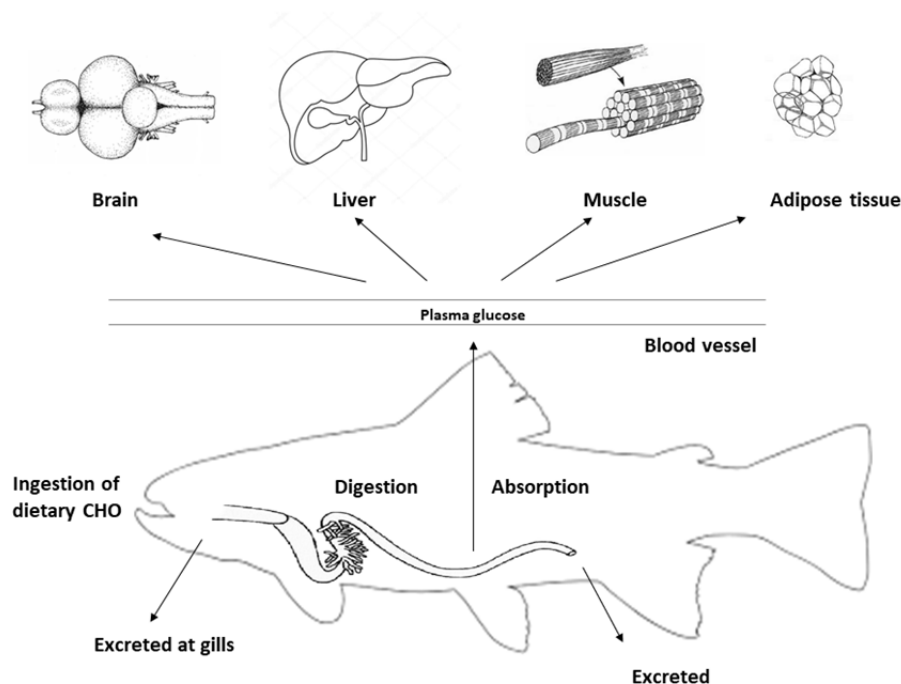


Figure 1-15 The digestive flux during dietary carbohydrates intake in teleost. Figure modified from (Rocha, 2015). CHO, Carbohydrates.

The digestion and absorption of nutrients are mostly dependent on enzyme activities that are involved in breakdown and assimilation of food (Klein et al., 1998). The primary enzyme responsible for carbohydrate digestion is α -amylase, which can hydrolyse the α -glycoside linkage of starch to produce glucose. This enzyme is synthesised in the exocrine pancreas and is secreted into the gut (Fish, 1960; Hemre et al., 2002). It has been showed that α -amylase widely exists in teleost, but its activity varies in different species. It is generally believed that carnivorous fish species possess relatively lower amylase activity than omnivorous species. However, the role of this enzyme remains questionable in carnivorous species due to the absence or low carbohydrates content in their natural food and the moderate activities reported in numerous species (Polakof et al., 2012). The herbivorous fish species can utilise part of the non-starch carbohydrates in the diet due to symbiosis with the gut microbiota whereas most fish species cannot (Krogdahl et al., 2005).

The absorption of glucose from intestinal lumen to the bloodstream is mediated by a Na^+ -dependent glucose carrier (Sglt1) present in the brush border/apical membrane of the enterocytes, which is followed by a facilitated Na^+ -independent diffusion through a glucose transporter Glut2 across the basolateral membrane to the blood (Bakke et al., 2010; Draai et al., 1990). Compared to mammals, teleost have lower capacity for glucose uptake in intestine. This could be possibly linked to the lower densities of glucose transporter or a lower amount of absorptive tissue (Collie and Ferraris, 1995). Moreover, glucose transport rates in carnivorous fish are lower than in carnivorous mammals (Buddington et al., 1987). When excessive carbohydrate intake induced hyperglycaemia that above the threshold level, excess glucose can be excreted through urine (glycosuria) or through gills (Bucking and Wood, 2005; Hemre and Kahrs, 1997).

After digestion and absorption, the transport of glucose from the bloodstream into the cells of brain, liver, muscle, adipose tissue and several other tissue/organs, and the subsequent glucose catabolic metabolism are essential steps for efficient glucose utilisation. The main metabolic pathways related to glucose utilisation include glucose phosphorylation (glycolysis), glucose production (gluconeogenesis), glycogen synthesis (glycogenesis) and breakdown (glycogenolysis), and glucose transport (Rocha, 2015). Fish have the whole enzymatic machinery for glucose metabolism (Polakof et al., 2012). However, their regulation by nutritional and hormonal factors is reported to be overall similar to mammals but with certain distinct features.

Insulin and glucagon are the two major pancreatic endocrine gluco-regulatory hormones in mammals as well as in teleost (Moon, 2004). Although the hormonal regulation of glucose metabolism is still not well understood in teleost, it has been demonstrated that the functional roles of insulin and glucagon on metabolism are in general similar to their mammalian orthologues (Moon, 2004). Insulin secretion is induced by feed intake regardless of dietary carbohydrate level, whereas glucagon is decreased after carbohydrates feeding (del Sol Novoa et al., 2004; Seiliez et al., 2008). Release of insulin can stimulate glucose uptake in the peripheral tissues, induce glycolysis, inhibit gluconeogenesis and enhance glycogenesis in liver, muscle and adipose tissues (Navarro et al., 2006), which further contribute to the downregulation of glycaemia (Polakof et al., 2012). By contrast, glucagon may promote cellular glucose production and increases plasma glucose through the activation of gluconeogenesis as well as glycogenolysis, and lipolysis (Moon, 1998; Plisetskaya and Mommsen, 1996). Apart from hormonal regulation, another key to the homeostatic control of glucose metabolism is the existence of glucosensors (*i.e.* Glut2, glucokinase, Sglt1, glycogen, ATP-sensitive inward rectified K⁺ channel, *etc.*) which can monitor the changes in circulating glucose level. Previous studies have demonstrated the presence of both central (*i.e.* brain) and peripheral (*i.e.* pancreatic β -cells, intestine) glucosensing mechanisms in teleost (Polakof et al., 2011a; Polakof et al., 2011b; Polakof and Soengas, 2013; Soengas, 2014).

1.4.2.2 Main hepatic glucose metabolic pathways

The liver is the principal organ for glucose homeostasis as well as the dominant regulator for intermediary metabolism (Klover and Mooney, 2004). Two main glucose metabolic pathways are glycolysis (glucose phosphorylation) and gluconeogenesis (*de novo* glucose synthesis) (**Fig. 1-16**).

Under aerobic conditions, glucose can be completely catabolised through glycolysis, the tricarboxylic acid (TCA) cycle and the respiratory chain for ATP production or through the pentose phosphate pathway for nicotinamide adenine dinucleotide phosphate (NADP) production. Glycolysis pathway is the major route for glucose utilisation, three key enzymes regulating the rate-limiting steps for glycolytic flux are glucokinase (Gck), 6-phosphofructo-1-kinase (Pfk) and pyruvate kinase (Pck) (Knox et al., 1980). Gk, the key enzyme that catalyse the first step of glycolysis, is considered a molecular marker for dietary carbohydrates in rainbow trout due to the significant induction in mRNA level of this gene after carbohydrates intake (Panserat et al., 2001a; Panserat et al., 2000a). Excessive glucose could be stored as

glycogen or converted to lipids. Under anaerobic conditions, the glucose mitochondria oxidative phosphorylation pathway is inhibited, and the pyruvate generating from glycolysis pathway will be further converted into lactate through the function of lactate dehydrogenase A (Ldha) instead of being converted into acetyl-CoA (Palmer and Clegg, 2014).

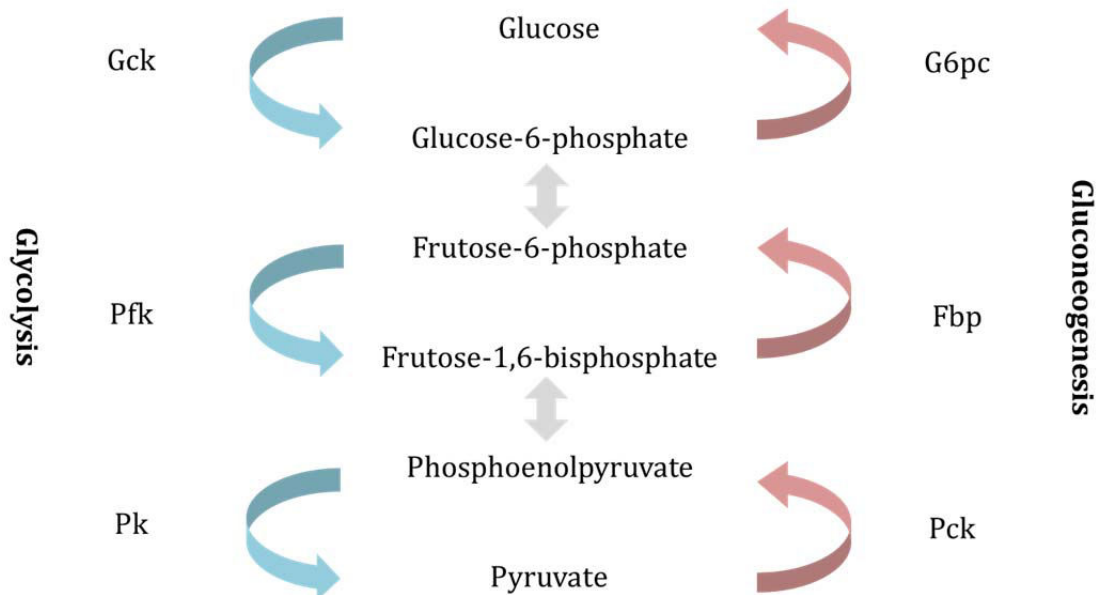


Figure 1-16 Key enzymes and the substrate cycles in the glycolic and gluconeogenic pathways in trout. Fbp: fructose-1,6-bisphosphatase; G6pc:glucose-6-phosphatase; Gck: glucokinase; Pck: phosphoenolpyruvate carboxykinase; Pfk: 6-phosphofructo-1-kinase; Pk: pyruvate kinase. Figure adapted from (Rocha, 2015).

Glucose requirements could be supplied by *de novo* glucose synthesis through gluconeogenesis (from lactate, glycerol and some amino acids) under fasting condition. Glycogen depletion may also provide some glucose but only to a limited extent at the beginning of fasting condition. Apart from the shared enzymes that catalyse the reversible reactions in glycolysis and gluconeogenesis, the three unidirectional rate-limiting steps are catalysed by phosphoenolpyruvate carboxykinase (Pck), fructose-1,6-bisphosphatase (Fbp) and glucose-6-phosphatase (**Fig. 1-16**) (Enes et al., 2009). Contrary to glycolysis which is a non-tissue specific pathway, gluconeogenesis takes place mainly in the liver (less in the kidney and intestine) (Kirchner et al., 2008; Knox et al., 1980).

In addition, the hepatic glucose concentration is maintained at equilibrium with glucose in blood through a specific glucose transporter Glut2 (Castillo et al., 2009; Krasnov et al., 2001). It seems to serve the bi-directional transport of glucose depending on the dietary or hormonal

status (Panserat et al., 2001b). Thus, the net flux between glucose production and glucose disposal is of great importance for regulating plasma glucose level. Although enzymes involved in all these glucose metabolic pathways are regulated under nutritional and hormonal regulation in teleost and in mammals, specific regulating mechanisms could be quite different (Hemre et al., 2002); (Polakof et al., 2012). For instance, food intake leads to repression of gluconeogenesis in some teleost species such as common carp, regardless of the carbohydrate content in the diets. This regulation seems to be similar to the mammals at least at the molecular level (Panserat et al., 2002). However, these inhibiting effects seem absent in carnivorous species including trout (Kamalam et al., 2016; Panserat et al., 2000b).

1.4.3 Hypotheses to explain the poor dietary carbohydrate use in rainbow trout

Over decades, numerous studies have been done in order to explain the poor dietary carbohydrate use in trout and some major hypotheses have been raised (Enes et al., 2009; Kamalam et al., 2016; Panserat et al., 2001a; Panserat et al., 2000a; Panserat et al., 2000b; Polakof et al., 2012).

1.4.3.1.1 Complex hormonal interplay

Previous studies have shown that although trout can secrete insulin to dietary carbohydrates, its efficiency of reducing glycaemia level is limited (Polakof et al., 2008). It seems certain amino acids have more insulinotropic potency than glucose in carnivorous teleost (Andoh, 2007). In addition, somatostatin, also known as growth hormone-inhibiting hormone, is a hormone that inhibits the secretion of several other hormones including insulin (Lamberts et al., 1997). One study showed that somatostatin secreting cells of trout pancreatic islets are more sensitive to glucose than the insulin-secreting cells (Eilertson and Sheridan, 1995). It was also reported that glucose stimulated somatostatin secretion in trout, which in turn increased glycaemia through the strong inhibition of insulin secretion and the stimulation of glucagon release (Harmon et al., 1991). A most recent study found that, the low capacity of insulin in reducing glycaemia is not because of the failure in inhibiting of hepatic glucose production, but due to the inhibiting effect of insulin on glucose disposal (Forbes et al., 2019).

1.4.3.1.2 Poor utilisation of dietary carbohydrates in muscle

Muscle is the largest peripheral tissue which accounts for almost 50% of the body weight in teleost (Moon, 2001). However, it was reported that there are relatively lower number of insulin receptors in trout muscle. Indeed, compared to mammals, fish have much fewer

insulin receptors per unit weight in all the major insulin sensitive tissues. Besides, the number of insulin receptors differs among species (less in carnivorous trout than in omnivorous carp) and also differs between tissues (less in muscle than in the liver) (Navarro et al., 1999; Parrizas et al., 1994). Another limiting factor for glucose utilisation in muscle is linked to glucose transport. Glut4 is the principle and the only insulin sensitive glucose transporter member in muscle of trout (Capilla et al., 2004). It located in the intracellular compartments in basal state and translocated to the plasma membrane upon insulin stimulation thus facilitating glucose uptake (Bryant et al., 2002). However, studies found that trout Glut4 showed lower affinity for glucose, lesser extent of insulin stimulated translocation (Capilla et al., 2009; Díaz et al., 2007). Moreover, it was reported that white muscle has a relatively lower glycolytic capacity compared to the liver, and that it was not regulated by dietary carbohydrates at least at mRNA level (Kamalam et al., 2012; Panserat et al., 2009).

1.4.3.1.3 No control of endogenous glucose production in the liver

As was mentioned above, the most striking difference between mammals and trout in glucose metabolic regulation is supposed to be the absence of hepatic gluconeogenesis inhibition after food intake with or without carbohydrates. In trout, on one hand carbohydrates intake sharply induces the Gck expression at mRNA level and/or protein level, which is the rate-limiting enzyme for the first step of glycolysis (Panserat et al., 2014; Polakof et al., 2012). It was surprisingly found that this induction is linked strictly to the carbohydrates intake other than insulin levels (Plagnes-Juan et al., 2008). On the other hand, the non-inhibition of gluconeogenesis could be possibly attributed to lost control of G6pc, which is responsible for the last step of gluconeogenic pathway for glucose production (Kamalam et al., 2012; Skiba-Cassy et al., 2013). The simultaneous upregulation of both Gck and G6pc might result in a futile cycle around glucose phosphorylation, which extends postprandial hyperglycaemia in trout. This hypothesis was reinforced when five G6pc encoding genes were identified in the genome of rainbow trout. Actually, three of them (*g6pca*, *g6pcb1a*, *g6pcb1b*) are inhibited whereas the other two *g6pcb2* genes (*g6pcb2a*, *g6pcb2b*) displayed an atypical upregulation by dietary carbohydrates in liver (Marandel et al., 2015). It can be thus postulated that maintenance of ohnologous *g6pcb2* pair in the genome may contribute to the poor use of dietary carbohydrate phenotype of trout.

1.4.3.1.4 Other hypotheses

Apart from the above major hypotheses, some other hypotheses also exist, which including i) lack of adaptive response in glucose digestion and absorption; ii) different neural regulatory network in brain; iii) poor lipogenic ability to store excess glucose in liver and adipose tissue, *etc.* (Kamalam et al., 2016; Panserat et al., 2009).

1.4.3.1.5 Hepatic DNA methylation and gluconeogenesis in trout

Among the above hypotheses, the non-inhibition of hepatic gluconeogenesis is of particular interest, since liver represents the most vital organ for metabolism. Recent studies have demonstrated that high dietary carbohydrates in trout can alter the hepatic epigenetic landscapes, leading to a global DNA hypomethylation as well as the promoter region of *g6pc* genes (Craig and Moon, 2013; Marandel et al., 2016a). These inspiring findings indicated that epigenetic mechanisms might play an important role in the poor use of dietary carbohydrate phenotype in trout. Thus, rainbow trout provides a special chance to be an experimental model to investigate the underlying relationships between dietary carbohydrates, poor glucose utilisation phenotype, and epigenetics. These studies may further contribute to our better understanding of what happens in carnivorous teleost when fed excessive dietary carbohydrates, and how to overcome this bottleneck to improve the dietary carbohydrate level in their feed formula.



CHAPTER II

HYPOTHESES & OBJECTIVES

CHAPTER 2 HYPOTHESES AND OBJECTIVES

In the current thesis, we set to address three major questions below:

2.1 Question I: How many DNA methylation-related genes are preserved in trout genome, and what are their evolutionary origins?

DNA methylation tools (*dnmt*, *tet* and *tdg* genes) have been well described in mammals in previous studies. However, increased copy numbers of DNA methylation tools have been found in teleost genomes due to additional whole genome duplication (WGD) events, and to date, their evolutionary history and phylogenetic relationships have remained unclear. Moreover, rainbow trout subjected to a recent fourth round of WGD event during evolution, leaving this species a genome of high complexity (**Fig. 2-1**).

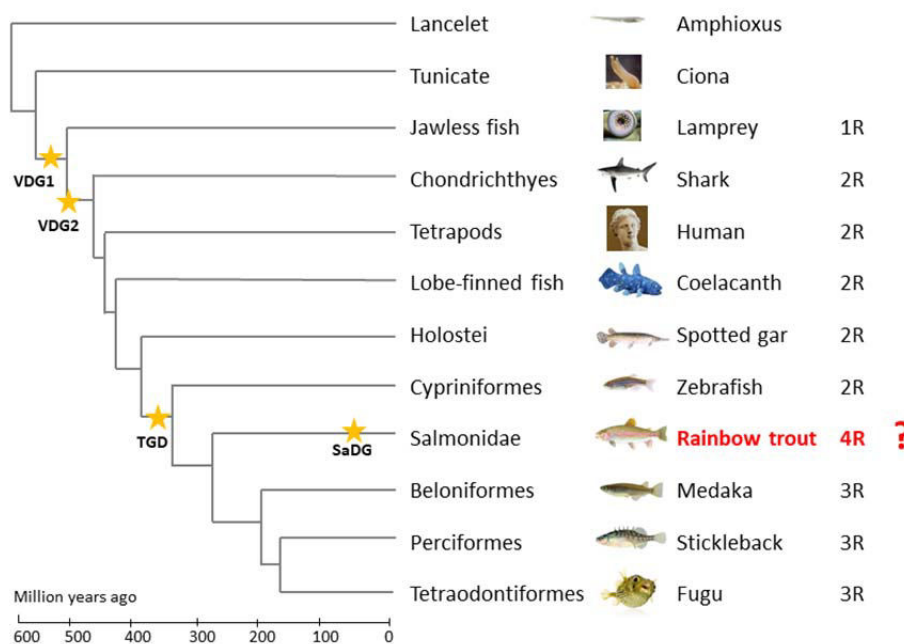


Figure 2-1 Whole genome duplication events during the evolution of chordates SaGD, salmonid specific genome duplication; VGD1, the first round vertebrate whole genome duplication; VGD2, the second round vertebrate whole genome duplication; TGD, teleost-specific genome duplication. nR represents the number of WGD events experienced during evolution (Ravi and Venkatesh, 2018)

It is generally believed that WGD can provide additional genetic materials and could be related to phenotypic diversity. Following the WGD event, the duplicated genes can be lost or

retained with three distinct outcomes: conservation of the ancestral gene functions, sub-functionalisation or neo-functionalisation. In order to well understand the DNA methylation mechanisms in trout, the first question that should be answered is to figure out how many DNA methylation/demethylation-related genes exist in trout, and what are their evolutionary origins. **We hypothesised that increasing copies numbers of DNA methylated genes could be kept in the genome of rainbow trout. There might be sub- or-neo-function for the duplicated genes.**

2.2 Question II: What are the nutritional factor(s) triggering the hepatic global DNA hypomethylation in trout after feeding a high-carbohydrate/low protein diet ? And what are the detail mechanisms involved in this demethylation process?

A previous study did by Marandel and collaborators observed a hepatic global DNA hypomethylation phenotype in trout after 4 days feeding with a high carbohydrate diet (Marandel et al., 2016a). However, it was impossible to clarify the exact causal factor for this phenotype because the increase in dietary carbohydrate content in the high carbohydrate (HighCHO) diet (0% to 30%) was compensated by a decrease in protein content (60% to 40%) in order to keep the same energy level. Additionally, the concomitant effect of hyperglycaemic status driven by the HighCHO diet also can't be excluded. Therefore, it is unknown what is the real factor triggering this hepatic global hypomethylation in trout. **We hypothesised that the hepatic global DNA hypomethylation phenotype could be induced by several factors: i) decrease in protein content; ii) increase in carbohydrate content; iii) a combination of both; or iv) hyperglycaemic environment.**

Moreover, previous studies demonstrated that the erasure of methylation can be achieved through either a passive or an active pathway (Bhutani et al., 2011). The passive demethylation process results from the inhibition or absence of DNMT1 function during DNA replication, whereas the active demethylation can occur through TET dioxygenase-mediated stepwise oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), followed by thymine DNA glycosylase (TDG)-dependent base excision repair (BER) or replication-dependent dilution (Maiti and Drohat, 2011; Weber et al., 2016). **We will thus investigate these 2 pathways to elucidate how the high carbohydrate/low protein diet induced global DNA hypomethylation in trout liver**

2.3 Question III: Can DNA hypomethylation phenotype and glucose metabolism be modified through metabolic programming strategy?

Metabolic programming is a hot topic of research in aquaculture. In rainbow trout, this strategy was shown to induce long-term alterations in glucose metabolism (Geurden et al., 2007; Geurden et al., 2014), as well as epigenetic landscapes (Panserat et al., 2017) through early dietary stimuli at first-feeding. **It can be hypothesised that metabolism programming has more competitive advantages than other strategies in modifying the hepatic global DNA hypomethylation and glucose metabolism thus improving the poor carbohydrate utilisation in trout.** However, results obtained in these previous studies showed that most of these programming effects were observed mainly in muscle. No persistent programming effects were noted in the liver in juvenile fish, despite the liver's pivotal role in glucose metabolism. The lack of programming effect in liver could be related to the improper experimental conditions used for programming. Moreover, previous studies showed that the atypical upregulation of *g6pcb2* phenotype by dietary carbohydrates were established as soon as the first-feeding stage (Marandel et al., 2016b), indicating that applying a stimulus at first-feeding may be too late to programme hepatic glucose metabolism (or at least gluconeogenesis) in trout in the long-term. Therefore, modification of the programming condition through targeting another 'earlier windows of metabolic plasticity', or choosing a more powerful stimulus should be considered.

Here we hypothesised that applying a stimulus before first-feeding, *i.e.*, during embryogenesis, may be more efficient for programming the hepatic glucose metabolism and the epigenetic landscapes in rainbow trout. Fish embryos and larvae depend exclusively on their endogenous yolk reserves until the first-feeding. Therefore, a non-nutritional stimulus (*i.e.*, temperature or hypoxia) appears to be an ideal choice, because embryos of the oviparous trout are directly exposed to environmental variations. Hypoxia has been well documented to affect DNA methylation and glucose metabolism (Palmer and Clegg, 2014; Pollard et al., 2008). **We hypothesised that hypoxic stimulus (either acute or chronic hypoxia) during early developmental stage alone and/or combined with a high carbohydrate dietary stimulus at first feeding may alter the hepatic global DNA hypomethylation phenotype and/or the glucose metabolism specifically in trout.**



CHAPTER III

RESULTS

CHAPTER 3 RESULTS

3.1 Flowchart of thesis design

In order to answer the three questions that have been put forward in the present thesis, the global design of the thesis consists of three major parts (**Fig. 3-1**):

Part1 *Evolutionary history of DNA methylation tools in chordates*. One publication (under review) was prepared from this part:

- *Publication1*: Liu, J., Hu, H., Panserat, S. & Marandel, L. Evolutionary history of DNA methylation related genes in chordates: new insights from multiple whole genome duplications (Under review in *Scientific Reports*)

Part2 *Determinants for hepatic global DNA hypomethylation by nutrition and the underlying mechanisms*. One publication (unpublished) was prepared from this part:

- *Publication2*: Liu, J., Herault C., Veron V., Panserat, S. & Marandel, L. Factors triggering hepatic global DNA hypomethylation phenotype in trout and the detail mechanisms (Redaction in progress)

Part3 *Metabolic programming effects of hypoxia during early development: impacts on epigenetic landscapes and glucose metabolism in trout juveniles*. This part consists of two sub-sections **Part3A** and **Part3B**, which focus on investigating the metabolic programming strategy by means of either an acute hypoxic stimulus or a chronic hypoxic stimulus during early life, respectively.

Two publications were produced from **Part3A**, which reported the short-term and long-term programming effects of acute hypoxic stimulus, respectively:

- *Publication 3*: Liu, J., Plagnes-Juan, E., Geurden, I., Panserat, S. & Marandel, L. (2017). Exposure to an acute hypoxic stimulus during early life affects the expression of glucose metabolism-related genes at first-feeding in trout. *Scientific Reports*, 7(1), 363
- *Publication 4*: Liu, J., Dias, K., Plagnes-Juan, E., Veron, V., Panserat, S. & Marandel, L. (2017). Long-term programming effect of embryonic hypoxia exposure and high-

carbohydrate diet at first feeding on glucose metabolism in juvenile rainbow trout.
Journal of Experimental Biology, 220(20), 3686-3694

Another one publication was produced from **Part3B**, which investigated the long-term programming effect of a chronic hypoxic stimulus during early development:

- *Publication 5*: Hu, H.*, Liu, J.*, Plagnes-Juan, E., Herman, A., Leguen, I., Goardon, L., Geurden, I., Panserat, S. & Marandel, L. (2018). Programming of the glucose metabolism in rainbow trout juveniles after chronic hypoxia at hatching stage combined with a high dietary carbohydrate: Protein ratios intake at first-feeding. *Aquaculture*. 488, 1-8. (*Co-first author)

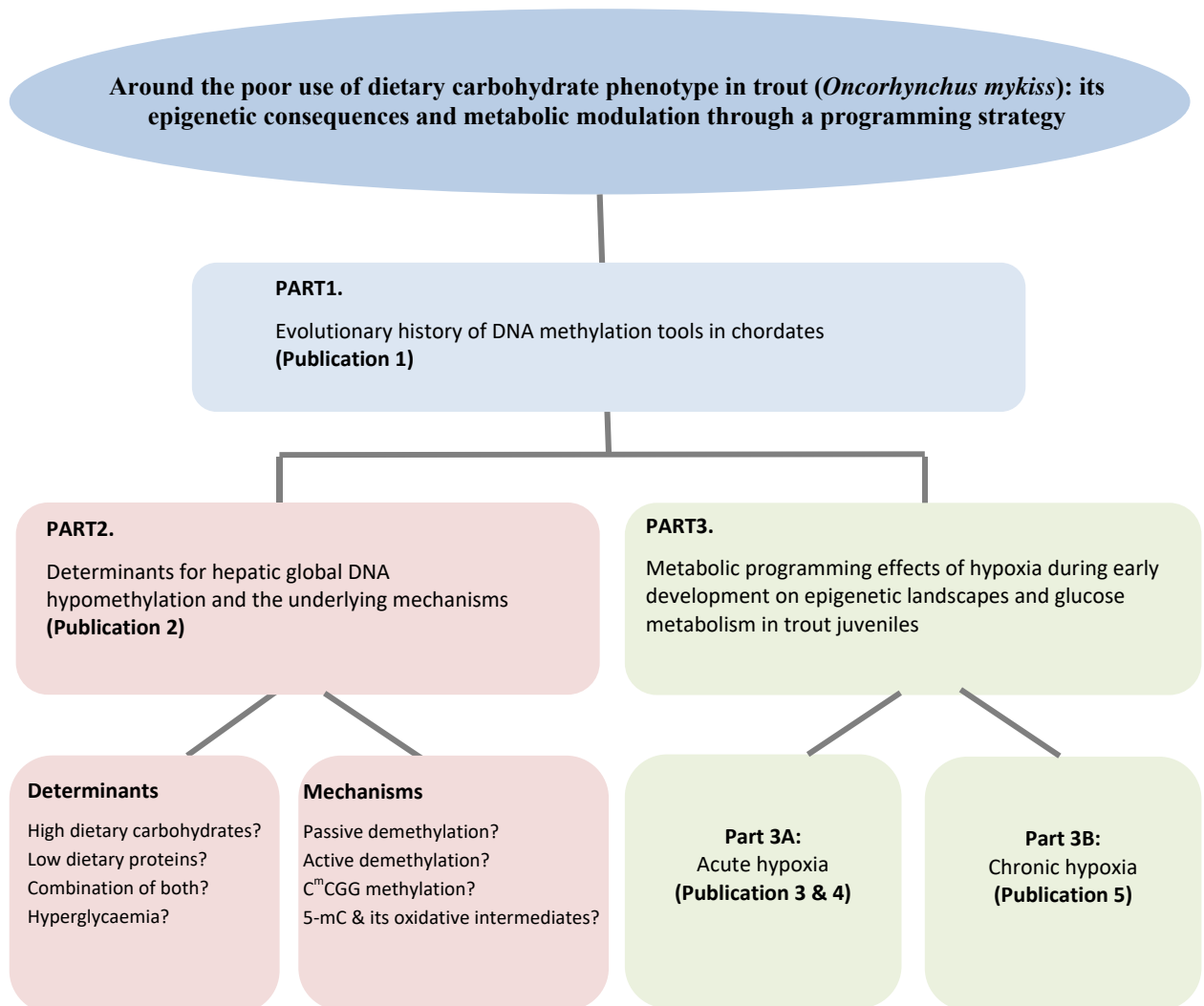


Figure 3-1 Flow chart of thesis design

3.2 Part 1 Evolutionary history of DNA methylation related genes in chordates: new insights from multiple whole genome duplications - Publication 1

Objectives

The present study aimed at **i)** identifying all the paralogous genes of *dnmt*, *tet* and *tdg* in trout genome; **ii)** clarifying their respective evolutionary history in chordates; **iii)** investigating their mRNA expression dynamics during gametogenesis and embryogenesis.

Experimental design

The present study consists of two research sections: **i) *in silico* analysis** of all the *dnmt*, *tet* and *tdg* gene sequences in rainbow trout and their orthologues in selected species in different taxa of chordates thereby conducting phylogenetic and syntenic analyses for these genes in the aim to clarify their evolutionary history; and **ii) qPCR analysis** of the mRNA levels of DNA methylation tools during gametogenesis and embryogenesis (**Fig. 3-2**). For gametogenesis experiment: two-year-old genitors were obtained and reared at 8 °C in INRA experimental facilities. Ovaries and testes were sampled (6 individuals per time point) during gametogenesis process in February, May, September and November, corresponding to pre-early vitellogenesis/ early spermatogenesis, vitellogenesis/ mid spermatogenesis, late vitellogenesis/ late spermatogenesis, and spawn stage, respectively. For ontogenesis experiment: embryos were collected on triplicate spawns intermittently before the fertilisation stage (oocyte) and during embryonic development: stages 5, 6, 7, 8, 10, 12, 15, 22, 23 and 31 (hatching alevins, 384°D (degree days)) according to Vernier (1969).

In silico analysis

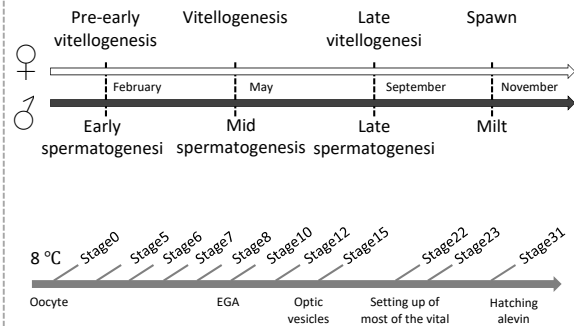
1. Sequence search

BLAST search of *dnmt*, *tet* and *tdg* gene sequences in rainbow trout and their orthologues in selected species in different taxa of chordates

2. Phylogenetic and syntenic analyses

Construct phylogenetic trees
Analyse the conserved syntenic block of *dnmt*, *tet* and *tdg*

Lab work



3. qPCR analysis

Measure the dynamic expression pattern of *dnmt*, *tet* and *tdg* during gametogenesis and embryogenesis in rainbow trout

Figure 3-2 Schematic overview of experimental design for the study of evolutionary history of DNA methylation related genes in chordates

Highlights

1. There were 2 *dnmt1*, 8 *dnmt3*, 7 *tet* and 4 *tdg* copies identified in the trout genome
2. Duplicates of *dnmt3* were preferentially fixed in genomes after successive WGD events
3. The 8 *dnmt3* paralogues were differentially transcribed during gametogenesis and embryogenesis processes in trout.

Conclusions

The fixation of multiple *dnmt3* genes in the genomes after WGD can lead to their sub- or neo-functionalisation. In addition, such fixation may contribute to novel mechanism related to the methylome of teleosts and in a largest extent to the diversification and plastic adaptation of the teleost.

Publication 1

Evolutionary history of DNA methylation related genes in chordates: new insights from multiple whole genome duplications

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ABSTRACT

DNA methylation is an important epigenetic mechanism involved in many biological processes, *i.e.* gametogenesis and embryonic development. However, increased copy numbers of DNA methylation related genes (*dnmt*, *tet* and *tdg*) have been found during chordate evolution due to successive whole genome duplication (WGD) events. Their evolutionary history and phylogenetic relationships remain unclear. The present study is the first to clarify the evolutionary history of DNA methylation genes in chordates. In particular, our results highlight the fixation of several *dnmt3*-related genes following successive WGD throughout evolution. The rainbow trout genome offered a unique opportunity to study the early evolutionary fates of duplicated genes due to a recent round of WGD at the radiation of salmonids. Differences highlighted in transcriptional patterns of these genes during gametogenesis and ontogenesis in trout indicated that they might be subjected to sub- or neo-functionalisation after WGD. The fixation of multiple *dnmt3* genes in genomes after WGD could contribute to the diversification and plastic adaptation of the teleost.

Introduction

DNA methylation is an important epigenetic mechanism involving the covalent binding of a methyl group to the 5th position of cytosine in CpG dinucleotides in vertebrates ¹. This mechanism is generally considered as a repressive epigenetic mark that inhibits gene expression ². DNA methylation plays a critical role in several biological processes such as embryonic development and gametogenesis ^{1,3,4}. The DNA methylation process is mediated by DNA methyltransferases (Dnmts): maintenance methyltransferase Dnmt1 ⁵ and *de novo* methyltransferase Dnmt3 ⁶. The erasure of methylation marks can be achieved either passively through the inhibition of Dnmt1 during DNA

replication and cell division ⁷, or actively through the action of ten-eleven translocation (Tet) dioxygenase family mediated iterative oxidation of 5-methylcytosine (5mC) and thymine DNA glycosylase (Tdg)-dependent base excision repair (BER) ⁸⁻¹⁰.

The DNA methylation/demethylation machinery has been well described in mammals. While *dnmt1* is generally identified as single-copy gene during evolution ¹¹, multiple *dnmt3* genes were found in vertebrates with different gains and losses among tetrapod lineages ¹². The mammalian *dnmt3* family consists of four members: *dnmt3a*, *dnmt3b*, *dnmt3c* and *dnmt3l* ^{13,14}. *dnmt3l* serves as a catalytically

inactive cofactor for *de novo* methylation, and is found to exist only in eutherian mammals and in some marsupials¹⁵, whilst *dnmt3c*, previously annotated as a pseudogene, was recently identified in rodent genomes¹⁴. In contrast, the discovery of active demethylation-related genes occurred fairly late, with three *tet* paralogs (*tet1*, *tet2* and *tet3*) and a single *tdg* gene found in mammalian genomes^{9,10,16}. The identification of well-conserved DNA methylation genes in vertebrates, including teleosts, suggests that these regulatory pathways may be conserved across vertebrates^{17,18}. However, due to the additional round of whole genome duplication (WGD) event that occurred before the radiation of the teleost lineage [TGD, teleost-specific genome duplication, 320 Mya (million years ago)], an increase in the number of copies of these genes has been found in teleost species¹⁹. For instance, the *de novo* methyltransferase *dnmt3* was shown to be more divergent in teleosts compared with mammals: despite the absence of *dnmt3l* in zebrafish (*Danio rerio*) genome, up to 6 *dnmt* genes were identified as orthologous to mammalian *dnmt3a* and *dnmt3b* genes²⁰⁻²². Similarly, 4 to 9 different *dnmt3* paralogs were reported to exist in the genome of other teleost species²³⁻²⁸.

It is generally accepted that a WGD event can provide additional genetic material for selection, and are thus associated with phenotypic diversity and evolutionary innovations²⁹. Alternatively, duplicated genes can also originate from small scale duplications (SSD) which can produce different kinds of adaptations compared to WGD³⁰. Following WGD or SSD events, duplicated genes can either be lost or retained with three distinct outcomes: conservation of the ancestral gene functions, sub-

functionalisation, or neo-functionalisation³¹. Through these processes, the fixation of extra copies of DNA methylation genes in teleost genomes may contribute to the diversification and plastic adaptation of teleosts. However, to characterise these adaptation, it is essential to first understand the evolutionary origin of these additional copies.

The increasing availability of sequenced genomes of teleost species facilitates to establish a comprehensive comparative study of DNA methylation genes among different taxa. However, few studies have been done to clarify the evolutionary history of *dnmt*, *tet* and *tdg* genes in vertebrates^{22-24,32}. Hence, the evolutionary history and the orthologous relationship of DNA methylation genes remain incomplete and unclear, especially when considering genomes with higher complexity, *i.e.* salmonid species, which experienced a fourth round of WGD (Salmonid specific genome duplication, SaGD, 100 Mya).

The present study aimed to refine the current knowledge concerning the evolutionary history of *dnmt* genes in vertebrates, and to update the existing story with all DNA methylation genes (*dnmt*, *tet* and *tdg*) for extended taxa within the chordate phylum. To conduct the present study, we selected representative species with sequenced genomes of different taxa from a WGD point of view. Rainbow trout (*Oncorhynchus mykiss*), a salmonid fish, was included as a model species, which is supposed to have the maximum copies of DNA methylation genes among chordates due to SaGD. To explore if WGD events lead to sub- or neo-functionalisation of DNA methylation genes, estimations of the expression patterns of DNA methylation genes were done during gametogenesis and early development in trout.

Results

Evolutionary history of dnmt genes in chordates

Dnmt1. Through analysing the genomes of representative species in Ensembl (Release 91, December 2017) and in NCBI, we found that the *dnmt1* gene was generally identified as a single copy in the chordate phylum (Table 1), except that 2 *dnmt1* genes were identified in trout and Atlantic salmon (*Salmo salar*). Phylogenetic analyses showed

that both *dnmt1* genes identified in trout and salmon grouped together with other vertebrate *dnmt1* orthologs, whereas the *dnmt1* genes of amphioxus (*Branchiostoma floridae*), ciona (*Ciona intestinalis*) and lamprey (*Petromyzon marinus*) rooted as outgroups in the tree (Supplemental Fig. S1A, *dnmt1* alignment in Supplemental Fig. S2). The

Table 1. Copy numbers of DNA methylation genes in target species in chordates

	<i>dnmt1</i>	<i>dnmt3</i>	<i>tet</i>	<i>tdg</i>
Amphioxus	1	1	1	1
Ciona	1	2 (?)	1	1
Lamprey	1	3 (A/A/A)	3 (1/2/3?)	1
Shark	1	2 (A/B)	3 (1/2/3)	1
Lizard	1	2 (A/B)	3 (1/2/3)	1
Chicken	1	2 (A/B)	3 (1/2/3)	1
Mouse	1	3 (A/B/C)	3 (1/2/3)	1
Spotted gar	1	3 (ab/ba/bb)	3 (1/2/3)	1
Zebrafish	1	6 (aa/ab/ba/bba/bbb1/bbb2)	3 (1/2/3)	2 (a/b)
Medaka	1	4 (aa/ba/bba/bbb)	3 (1/2/3)	2 (a/b)
Stickleback	1	5 (aa/ab/ba/bba/bbb)	3 (1/2/3)	2 (a/b)
Fugu	1	5 (aa/ab/ba/bba/bbb)	3 (1/2/3)	2 (a/b)
Tetraodon	1	5 (aa/ab/ba/bba/bbb)	3 (1/2/3)	2 (a/b)
Trout	2	8 (aa/ab1/ab2/ba1/ba2/bba1/bba2/bbb)	7 (1a/1b/2a/2b/2c/3a/3b)	4 (aa/ab/ba/bb)

subsequent syntenic analysis showed that the 2 *dnmt1* genes in trout were included in the same syntenic group conserved among vertebrates (*angptl6-eif3g-dnmt1-s1pr2-cdc37-tyk2-raver1*, **Supplemental Fig S1B**). We thus annotated them as *dnmt1a* and *dnmt1b* according to ZFIN (The Zebrafish Information Network) Nomenclature. Further analysis of conserved domain search showed that these two *dnmt1* encoding protein sequences in trout both have all the conserved domain features as their mammalian DNMT1 orthologs (DMAP1-binding domain, DNMT1-RFD domain, zf-CXXC domain, BAH domain, and Dcm domain). They also share identical conserved motifs in C-terminal catalytic domain (**Supplemental Fig. S2**).

Dnmt3. We first evaluated the evolutionary history of *dnmt3* in non-vertebrate chordate species. Through a BLAST search against the amphioxus genome, we found that both *B. floridae* and *B. lanceolatum* had only one *dnmt3* gene located on BRAFLscaffold_185, and sc_0000092, respectively. In ciona (*C. savignyi*), two putative *dnmt3* homologs were identified (**Table 1**), with 45.5% of their identity shared in their protein sequences. Further analysis of conserved domain search showed that the *dnmt3*-related protein sequence in *B. lanceolatum* and one of the *dnmt3* in *C. savignyi* (ENSCSAVP00000020042) have all the domain features specific for *dnmt3*: PWWP domain, ADD

domain and Dcm domain (**Supplemental Fig. S3**). But the other *dnmt3* in *C. savignyi* (ENSCSAVP00000020042) has only the Dcm domain in its protein sequence. The conserved *dnmt3* amino acid sequences (sequences alignment including PWWP, ADD and Dcm domain) of representative species in chordates were used to perform phylogenetic analysis (alignment in **Supplemental Fig. S3**), results showed that amphioxus and the ciona *dnmt3* rooted as outgroup in the tree, whereas vertebrate *dnmt3a* and *dnmt3b* related sequences formed two separate groups (**Fig. 1**). Percentage identity matrix was calculated between this two *dnmt3* related sequences in ciona and tetrapods, which showed that ciona sequences seemed no more related to *dnmt3a* than to *dnmt3b* (**Supplemental Table S1**). In order to clarify the specific timing for when *dnmt3a* and *dnmt3b* genes arose, we did a *dnmt3* syntenic analysis in chordates using Genomicus software v01.01 (www.genomicus.biologie.ens.fr, Supplemental Fig. S4). We showed that the synteny around *dnmt3* genes in amphioxus and ciona was not well conserved except for *col21a1* and *akap7*, which were found on reftig_1 and reftig_44 in ciona, respectively. In vertebrates, *dnmt3a* and *dnmt3b* genes were included in two distinct syntenic groups: *ak7-abhd1-col21a1-akap7-epb4112-sl3a1-zbtb24* and *pcsk2-dusp15-pofut1-b4galt5-zbtb46*, respectively.

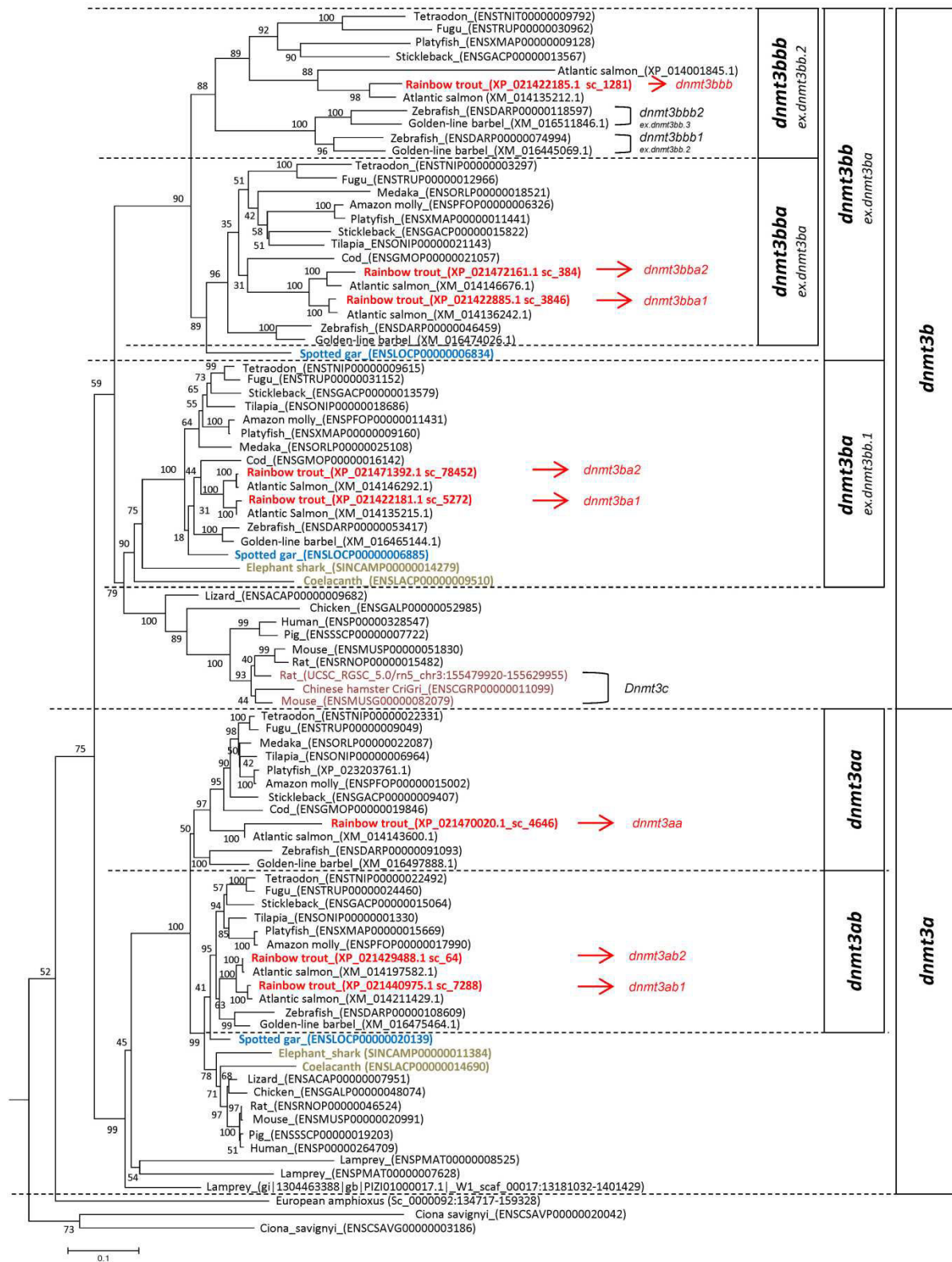


Figure 1. Phylogeny of dnmt3 in chordates. The phylogenetic tree was built by the Neighbor-Joining (NJ) method based on the Jones-Taylor-Thornton (JTT) model in MEGA7. The alignment of the conserved functional domains (PWWP, ADD and Dcm) of Dnmt3 amino acid sequences were used to construct the tree. The optimal tree with the sum of branch length = 14.78071478 is shown. A Gamma distribution (G) was used to model evolutionary rate differences among sites. The reliability of the inferred trees was estimated by the bootstrap method with 1,000 replications. All accession numbers are specified in parentheses.

We then investigate the evolutionary history of *dnmt3* in vertebrates. In jawless vertebrate, lamprey, two *dnmt3* genes annotated as *dnmt3a* (ENSPMAG00000007710 and ENSPMAG00000006895) were identified in the Ensembl database. However, we identified a 3rd sequence in the lamprey genome assembly in NCBI (NCBI, GCA_002833325.1). We identified this sequence (W1 scaf_00017) as a novel *dnmt3* in lampreys. This new *dnmt3* gene and ENSPMAG00000007710 in lamprey have all the conserved domain features for *dnmt3*, whereas the ENSPMAG00000006895 has no PWWP domain (Supplemental Fig. S3). Percentage identity matrix (Supplemental Table S1) and phylogenetic analysis (Fig. 1) confirmed that all these three *dnmt3* related genes in lamprey shared higher conservation with *dnmt3a* gnathostoma genes than with *dnmt3b*. Syntenic analysis results also showed a partial syntenic conservation of the first group cited above around *dnmt3* genes found in lampreys, confirming that these genes were related to the *dnmt3a* gene in other vertebrates (Supplemental Fig. S4). We also verified this result in SIMRBASE. Noteworthy, there is another gene PMZ_0040501-RA annotated as *dnmt3b* in this database. However, we found that this

latter sequence contains only a FYVE domain, and clustered with the mammalian *DNMT3L* orthologs (Data not shown).

In jawed vertebrates, only one *dnmt3a* gene was identified in tetrapods, shark, coelacanth and spotted gar, but *dnmt3a* pseudogenes were identified in humans (ENSG00000224071, chr.2) and mice (ENSMUST00000192011.1, chr.3, Supplemental Fig. S4). By contrast, two *dnmt3a* genes were found in most of the teleost species (Table 1), which were well grouped as two independent clusters, *dnmt3aa* and *dnmt3ab*, in phylogenetic tree (Fig. 1). Both duplicated *dnmt3a* genes in teleost shared conserved synteny with other vertebrates (Fig. 2). Exceptionally, one *dnmt3aa* gene and two *dnmt3ab* genes were found in trout and salmon, whereas *dnmt3ab* was not found in medaka. Conserved domain analysis showed that no PWWP domain was identified in Dnmt3aa of salmon. Besides, rainbow trout Dnmt3aa lost one of the 6 conserved motifs (I, IV, VI, VIII, IX and X), motif IX, in its catalytic region, and its ADD domain sequence also showed less conserved compared to other DNMT3 related sequences (Supplemental Fig. S3).

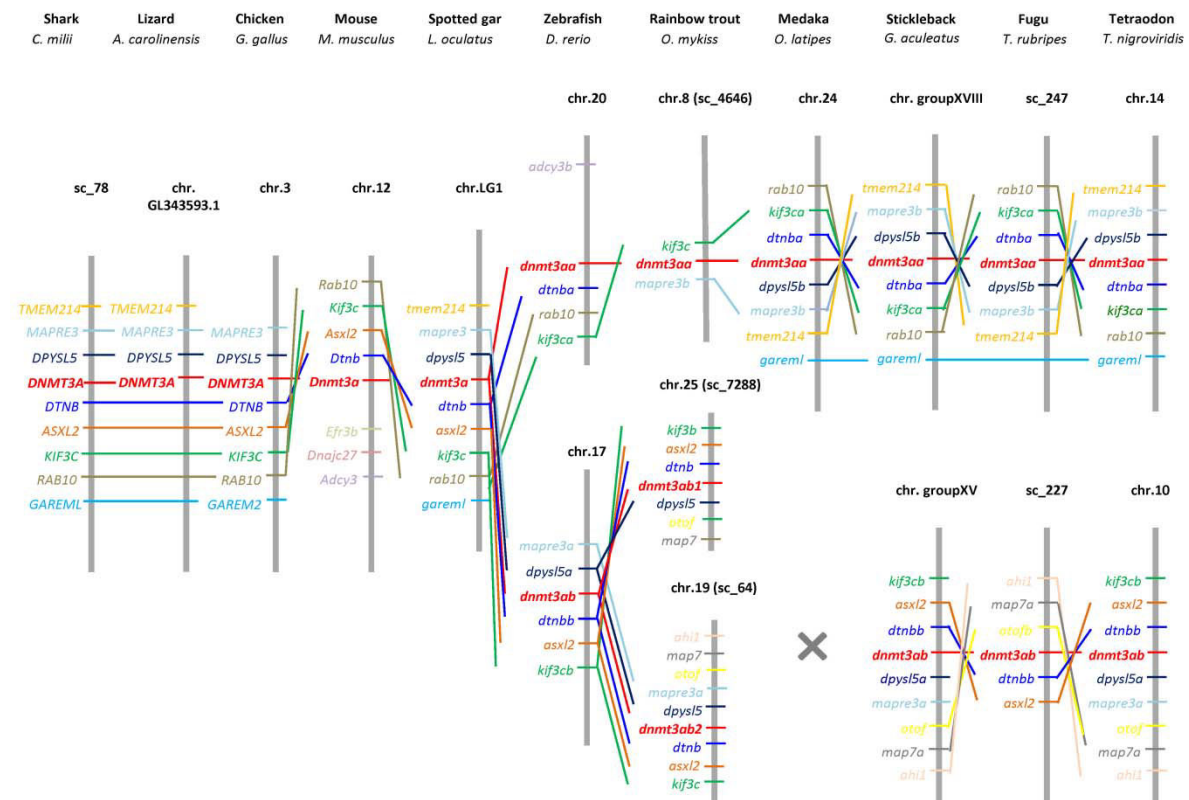


FIGURE 2. Conserved synteny around the *dnmt3* loci in chordates. Data were collected with Genomicus software version 01.01 and NCBI. chr., chromosome; sc., scaffold.

Concerning *dnmt3b*, only one *dnmt3b* related gene was found in non-teleost vertebrates except for the spotted gar, whose genome contained 2 *dnmt3b* related genes in Ensembl annotated as *dnmt3ba* and *dnmt3bb.1*. The murine specific *Dnmt3c* genes were grouped together, and rooted with the *Dnmt3b* genes of rodents in phylogenetic tree (Fig. 1). In most of the teleost species, there were three *dnmt3b* genes identified in Ensembl, namely *dnmt3ba*, *dnmt3bb.1* and *dnmt3bb.2*. Phylogenetic tree showed that the *dnmt3bb.1* in spotted gar clustered with the teleost *dnmt3bb.1* genes, the elephant shark and the

coelacanth *dnmt3b* orthologs and then rooted together with the tetrapod *dnmt3b* genes, whereas the gar *dnmt3ba* rooted together with teleost *dnmt3ba* and *dnmt3bb.2* genes as a separate branch (Fig. 1). We thus proposed to rename *dnmt3bb.1* and *dnmt3ba* in the spotted gar as *dnmt3ba* and *dnmt3bb* and the previous *dnmt3bb.1*, *dnmt3ba* and *dnmt3bb.2* in teleosts as *dnmt3ba*, *dnmt3bba* and *dnmt3bbb*, respectively according to ZFIN Nomenclature guidelines (Table 2). These genes will be referred by their new nomenclature henceforth in this paper.

Table 2. Proposed nomenclature for *dnmt3* and *tdg* genes

Species	Name proposed in Ensembl	Name proposed by Simoda et al.(2005)	Name proposed by Campos et al.(2012)	Proposed new nomenclature
Spotted gar	<i>dnmt3aa</i>			<i>dnmt3a</i>
	<i>dnmt3ba</i>			<i>dnmt3bb</i>
	<i>dnmt3bb.1</i>			<i>dnmt3ba</i>
	<i>tdg.1</i>			<i>tdg</i>
Zebrafish	<i>dnmt3aa</i>	<i>dnmt8</i>	<i>dnmt3a2</i>	<i>dnmt3aa</i>
	<i>dnmt3ab</i>	<i>dnmt6</i>	<i>dnmt3a1</i>	<i>dnmt3ab</i>
	<i>dnmt3bb.1</i>	<i>dnmt4</i>	<i>dnmt3b1</i>	<i>dnmt3ba</i>
	<i>dnmt3ba</i>	<i>dnmt7</i>	<i>dnmt3b2</i>	<i>dnmt3bba</i>
	<i>dnmt3bb.2</i>	<i>dnmt3</i>	<i>dnmt3b3</i>	<i>dnmt3bbb1</i>
	<i>dnmt3bb.3</i>	<i>dnmt5</i>	<i>dnmt3b4</i>	<i>dnmt3bbb2</i>
	<i>tdg1</i>			<i>tdga</i>
	<i>tdg2</i>			<i>tdgb</i>
Trout				Proposed new nomenclature
	scaffold_644	chr.12		<i>dnmt1a</i>
	scaffold_1433	chr.13		<i>dnmt1b</i>
	scaffold_4646	chr.8		<i>dnmt3aa</i>
	scaffold_7288	chr.25		<i>dnmt3ab1</i>
	scaffold_64	chr.19		<i>dnmt3ab2</i>
	scaffold_5272	chr.16		<i>dnmt3ba1</i>
	scaffold_78452	chr.9		<i>dnmt3ba2</i>
	scaffold_3846	chr.16		<i>dnmt3bba1</i>
	scaffold_384	chr.9		<i>dnmt3bba2</i>
	scaffold_1281	chr.16		<i>dnmt3bbb</i>
	scaffold_18888 & 2097	chr.1		<i>tet1a</i>
	scaffold_189 & 12473	chr.23		<i>tet1b</i>
	scaffold_1163	chr.19: 4604994..4635364		<i>tet2a</i>
	/	chr.19: 4726729..4738210		<i>tet2b</i>
/	chr.10		<i>tet2c</i>	
scaffold_21498 & 36080	chr.6		<i>tet3a</i>	
scaffold_605	chr.11		<i>tet3b</i>	
scaffold_2682	chr.4		<i>tdgaa</i>	
scaffold_82	chr.2		<i>tdgab</i>	
scaffold_2682	chr.4		<i>tdgba</i>	
scaffold_82	chr.2		<i>tdgbb</i>	

Evolutionary history of DNA demethylation genes: *tet* and *tdg* genes

Tet. We identified one *tet* related sequence in each genome of amphioxus (BRAFLscaffold_344 in *B. floridae*; sc_00000095 in *B. lanceolatum ill*) and *ciona* (chromosome 12 in *C. intestinalis*; refig_54 in *C. savignyi*) through a BLAST search (**Table 1**). Our results also showed that, most of the jawed vertebrates have three *tet* genes, namely *tet1*, *tet2* and *tet3* (**Table 1**) which were included in three distinct syntenic groups conserved among vertebrates (**Supplemental Fig. S5**). Interestingly, in amphioxus, the only *tet* gene was included in the *wnt8b-scd-tet-rassf-tmem2-cisd2-fam13-cdh-hnrnpd* syntenic group. Genes from this syntenic group were also found syntenic with *tet1*, *tet2* and to a lesser extent with *tet3* vertebrate genes, suggesting that these 3 genes shared a common ancestor. The phylogenetic analysis showed that *tet1/2/3* formed three separate groups in the tree, whereas the amphioxus and *ciona* *tet* sequences rooted as an outgroup (**Supplemental Fig. S6**; protein alignment in **Supplemental Fig. S7**).

In lamprey, 3 hits were found on chromosomes scf_00044, scf_03335 and scf_00172, respectively, in the Genbank assembly GCA_002833325.1. Further research in SIMRBASE confirmed this result. However, because the *tet* sequences on scf_00172 and scf_03335 were aligned to different region of putative *tet* gene on scf_00044, thus it was impossible to include 3 of them together in phylogenetic tree. Our phylogenetic analysis showed that, the putative *tet* gene in lampreys on scf_03335 clustered with *tet3*, whereas the other putative *tet* gene on scf_00044 rooted together with *ciona* and amphioxus as an outgroup in the tree but the bootstrap value for this outgroup was very low (*i.e.* 18, **Supplemental Fig. S6**). Interestingly, the conserved syntenic group of both *tet1* and *tet2* in vertebrates were found on scf_00044.

In trout, a total of seven *tet* paralogs were identified, with two orthologs of *tet1*, three orthologs of *tet2* and two orthologs of *tet3* (**Table 1**). *tet1* and *tet3* most probably duplicated before or around the salmonid radiation following the SaGD, giving rise respectively to *tet1a* and *tet1b*, and *tet3a* and *tet3b*. In the case of *tet2*, as previously mentioned, 3 duplicates (annotated as *tet2a*, *tet2b* and *tet2c*, respectively; **Table 2**) were identified in trout genomes. In trout, *tet2a* (XM_021573348.1) and

tet2b (XM_021573351.1) were located close to one another on chromosome 19 (**Supplemental Fig. S5**) and shared extremely high identity in the cDNA sequence (96.5%), but *tet2b* was found to be only half the length of *tet2a*. The *tet2b* sequence corresponded to exons 6 to 12 of *tet2a*. Similarly, *tet2c* (XM_021617281.1) was found to be closely related to *tet2a* (89.6% identity), but again, the *tet2c* sequence was shorter than *tet2a* and corresponded to exons 1 to 5 of *tet2a*. There was thus no shared region between *tet2b* and *tet2c*, making it impossible to include the three *tet2* paralogs of trout together in the phylogenetic analysis (**Supplemental Fig. S6**). However, the high sequence identity shared by these 3 sequences and the conserved synteny around these 3 loci strongly confirmed that these genes were paralogs. Analysis in salmon also highlighted the 3 *tet2* related sequences (XP_014050290.1, XP_014065146.1 and XP_014042431.1) within its genome. Further study of conserved domain search confirmed that that Tet2b and Tet2c share only part of the sequence with Tet2a sequence in trout, with Tet2b lacking the cysteine-rich domain whereas Tet2c lacking the DBSH domain in the C-terminal catalytic domain (**Supplemental Fig. S7**). Moreover, in most of the studied species, all the conserved motifs in catalytic domain of Tet families (*i.e.* iron and 2-OG binding sites) can be found (**Supplemental Fig. S7**), except that the Fe binding, 2-OG binding and 5mC binding sites at the end of C-terminal catalytic domain were not found in amphioxus, two putative *tet* (located on scf_00044 and scf_03335) in lamprey and Tet1a of salmon.

Tdg. Finally, with regards to *tdg* locus, our results showed that amphioxus, *ciona*, lamprey, shark, tetrapods and spotted gar had one copy of *tdg* in their genome (**Table 1**). In most of teleost species, there were two *tdg* paralogs (**Table 1**). Our phylogenetic analysis demonstrated that these two genes were co-orthologous to non-teleost *tdg* genes (**Supplemental Fig. S8A**, *tdg* protein alignment in **Supplemental Fig. S9**). The syntenic analysis (**Supplemental Fig. S8B**) showed that these 2 genes were side-by-side on the same chromosome and included in a syntenic group (*appl2-nuak1b-rps13-pik3c2a-tdgb-tdg-samm50-api5-nt5dc3-hsp90b1*) in teleosts, which is similar to the syntenic block of *tdg* in non-teleost species (*i.e.* *nuak1*, *appl2*, *nfyb*, *hsp90b1*, *nt5dc3*, and *samm50*). On the other hand, salmonids retained duplicates of *tdga* and *tdgb* genes (**Table 1**), which were therefore annotated as *tdgaa*, *tdgab*, *tdgba* and *tdgbb* (**Table 2**). Phylogenetic analysis showed that

the duplicated *tdga* and *tdgb* genes in salmon and trout were well clustered with their respective orthologs in other teleosts. The syntenic region around *tdga/tdgb* in trout was similar to those in other teleost species, with *tdgaal/tdgba* and *tdgab/tdgbb* arranged in tandem on chromosome 4 and 2, respectively (Supplemental Fig. S8B). Conserved domain search demonstrated that the functional sites were highly conserved among all the analysed *tdg*-related sequences (Supplementary Fig. S9).

Expression pattern of DNA methylation genes during gametogenesis and embryogenesis in trout

In the Phylofish database³³ we found that the high expression of DNA methylation genes in reproduction and development-related tissues seems to have been conserved throughout chordate evolution (data not shown). Therefore, in order to explore the potential different function of duplicated DNA methylation genes after WGD, we investigated their expression patterns during gametogenesis (Fig. 4A) and embryogenesis (Fig. 5A) in trout, which has the maximum DNA methylation genes fixed in its genome.

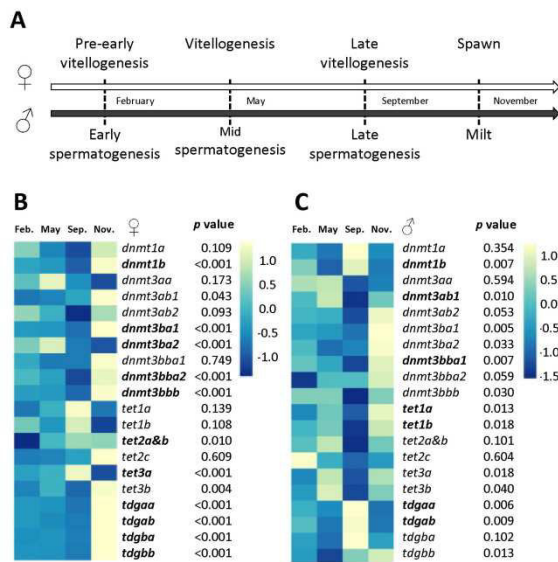


Figure 4. Expression patterns of DNA methylation genes during gametogenesis in trout. Experimental design (A). Heatmaps were used to depict the mRNA levels of DNA methylation genes during oogenesis (B) and spermatogenesis (C) with the mean value of each stage (n=6). When there is a significantly difference among groups (p < 0.05), the gene's name is in bold.

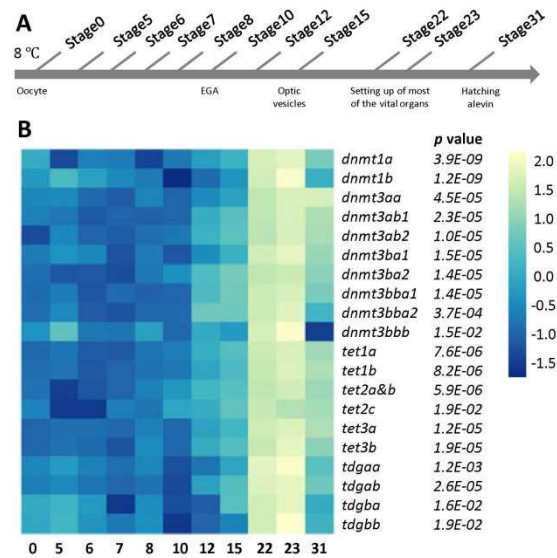


Figure 5. Expression patterns of DNA methylation genes during ontogenesis in trout. (A) Experimental design: Stages numbers referred to the Vernier developmental table⁴⁶. (B) The mRNA levels of DNA methylation genes during ontogenesis were shown in heatmap with the mean value of each stage. Data were subjected to log transformation. EGA, embryonic genome activation.

During oogenesis, we observed that *dnmt1b*, *dnmt3ba1*, *dnmt3bba2*, *dnmt3bbb* exhibited a similar expression pattern (Fig. 4B, mean ± SD data in Supplemental Table S4), which remained steady from February to May (pre-early vitellogenesis and vitellogenesis stages), was halved in September (late vitellogenesis stage), and increased several fold in November (spawn stage). In contrast, the mRNA level of *dnmt3ba2* was significantly lower in September and November compared to February and May in trout ovaries (Fig. 4B). No significant difference was observed in the mRNA levels of *dnmt1a*, all *dnmt3a* paralogs and *dnmt3bba1* during oogenesis (Fig. 4B). Regarding *tet* and *tdg* genes, mRNA levels of both *tet1* orthologs, *tet2c* and *tet3b* were not significantly different among different stages. As for *tet2a&b* (we were not able to design discriminating primers to separately amplify *tet2a* and *tet2b*), the mRNA level increased slightly but significantly during oogenesis (Fig. 4B). The same pattern was observed for *tet3a* except in spawned oocytes (November) in which *tet3a* mRNA level was lower than the other stages. All *tdg* paralogs displayed a similar pattern with a strong increase in mRNA levels at the spawn stage (November) (Fig. 4B).

In males, we observed that the expression profile of DNA methylation genes fluctuated during spermatogenesis (Fig. 4C, mean \pm SD data in Supplemental Table S5). Increased mRNA level of *dnmt1b* was found in testes sampled in September (late spermatogenesis stage), whereas the expression of *dnmt3ab1* and *dnmt3bba1* decreased to its lowest level in September, and increased again to a high level in November (milt stage, Fig. 4C). No significant difference was found in mRNA levels for other *dnmt* genes during spermatogenesis (Fig. 4C). There was no significant difference in all *tet* genes in testes whatever the gonadal developmental stages, except for *tet1 ohnologs*, which displayed a significantly lower mRNA abundance in September than in earlier stages, but increased to a higher level in November (Fig. 4C). For *tdg* genes, *tdga* ohnologs displayed similar expression patterns and remarkably reached a maximum in September and then decreased to a low level in November. No significant difference was found in the mRNA level of *tdgb ohnologs* across all stages (Fig. 4C).

During trout ontogenesis (Fig. 5A), our results showed that mRNA levels for most DNA methylation genes displayed a similar expression pattern, with no or low mRNA levels from stages 0 to 15, followed by a remarkable increase to reach the maximum expression at stage 22/23, and then a decrease after hatching (Fig. 5B, mean \pm SD data in Table S6). Exceptionally, relatively high mRNA abundances of *dnmt1b*, *dnmt3bbb* and *tdgb* ohnologs were observed at the beginning of ontogenesis. Besides, significantly high mRNA level of *dnmt3aa* and low mRNA level of *dnmt3bbb* were found at stage 31.

Discussion

In silico analysis of DNA methylation genes in the present study showed that only one *dnmt1* gene was fixed in the genomes of most species in chordates, which was in accordance with previous studies^{34,35}. A recent study concerning the molecular evolution of *dnmt1* in vertebrates demonstrated that an additional SSD event occurred before the radiation of major marsupial groups, giving rise to the fixation of two *dnmt1* copies in marsupials¹¹. Here, we also identified two *dnmt1* genes in trout and salmon, which were supposed to arise from the SaGD thus being ohnologous genes.

Similar results were found for *tet1*, *tet2*, and *tet3*, which represent as single-copy gene across chordate

evolution, except that amphioxus and ciona have only one *tet* related gene, whereas trout has 7 *tet* related genes. Previous studies did by Zhang et al.³⁶ failed to identify any *tet* related genes in lamprey genome, probably due to the fact that their analyses were done with lamprey somatic genome, which contains only a part of the germline genome due to the programmed genome rearrangement event that happened specifically in lamprey³⁷. In the present study, we identified 3 putative *tet* genes in lamprey, 1 of them are likely to be ortholog of *tet3*, but with a remaining question about the identity of the other two genes. So it still remains elusive to identify the origin of *tet1/2/3* during evolution. In terms of the fixation of multiple *tet* genes, especially the 3 *tet2* genes in salmonid species, it could be hypothesised that a lineage-specific duplication of *tet2* and syntenic genes may have occurred following the SaGD event.

Concerning *tdg* genes, our results showed an increase in the copy number of this gene during chordate evolution. Results in this study suggested that, a single copy of *tdg* was present at least in the gnathostomata ancestor, and a duplication of the *tdg* gene occurred before or around the teleost radiation, which resulted in the presence of the duplicated genes *tdga* and *tdgb* in the ancestral teleost. These results were in contrast with the previous hypothesis proposed by Best et al.²⁸, which stated that the two *tdg* paralogs derived from a lineage-specific duplication event²⁸. This is the reason why in the present study we renamed *tdg1* and *tdg2* as *tdga* and *tdgb*, respectively according to ZFIN Nomenclature guidelines. On the other hand, salmonids possess duplicates of *tdga* and *tdgb* genes, which most probably duplicated before or around the salmonid radiation and were retained following the SaGD.

Finally, the most important work in the present study should be the clarification of the evolutionary history of *dnmt3* genes in chordates. First, our investigation of the origin timing of *dnmt3a* and *dnmt3b* takes advantage of the inclusion of cephalochordate, tunicate and agnatha data. Campos et al. suggested that the ancestral *dnmt3a* and *dnmt3b* genes seem to have arisen during the VGD2 (second vertebrate whole genome duplication) event²², but the identification of multiple *dnmt3* genes in ciona and more specifically the confirmation of several *dnmt3a* genes in lamprey in the present study challenged this view. Several authors are now in support of the idea that one round of genome

duplication (VDG1) occurred during the evolution of jawless vertebrates from chordate invertebrates, but whether the VGD2 occurred before or after the cyclostome-gnathostome split remains a controversy^{38,39}. It can be thus hypothesised that *dnmt3a* and *dnmt3b* arose before the radiation of the lamprey ancestor, probably during VGD1 event. The *dnmt3b* seems to be lost in lamprey, whereas the 3 *dnmt3a* exist in its genome suggested that lamprey *dnmt3a* experienced several duplication events either through VGD2 followed by another SSD event, or through other possible duplication scenarios *i.e.* triplication, chromosome-scale duplications³⁷.

On the other hand, we refined the evolutionary history of *dnmt3* in vertebrates, especially for *dnmt3b*. A previous study proposed that *dnmt3bba* (*ex. dnmt3b2/dnmt3ba*) was derived from TDG, whereas *dnmt3ba* (*ex. dnmt3b1/dnmt3bb.1*) and *dnmt3bbb* (*ex. dnmt3b3/dnmt3bb.2*) arose from a common ancestor through lineage-specific duplication after TDG²². This conclusion, however, took only tetrapod and teleost sequences into account. Thus through the inclusion of the missing link which was previously described as the bridge from tetrapods to teleosts⁴⁰, namely the spotted gar, as well as shark and coelacanth, we clarified and reviewed the phylogenetic relationship between *dnmt3b* paralogs in teleosts. Our results showed that 2 *dnmt3b* genes exist in the genome of gar; one of them (*ex. dnmt3bb.1*) clustered together on one hand with *dnmt3b* orthologs in shark, coelacanth and tetrapods and one the other hand with teleost orthologs, while the other *dnmt3b* gene in gar clustered with *ex-dnmt3ba* and *ex-dnmt3bb.2* in teleosts. We thus hypothesised that the ancestral *dnmt3b* duplicated at VGD2, both duplicates were fixed at least in holostei, whereas one copy was lost in gnathostomes. Although we cannot exclude the possibility that the *dnmt3ba/bb* might arise from a punctual duplication occurred in ancestral holostei, we are much in favour of the former hypothesis. In addition, we demonstrated a closer phylogenetic relationship between *ex-dnmt3ba* and *ex-dnmt3bb.2* than between these genes and *ex-dnmt3bb.1*, which is in accordance with the previous studies^{22-24,41}. This is the reason why we proposed new nomenclatures for these genes. Besides, we found two *dnmt3bbb* (*ex. dnmt3bb.2*) paralogs (*ex. dnmt3/dnmt3bb.2* and *ex. dnmt5/dnmt3bb.3*) in zebrafish and another cyprinid fish, indicating that these two genes may arise from a lineage-specific duplication in cyprinid ancestor, as was reported before²².

In summary, we shed new light onto the evolutionary history of complex *dnmt3* among chordates. Based on our findings, we proposed a model for the evolutionary history of these genes in chordates depicted in **Figure 6**.

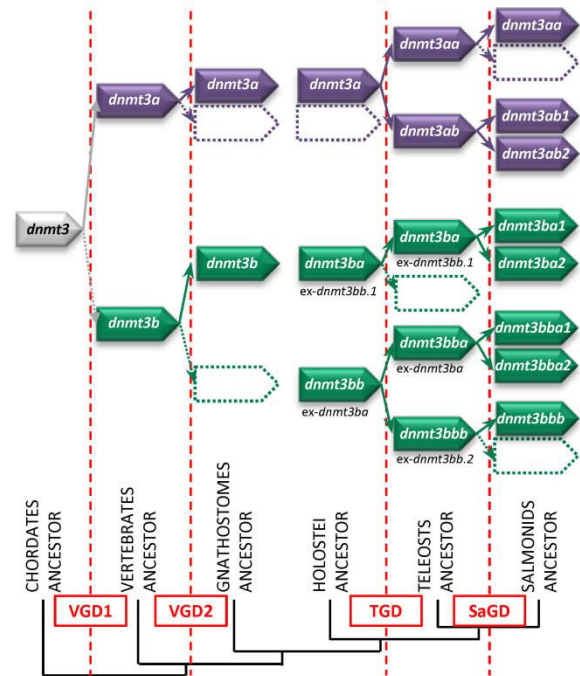


Figure 6. Proposed evolutionary history model of *dnmt3* in chordates. SaGD, salmonid specific genome duplication; TGD, teleost-specific genome duplication. VGD1, the first round vertebrate whole genome duplication; VGD2, the second round vertebrate whole genome duplication.

Our findings prove that DNA methylation genes seemed to have conserved their importance during gametogenesis in teleosts as in mammals. However, the increasing number of DNA methylation related genes in teleosts raises the question of their divergence in term of expressional territories and functions.

In the present study, we demonstrated that most of the identified ohnologous/paralogous genes displayed divergent expression pattern during oogenesis or spermatogenesis (*i.e.* among the *dnmt3b* encoding genes), suggesting that after duplication these genes encountered a sub- or neo-functionalisation. This hypothesis might be supported by our analyses of the branch test of dN/dS ratio for duplicated *dnmt*, *tet*, and *tdg* genes in trout (**Supplemental Fig. S10**), which demonstrated that these genes evolved differently. For instance, among the 6 *dnmt3* paralogs in trout, the *dnmt3aa* and *dnmt3bba2* exhibited higher dN/dS ratio compared to

the other paralogs, indicating that these genes accumulated more non-synonymous substitutions and evolved faster (**Supplemental Fig. S10B**).

In addition, results obtained from the present study showed that these duplications also led to new expression patterns compared to what was previously described in other teleosts or mammals. During oogenesis, *dnmt1b* was found maternally expressed in trout oocytes whereas its zebrafish ortholog *dnmt1* was not found in mature oocytes⁴². Moreover trout *tet3a* expression pattern strongly differed from those previously described for the mammalian *tet3*⁴³. To date, little is known concerning methylome modification during gametogenesis in fish. Nonetheless, low mRNA levels of *dnmt1b*, *dnmt3ba1*, *dnmt3ba2*, *dnmt3bbb* together with the high mRNA level of *tet3a* in September (late vitellogenesis stage) may possibly coincide with a demethylation event. Additionally, our results highlighted a significant increase in mRNA levels for genes involved in both methylation and demethylation (all *tdg* paralogs) pathways of mature oocytes, indicating that they may be important for early embryonic development before zygotic genome activation.

In terms of spermatogenesis, a previous study in zebrafish reported that the testicular transcription of *dnmt1* and *dnmt3* was correlated with global DNA methylation level⁴⁴. In trout, the higher mRNA levels of *dnmt3ab1* and *dnmt3bba1* observed at the end of spermatogenesis may indicate that a *de novo* methylation event occurred before the release of sperm. Moreover, *tet1* ohnologs were the only tet encoding genes that differentially regulated during spermatogenesis in trout, suggesting an important role of *tet1* genes as was previously proposed in mammals⁴⁵. Overall, our results suggest that late spermatogenesis stage may be as a possible “active phase” for the transcriptional regulation of DNA methylation genes, as most of the affected DNA methylation genes exhibited either their highest (*dnmt1b*, *tdgaa*, and *tdgab*) or lowest (*dnmt3ab1*, *dnmt3bba1* and *tet1b*) mRNA level at this stage.

Concerning early development, we demonstrated that all DNA methylation genes have similar expression pattern with an increase of mRNA levels after the embryonic genome activation (stage 10) and strongly increased from stage 15, which corresponds to the developmental stage of visible optic vesicles⁴⁶. This observation could possibly be explained by the indispensable function of DNA methylation in lens development as was previously described in

zebrafish⁴⁷. The peak of expression was observed occurred during the setting up of vital organs (stage 22, 23). This result is in accordance with previous studies, which showed that the expression of DNA methylation genes was largely increased during the organogenesis period in teleosts^{18,48-50}. The high expression of DNA methylation related genes at these specific stages maybe explained by the phylotic stage related widespread DNA demethylation of enhancers of regulatory genes during embryogenesis⁵⁰. However this latter publication was conducted on zebrafish, and comparative embryology of 2 different ectotherm species bred at different temperature remained elusive without performing histological analysis.

Looking at the results in detail we showed that some ohnologous/paralogous genes displayed divergent expression patterns at specific stages, suggesting a neo- or sub-functionalisation after duplication. For some of these genes, expression patterns diverged from what was previously described in other species, which is in accordance with previous studies, indicating that DNA methylation related genes could be differentially regulated from species to species^{22-24,48}. Indeed, in contrast to what was previously described in zebrafish and sole, we did not observe a peak in the expression of *dnmt3b* paralogs during the blastula or gastrula stages^{22,23,51}, which were in favour of neo-functionalisation of these genes in trout during development.

On the other hand, early embryonic expression of *dnmt1* was in accordance with previous studies in other vertebrate species^{23,52-54}, suggesting that the vital function of methylation maintenance during this period was conserved during evolution. Besides, the high mRNA level of *dnmt3aa* in hatched alevins as was previously showed in several teleosts^{22,23,48} may indicate a special role of this gene in alevins compared to the other DNA methylation genes.

Conclusion

The present study is the first to analyse the evolutionary history of DNA methylation genes (*dnmt*, *tet* and *tdg*) in chordates. Results showed that while *dnmt1*, *tet1*, *tet2*, and *tet3* remain as a single copy gene in the genomes of most species of chordates, *dnmt3* and *tdg* duplicates were preferentially fixed in genomes after successive WGD events, increasing their copy numbers during evolution. The case study for trout revealed that these

duplicated genes were regulated independently during gametogenesis and embryogenesis, suggesting that they may have suffered sub- or neo-functionalisation after WDG.

Methods

Ethical issues and approval

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (Decree 2001-464, 29 May 2001 and Directive 2010/63/EU, respectively). This protocol and the project as a whole were approved by the French National Consultative Ethics Committee (201610061056842).

In silico analysis

Paralogs for DNA methylation genes (*dnmt*, *tet* and *tdg*) in trout were identified in in NCBI assembly database (GCF_002163495.1) using the BLAST tool. Orthologous genes of DNA methylation genes and deduced amino acid sequences for other species were identified from the Ensembl genome browser (Ensembl release 91, December 2017, <http://www.ensembl.org>) or NCBI database with BLAST tool. The corresponding genes in amphioxus, elephant shark were identified in their respective genome database (<https://genome.ucsc.edu/>) or NCBI database through BLAST searches. SIMRBASE (<https://genomes.stowers.org/search/loc>), a database which provides preliminary annotations to characterise the latest lamprey assembly (GCA_002833325.1) was also used to verify the results in lamprey. All the BLAST searches were performed using TBLASTN method with default algorithm parameters: expect threshold was set as 10; word size was set as 6; and low complexity regions were filtered. All the identified sequences were subsequently subjected to conserved domain search in NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to further confirm their identities and check if there is any alteration in conserved functional sites.

Phylogenetic analyses were carried out using MEGA package version 7 software⁵⁵ with the deduced amino acid sequences of DNA methylation genes. All the protein sequences were aligned with the MUSCLE method in MEGA software before constructing phylogenetic tree. Phylogenetic trees of Dnmt1, Tet and Tdg were built using Maximum Likelihood method and the whole protein sequences,

whereas the phylogenetic tree of Dnmt3 was constructed using Neighbour-Joining (NJ) method with the protein alignment containing only the conserved functional domains (PWWP, ADD and Dcm). The reliability of the inferred trees was estimated using the bootstrap method with 500 or 1000 replications in Maximum likelihood method and NJ method, respectively. New gene annotations were allocated according to ZFIN Nomenclature guidelines (<http://zfin.org/>).

Syntenic analyses were performed with the Genomicus software, version 01.01 (www.genomicus.biologie.ens.fr) to confirm identities of the target genes in various vertebrates. The gene loci of DNA methylation genes in lampreys, elephant sharks and trout and their syntenic genes were identified using the BLAST tool against their corresponding assembly databases in NCBI.

In order to have a preliminary idea about the prediction of sub- or neo-functionalisation among multiple paralogues, the ratios between non-synonymous and synonymous differences (dN/dS) were also calculated with the codeml program in the PAML package version 4.9i⁵⁶. The aligned amino acids sequences of *dnmt1*, *dnmt3*, *tet1*, *tet2*, *tet3* and *tdg* genes in selected representative species were used to guide the alignment of the nucleotides sequences by PAL2NAL to get codon alignments of these genes⁵⁷. We further built phylogenetic trees with their respective codon alignment of these genes using Maximum likelihood method. The codon alignments and phylogenetic trees were used as the input file for codeml program. Branch tests with the free-ratios model (Alternative hypothesis: heterogeneity of dN/dS among branches) and model 0 (Null hypothesis: homogeneous dN/dS across all sites and branches) were performed to calculate the dN/dS for each of the branches of the gene tree. The comparison between two models was conducted using a likelihood ratio test (LRT) followed by a chi-squared test of significance⁵⁶.

Fish and experimental design

For gametogenesis experiment: two year old genitors were obtained and reared at 8°C in INRA experimental facilities. Ovaries and testes were sampled (6 individuals per time point) during gametogenesis process in February, May, September and November, corresponding to pre-early vitellogenesis/ early spermatogenesis, vitellogenesis/ mid spermatogenesis, late vitellogenesis/ late

spermatogenesis, and spawn stage, respectively (**Fig. 4A**). For ontogenesis experiment: embryos were collected on triplicate spawns intermittently before the fertilisation stage (oocyte) and during embryonic development: stages 5, 6, 7, 8, 10, 12, 15, 22, 23 and 31 (hatching alevins, 384^oD (degree days)) according to Vernier Table ²⁸. Embryos were directly snap-frozen whereas alevins and fish were sacrificed by terminal anaesthetisation with a benzocaine bath (60 mg·L⁻¹) prior to sampling and subsequently frozen in liquid nitrogen. Samples were stored at -80 °C until further analysis (**Fig. 5A**).

Total RNA extraction, cDNA synthesis and RT-qPCR analysis

Total RNA, cDNA synthesis and RT-qPCR analysis on whole embryos (30 embryos extracted together per spawn) and whole alevins (6 alevins extracted individually per spawn) was performed as previously described ⁵⁸. Data were normalised to the exogenous luciferase transcript abundance in samples diluted at 1:25 ⁵⁹.

For gonads, total RNA and cDNA synthesis were performed as previously described ⁵⁹. However, samples were diluted at 1:40, and the geometric mean of several reference genes: *β-actin*, elongation factor 1α (*ef1a*), 18S ribosomal RNA (*18S rRNA*), 40S ribosomal protein S6 (*rps16*), and 60S Ribosomal Protein L27 (*rpl27*) was used as a normalisation for qPCR data during gametogenesis using the E method (Light Cycler software) as previously described ⁶⁰⁻⁶². The primer sets used for analysis are listed in Supplemental **Table S2** (target genes) and Supplemental **Table S3** (reference genes).

Statistical analysis

The qPCR data were presented as heatmap with mean value of each condition using ClustVis (<https://biit.cs.ut.ee/clustvis/>). Normality of distributions was assessed using the Shapiro-Wilk test using R (v3.4.0)/Rcmdr Package. Data were analysed by a Kruskal-Wallis non-parametric test followed by a Tukey test as *post hoc* analysis.

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Author contributions

L.M. designed the study. L.M. and J. L. managed the study and performed *in silico* analysis. J.L. and H.H. performed qPCR analysis. J.L. did statistical analysis and wrote the manuscript. L.M. and S.P. contributed to the manuscript corrections.

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Competing interests

The authors declare no competing interests.

Supplemental material

https://drive.google.com/file/d/1KU6QiKgkfdtgyw0BLZRkdt_P4Iko-WeL/view?usp=sharing

3.3 Part 2: Factors triggering hepatic global DNA hypomethylation phenotype in trout fed a high carbohydrate-low protein diet and the detail mechanisms - Publication 2

Objectives

The objectives of the present study include: i) the identification of what are the factors triggering the hepatic global DNA hypomethylation in trout fed a high carbohydrate-low protein diet (**What factor(s)**: high dietary carbohydrates, low dietary proteins, or hyperglycaemia; ii) the exploration of the detail mechanisms underlying the demethylation process (**How**: through passive or active pathway).

Experimental design

The present study consists of 3 experimental trials: Feeding trial 1 (**Fig. 3-3a**), Feeding trial 2 (**Fig. 3-3b**) and intraperitoneal injection trial (**Fig. 3-3c**). Feeding trial 1 aimed at monitoring how it was achieved (*i.e.* through an active or passive demethylation pathway). In addition, another independent experiment including a feeding trial and an injection trial aimed at investigating the effects of protein/carbohydrates (Feeding trial 2) and the effects of hyperglycaemia (Glucose injection trial), respectively.

For all of the 3 trials, trout juveniles were firstly subjected to 4-day fasting. Samplings (plasma and liver) were done at the end of fasting (Sampling 1 in Feeding trial 1&2, T0 in injection trial). Subsequently, **trout in feeding trial 1** were fed for 4 days with either a high protein/low carbohydrate [HP-LC, 60% crude protein (CP), 0% carbohydrate (CHO)] diet or a low protein/high carbohydrate (LP-HC, 40% CP, 30% CHO) diet; **trout in feeding trial 2** were fed for 4 days with either a control (CTR, 40% CP, 0% CHO), high carbohydrate (HC, 40% CP, 30% CHO), or low protein (LP, 20% CP, 0% CHO) diet. Samplings (plasma and liver) were done at 6 h after the last meal in both feeding trials. In **glucose injection trial**, fasted trout were injected with glucose (750mg/kg body weight) or saline solution. Samplings (plasma and liver) were done at 6 h and 24 h post injection.

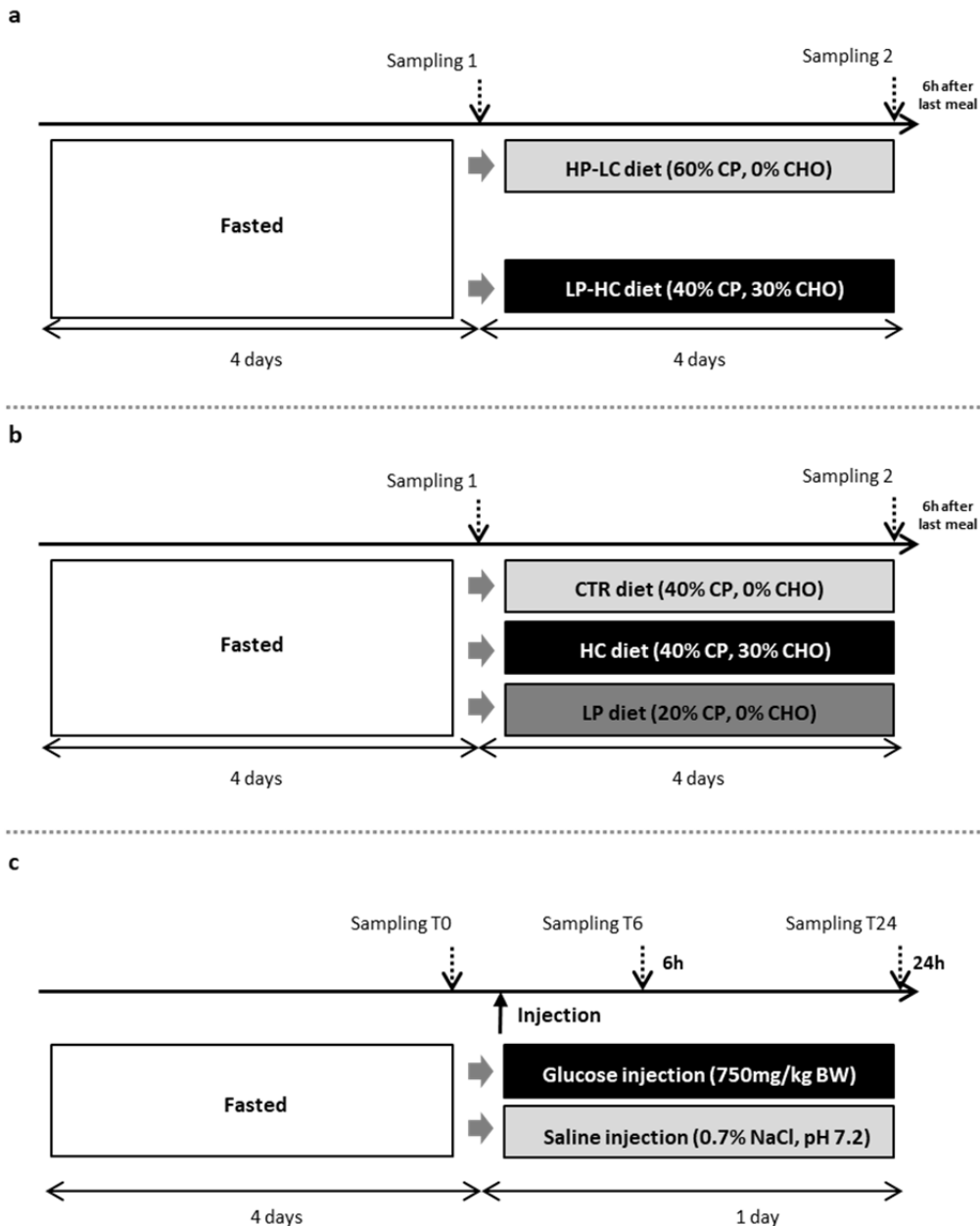


Figure 3-3 Experimental design of feeding trial 1(a), feeding trial 2 (b) and intraperitoneal glucose injection trial (c). CTR, control; HC, high carbohydrates; LP, low protein; BW, wet body weight. CP, crude protein; CHO, carbohydrate; T0, before injection; T6, 6 h after injection; T24, 24 h after injection

Highlights

1. Hepatic global 5-mdC level was significantly lower in trout fed the LP-HC diet compared to fasted ones and those fed the HP-LC diet in feeding trial 1. Hepatic global 5-mdC level was lower in HC and LP groups compared to Fasted and CRT groups in feeding trial 2.

2. Hepatic global 5-cadC level was significantly higher in trout fed the LP-HC diet compared to fasted ones and those fed the HP-LC diet in feeding trial 1. Hepatic global 5-cadC level was significantly higher in trout fed the LP diet compared to fasted ones and those fed the CTR diet in feeding trial 2.
3. Global C^mCGG methylation was not affected by nutritional status or dietary composition.
4. Glucose injection induced hyperglycaemia at 6 h post injection and hepatic global DNA hypomethylation in trout liver at 24 h post injection, which mainly occurred at C^mCGG sequences, a common indicator of CpG methylation (c).
5. The alterations in DNA methylome were barely correlated to the changes in mRNA level of DNA methylation related genes (except for *dnmt3aa* in feeding trial 2), nor did the changes in abundance of SAM/SAH and aminothiols.

Conclusions

The nutritional transition between fasted (4 days) and refed (4 days) fish induced a DNA demethylation which did not occur at CpG sites included in C^mCGG sequences. A 4-day dietary low protein feeding can be associated with global hypomethylation in liver of trout. This dietary factor caused loss of 5-mdC content was demonstrated to occur through active demethylation pathway, and lead to an accumulation of 5-cadC level. Closed profiles were found in the presence digestible dietary carbohydrates which suggested that this nutrient, probably in interaction with proteins, can also initiate a global hypomethylation of DNA. Even if hyperglycaemia does not seem to have an impact on DNA methylation profiles observed in feeding trials, our results showed that hyperglycaemia induced by glucose injection at 6 h after injection, is correlated with a global hepatic C^mCGG hypomethylation at 24 h after injection. The detailed mechanisms of these demethylation processes remain to be elucidated.

Publication 2

Factors triggering hepatic global DNA hypomethylation phenotype in trout fed a high-carbohydrate diet

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ABSTRACT

The present study aimed at identifying the causal factors (*i.e.* dietary high carbohydrates, dietary low proteins and hyperglycaemia) and the underlying mechanisms (*i.e.* active or passive demethylation) for the hepatic global DNA hypomethylation in trout after 4-day feeding of a low protein/high carbohydrate [LP-HC, 40% crude proteins (CP), 30% carbohydrates (CHO)] diet. Two feeding trials and one intraperitoneal injection trial were included in the present study. In feeding trial 1, trout were subjected to 4-day fasting followed by 4-day feeding of either a high protein/low carbohydrate [HP-LC, 60% CP, 0% CHO] diet or a low protein/high carbohydrate (LP-HC, 40% CP, 30% CHO) diet. In feeding trial 2, after 4-day fasting, trout were fed with either a control (CTR, 40% CP, 0% CHO), high carbohydrate (HC, 40% CP, 30% CHO), or low protein (LP, 20% CP, 0% CHO) diet for 4 days. In injection trial, trout were fasted for 4 days and then injected with glucose (750 mg/kg body weight) or saline solution. Blood and liver samples were collected at 6 h after the last meal in feeding trials, or at 0 h, 6 h and 24 h post injection in injection trial. Glycaemia was measured in blood samples; global C^mCGG methylation, global content of 5-mdC and its oxidised intermediates were measured in the liver. We also measured the mRNA levels of DNA methylation/demethylation-related genes and the abundance of SAM/SAH and aminothiols in the liver. Our results showed that the nutritional transition between fasting (4 days) and refeeding (4 days) induced global DNA demethylation which mainly occurred at non-C^mCGG sites. Besides, a 4-day dietary low protein feeding can induce global hypomethylation in the liver of trout. This dietary factor caused loss of hepatic 5-mdC content may occur through an active demethylation pathway, and lead to an accumulation of 5-cadC level. Closed profiles were found in the presence digestible dietary carbohydrates. This finding suggested that dietary carbohydrates, probably in interaction with proteins, can also initiate global DNA demethylation in the liver. Glucose injection induced hyperglycaemia at 6 h post injection and hepatic global C^mCGG hypomethylation in trout at 24 h post injection. These alterations in DNA methylome were barely correlated to the changes in the mRNA levels of DNA methylation/demethylation related genes, nor did the changes in the abundance of SAM/SAH and aminothiols. However, a significant higher SAM/SAH ratio was observed both in trout fed the LP-HC diet in feeding trial 1 and in trout fed the HC diet in feeding trial 2, which is supposed to be due to an increase of SAM. We hypothesised that the accumulation of SAM in hypomethylated liver may also reflect its non-utilisation because of the decrease even arrest of DNA methylation process. In conclusion, dietary high carbohydrates, dietary low proteins and hyperglycaemia can affect hepatic global DNA methylation level but probably in different manner. The detail mechanisms of these demethylation processes remain to be elucidated.

Keywords:

Epigenetics; Global DNA methylation; High dietary carbohydrates; Low dietary proteins; Glucose injection

Introduction

DNA methylation is one of the most comprehensively studied epigenetic mechanisms, which plays crucial roles in various biological processes (Bird, 2002; Dean, 2014; Jones et al., 2015). It is a covalent modification in which a methyl group (CH₃) is added to the 5th position of the pyrimidine ring of a cytosine (5-mC). In vertebrates, the majority of methylated cytosines occur on cytosine positioned in the 5' side of a guanine (namely CpG dinucleotides), and the CpG methylation mark is symmetrically presented on both strands of DNA (Lyko, 2018). It has been widely acknowledged that methylation on the CpG:GpC palindromic sequence is generally associated with gene silencing and a compacted chromatin structure. Methylated cytosines can also occur on non-CpG sites such as CpA, CpT and CpC dinucleotides (Grandjean et al., 2007; Metzger and Schulte, 2016). In contrast to CpG methylation, non-CpG methylation marks are asymmetric and strand-specific (Guo et al., 2014). These strand-specific marks have been initially reported in plants (Meyer et al., 1994) and subsequently in mammals (Hao et al., 2016; Jang et al., 2017; Ramsahoye et al., 2000; White et al., 2002). Although non-CpG methylations account for a smaller fraction in mammalian genomes and their functional roles remain less understood, evidence has proved that they may also participate in gene regulation and mostly in metabolic context (Barres et al., 2009). Indeed, recent studies have highlighted that non-CpG methylation preferentially occurred in genomic region characterised by low density CpGs distribution and seems more prone to environmental stimulus (Fuso and Lucarelli, 2019). Besides, methylation of non-CpG was suggested to be a mechanism of regulating DNA function in response to environmental changes in non-divided cells such as skeletal muscle and neuro cells (Patil et al., 2014). Recently, non-CpG methylation involving transcriptional regulation of specific genes has also been reported in teleost species (Koganti et al., 2017; Shang et al., 2015).

The DNA methylation process is mediated by DNA methyltransferase (Dnmt) family: maintenance methyltransferase Dnmt1 (Hermann et al., 2004) and *de novo* methyltransferase Dnmt3 (Okano et al., 1998). By contrast, the demethylation process can be achieved through either passive or active

demethylation pathway. The passive demethylation occurs when there is a lack or inhibition of Dnmt1 during DNA replication and cell division (Kagiwada et al., 2013). The active demethylation pathway is accomplished through the ten-eleven translocation (Tet) dioxygenase family mediated iterative oxidation of 5-mC into 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), followed by thymine DNA glycosylase (Tdg)-dependent base excision repair (BER) or replication-dependent dilution (He et al., 2011; Ito et al., 2010; Tahiliani et al., 2009) leading to Cytosine. Notably, accumulating evidence indicates that these DNA demethylation oxidative intermediates are dynamic and could themselves carry regulatory functions (Kellinger et al., 2012; Song and He, 2013; Sun et al., 2014).

It has been widely accepted that aberrant DNA methylation status, *i.e.* DNA hyper- or hypomethylation, can modulate gene expression and genome stability thereby leading to several metabolic disorders and diseases in mammals (Luttmer et al., 2013; Robertson, 2005). Previous studies showed that DNA hypomethylation can be induced by dietary manipulations, *i.e.* protein restriction or carbohydrate excess in mammals (Lillycrop et al., 2007; Yamazaki et al., 2016) as well as in teleost (Craig and Moon, 2013). In addition, other non-nutritional factors such as hyperglycaemia and pollutant exposure, have also been reported to influence DNA methylation pattern (Luttmer et al., 2013; Olsen et al., 2012; Pacchierotti and Spanò, 2015; Tros et al., 2014). Nonetheless, the detail mechanisms through which these environmental factors affect the methylation profile are yet to be fully understood.

Rainbow trout (*Oncorhynchus mykiss*), a carnivorous salmonid species, is considered as a “poor user” of dietary carbohydrates due to the prolonged postprandial hyperglycaemia after a high carbohydrate (HC) meal (30%) and growth retard when feeding a HC diet in the long term (Panserat et al., 2013; Polakof et al., 2012). The low capacity of dietary carbohydrate utilisation in trout represents a big constraint for further exploiting this promising ingredient as fish meal substitutions, thus hindering the sustainable development of salmonids culture (Kamalam et al., 2016). Recent studies showed that dietary high carbohydrates induced remodelling of the hepatic epigenetic landscapes in trout, *i.e.* global

DNA hypomethylation and hypomethylation of specific CpG sites at the promoter region of genes involved in gluconeogenesis pathway (Marandel et al., 2016). These results indicate that DNA methylation mechanism may play an important role in the low carbohydrate utilisation and “glucose-intolerant” phenotype in trout. Indeed, the hepatic global DNA hypomethylation phenotype was induced only 4 days after feeding of a carbohydrate-rich diet (Marandel et al., 2016). However, detail mechanisms (*i.e.* through passive or active demethylation pathway) underlying these alterations in global DNA methylome are unclear. Moreover, if an active demethylation process occurred, people may wonder about the variation in the levels of the oxidative intermediates of 5-mC (5-hmC, 5-fC and 5-caC). In other word we can wonder if the active demethylation process is completed or lead to an accumulation of one or the other oxidative intermediates of 5-mC. Indeed, several studies published in the recent years tended to highlight emerging regulatory roles of these intermediaries on gene expression (Wang et al., 2015).

Furthermore, it was impossible to clarify the exact causal factor for this phenotype because the increase in dietary carbohydrate content in the high carbohydrates (HighCHO) diet (0% to 30%) was compensated by a decrease in protein content (60% to 40%) in order to keep the same energy level. Additionally, the concomitant effect of hyperglycaemic status driven by the HighCHO diet also cannot be excluded. Therefore, it is unknown what is the real factor triggering this hepatic global DNA hypomethylation in trout, *i.e.* decrease in protein content, increase in carbohydrate content, a combination of both, or hyperglycaemic environment alone?

In order to answer the above questions, two main objectives were set in the present study. We explore the causal factors/detailed mechanisms for the hepatic global DNA hypomethylation phenotype in trout through introducing a control diet and glucose injection to separately compare the effects of dietary high carbohydrates, dietary low proteins and hyperglycaemia.

Methods

Ethical issues and approval

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (Décret 2001-464, 29 May 2001 and Directive 2010/63/EU, respectively). This protocol and the project as a whole were approved by the French National Consultative Ethics Committee (reference number APAFIS#2017010412054815_v8).

Experimental design

The experimental design of the previous experiment did by Marandel *et al.* could be found in previous publication (Marandel et al., 2016), and we will refer this experiment as Feeding trial 1 in the present study (Fig. 1a). In order to precisely describe the dietary treatments of this experiment, we will refer to the NoCHO (60% protein, 0% carbohydrates) and HighCHO (30% carbohydrates, 40% protein) group in the previous study as HP-LC (high protein-low carbohydrates) and LP-HC (low protein-high carbohydrates), respectively in the present study (Table 1).

Table 1 Formulation and proximate composition of the two experimental diets used (NoCHO and HighCHO diets) in feeding trial 1 (Marandel et al., 2015).

Ingredients %	Diets	
	LC-HP (ex. NoCHO)	HC-LP (ex. HighCHO)
Fish meal ¹	90.85	55.9
Fish oil ²	5.15	10.1
Starch ³	0	30
Vitamin mix ⁴	1	1
Mineral mix ⁵	1	1
Alginate ⁶	2	2
<i>Proximate composition</i>		
Dry matter (DM, % diet)	95.46	94.69
Crude proteins (% DM)	61.3	39.08
Crude lipids (% DM)	18.86	17.7
Gross energy (kJ/g DM)	22.05	21.76
Ash (% DM)	17.73	11.38
Carbohydrates (% DM)	<1.0	28.4

^{1,2} Sopropeche, Boulogne-sur-Mer, France. ³ Gelatinized corn starch (Roquette, Lestrem, France). ⁴ Supplied the following (kg⁻¹ diet): DL- α tocopherol acetate 60 IU, sodium menadione bisulphate 5 mg, retinyl acetate 15000 IU, DLcholecalciferol 3000 IU, thiamin 15 mg, riboflavin 30 mg, pyridoxine 15 mg, vit. B₁₂ 0.05 mg, nicotinic acid 175 mg, folic acid 500 mg, inositol 1000 mg, biotin 2.5 mg, calcium panthothenate 50 mg, choline chloride 2000 mg. ⁵ Supplied the following (kg⁻¹ diet): calcium carbonate (40% Ca) 2.15 g, magnesium oxide (60% Mg) 1.24 g, ferric citrate 0.2 g, potassium iodide (75% I) 0.4 mg, zinc sulphate (36% Zn) 0.4 g, copper sulphate (25% Cu) 0.3 g, manganese sulphate (33% Mn) 0.3 g, dibasic calcium phosphate (20% Ca, 18% P) 5 g, cobalt sulphate 2 mg, sodium selenite (30% Se) 3 mg, potassium chloride 0.9 g, Sodium chloride 0.4 g. ⁶ Louis François, Marne-la-Vallée, France

Another feeding trial (feeding trial 2, **Fig. 1b**) and an intraperitoneal glucose injection trial (**Fig. 1c**) were designed based on feeding trial 1 to test the effects of dietary factors (high dietary carbohydrates or low dietary proteins) and hyperglycaemia on

global hepatic DNA methylation. Fish were obtained and reared in INRA experimental facilities (Donzacq, France) with a flow-through rearing system supplied with natural spring water (18 °C) under a natural photoperiod.

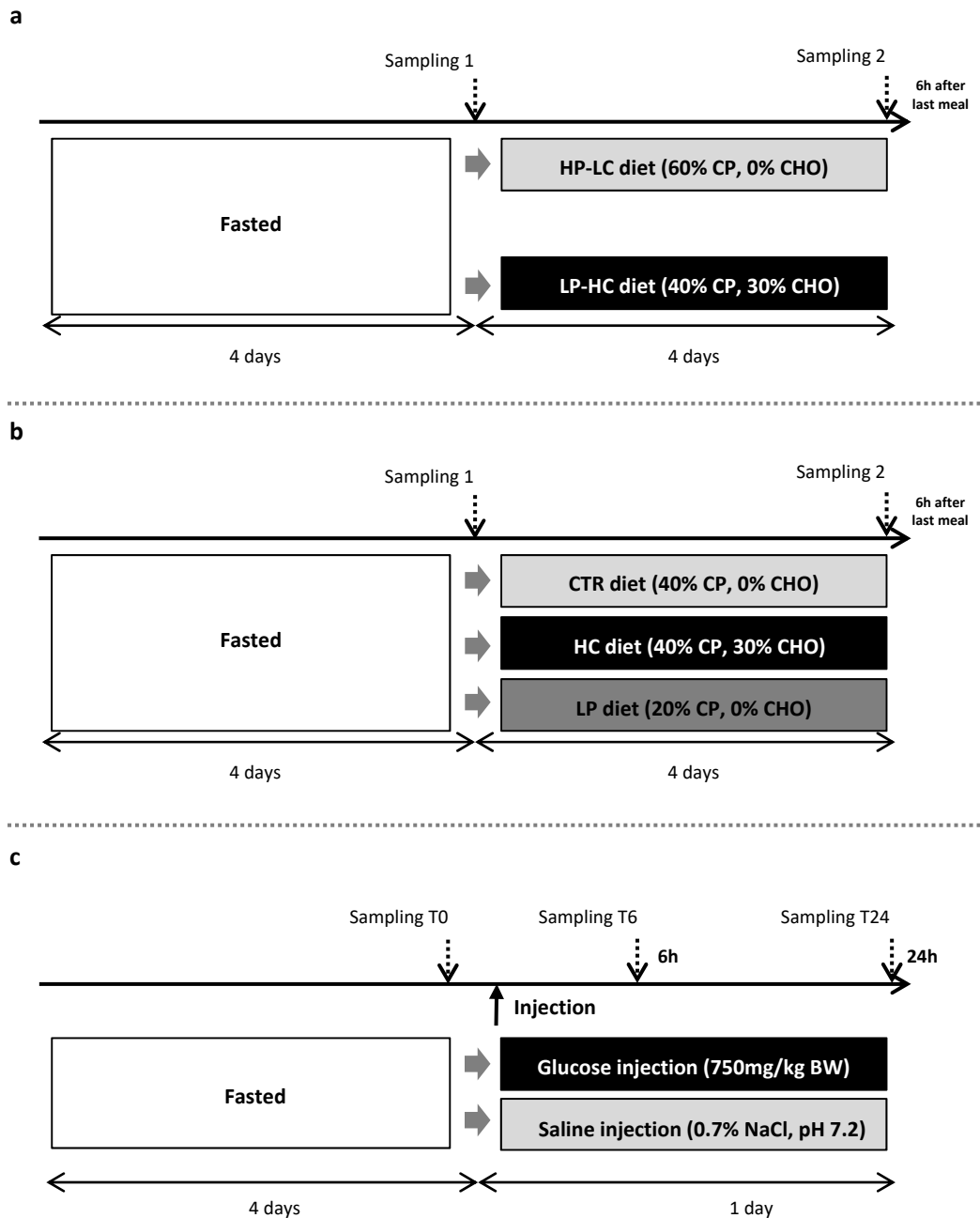


Figure 4 Experimental design of feeding trial 1(a), feeding trial 2 (b) and intraperitoneal injection trial (c). CTR, control; HC, high carbohydrates; LP, low protein; BW, wet body weight. CP, crude protein; CHO, carbohydrate; T0, before injection; T6, 6h after injection; T24, 24h after injection

Table 2. Formulation and proximate composition of experimental diets used in this experiment

Ingredient g/100g diet	CTR	HC	LP
Fish meal ¹	50.3	50	23.9
Gelatinised starch ²	-	32	-
Crude starch ³	20	-	20
Cellulose	11.8	-	36.3
Fish oil ⁴	9	9	10.8
CPSP90 ⁵	5	5	5
Vitamin mix ⁶	1	1	1
Mineral mix ⁷	1	1	1
Alginate ⁸	2	2	2

Proximate composition

DM (% diet)	98	94.6	97.9
Crude proteins (% DM)	42	41.8	23
Crude lipids (% DM)	14.1	13.7	13.3
Gross energy (KJ g ⁻¹ DM)	21.1	21.4	20.8
Ash (% DM)	10.4	10.4	5.9
Carbohydrates (% DM)	16.4	25.6	16.6

Note: CTR, Control diet; HC, high carbohydrates diet; LP, low protein diet. ¹ Sopropheche, Boulogne-sur-Mer, France; ² gelatinised wheat starch (Roquette, Lestrem, France); ³ Roquette, Lestrem, France; ⁴ Sopropheche, Boulogne-sur-Mer, France; ⁵ Sopropheche, Boulogne-sur-Mer, France; ⁶ supplying (kg⁻¹ diet): 60 IU DL- α -tocopherol acetate, 5 mg sodium menadione bisulphate, 15,000 IU retinyl acetate, 3000 IU DL-cholecalciferol, 15 mg thiamine, 30 mg riboflavin, 15 mg pyridoxine, 0.05 mg vitamin B₁₂, 175 mg nicotinic acid, 500 mg folic acid, 1000 mg inositol, 2.5 mg biotin, 50 mg calcium pantothenate and 2000 mg choline chloride; ⁷ supplying (kg⁻¹ diet): 2.15 g calcium carbonate (40% Ca), 1.24 g magnesium oxide (60% Mg), 0.2 g ferric citrate, 0.4 mg potassium iodide (75% I), 0.4 g zinc sulphate (36% Zn), 0.3 g copper sulphate (25% Cu), 0.3 g manganese sulphate (33% Mn), 5 g dibasic calcium phosphate (20% Ca, 18% P), 2 mg cobalt sulphate, 3 mg sodium selenite (30% Se), 0.9 g potassium chloride and 0.4 g sodium chloride; and ⁸ Louis François, Marne-la-Vallée, France.

In feeding trial 1, the average body weight of trout was around 70 g. For both new trials, trout juveniles (~60g body weight in feeding trial 2 and ~200g body weight in injection trial) were subjected to a 4-day food deprivation to clear up the effect of previous diet and reach a basal metabolic level. For the feeding trial 2, the first sampling was conducted after fasting. During the following 4 days, the fish were fed with either a Control diet [CTR; 40% Crude protein (CP), 0% carbohydrate (CHO)], High carbohydrates diet (HC; 40% CP, 30% CHO), or Low protein diet (LP; 20% CP, 0% CHO) twice per day. The last sampling was conducted 6 h after the last meal (**Fig. 1b**). The detail composition of the experiment diets was shown in Table 2. At each sampling point, 9 fish individuals of each treatment were randomly chosen and blood and liver were collected. For the injection trial, the first sampling was conducted after 4-day fasting (T0, n = 4 fish), and then fish were intraperitoneally injected with 30% glucose solution (750mg/kg body weight) or 0.7% saline solution (pH=7.2). Samplings were also done at 6h and 24h post injection. At each sampling point,

5 fish from each treatment were randomly chosen, and blood and liver were collected (**Fig. 1c**).

For all samplings, fish were first anaesthetised in benzocaine and then euthanized in an overdose benzocaine bath before handling. Blood samples were collected from the caudal vasculature and centrifuged (3000 g, 5 min). The recovered plasma was frozen and stored at - 20 °C until analysis. Liver was dissected and immediately frozen in liquid nitrogen, and stored in - 80 °C for further analysis.

Chemical composition of the diets

The approximate composition of the diets was analysed as follows: dry matter was determined after drying to constant weight at 105 °C; crude protein (N \times 6.25) was determined by the Kjeldahl method after acid digestion; crude lipid was determined by petroleum ether extraction (Soxtherm); gross energy was measured in an adiabatic bomb calorimeter (IKA, Heitersheim Griebelmer, Germany); ash was estimated through incinerating in a muffle furnace for 6 h at 600 °C.

Glycaemia analysis

Plasma glucose was analysed with Glucose RTU (BioMerieux) kits according to the recommendations of each manufacturer.

*DNA extraction and global DNA methylation analysis**DNA extraction*

DNA extraction was performed on liver tissues. 10 mg liver samples were subjected to 37 °C overnight digestion under agitation (250 rpm) in 1 mL TNES buffer (pH=8; 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea, Tris-HCl 10mM) supplemented with 100 μ g proteinase K (P6556, Sigma-Aldrich). 1 mL phenol-chloroform-isoamyl alcohol (25 : 24 : 1) was added to each sample, and mixed gently for 15 min followed by a 10000 g centrifuge at room temperature for 15 min. 100 μ L 5M NaCl and 1 mL 100% ice-cold ethanol was added to the supernatant, and centrifuge for 15 min at 10000 g, 4 °C. The pellets were washed in 1 mL 75% ice-cold ethanol and centrifuge for 15 min at 10000 g, 4 °C again. Finally the pellets were dried and re-suspended in DNase-free water and treated with RNase (A7973, Promega, Charbonnières les bains,

France) for 1 h at 37 °C. DNA was quantified by NanoDrop (Thermo Fisher, USA) and the quality (high molecular weight) was verified on 1% agarose gel.

Global CpG methylation by LUMA

Global CpG methylation pattern was assessed at Epigenomics core facility at the University of Paris Diderot using the LUMInometric Methylation Assay (LUMA) method. Each DNA sample was performed in two runs with 1 µg DNA (measured by Qubit) required for each run. Briefly, LUMA utilises a combination of two DNA restriction digest reactions performed in parallel and subsequent pyrosequencing reactions to fill-in the protruding ends of the digested DNA strands. One digestion reaction is performed with the CpG methylation-sensitive enzyme HpaII; while the parallel reaction uses the methylation-insensitive enzyme MspI, which will cut at all CCGG sites. The enzyme EcoRI is included in both reactions as an internal control. Both MspI and HpaII generate 5'-CG overhangs after DNA cleavage, whereas EcoRI produces 5'-AATT overhangs, which are then filled in with the subsequent pyrosequencing-based extension assay. The measured light signal calculated as the HpaII/MspI ratio is proportional to the amount of unmethylated DNA present in the sample. LUMA gives an estimation of the CpG DNA methylation level.

Global cytosine methylation and its other oxidative intermediates

DNA hydrolysis

DNA samples (previously treated with RNase) were subjected to a hydrolysis process to generate single nucleosides with DNA Degradase Plus™ kit (ZYMO RESEARCH) following the manufacturer's instruction. Briefly, the reaction mix consists of 1 µg DNA sample (measured by Nanodrop), 2.5 µL 10X DNA Degradase Plus™ Reaction Buffer, 1 µL DNA Degradase Plus™ (5 units/µL) and ddH₂O up to 25 µL total volume. The reaction mixes were incubated at 37 °C for 2 h followed by heat-inactivation at 70 °C for 20 min.

HPLC & chromatography

HPLC analysis was performed according to the protocol of Kovatsi *et al.* and Zauri *et al.* using a Waters® Alliance System (2695 separations module) equipped with a Waters® 2695 Alliance autosampler and a 100 µL injection loop, coupled with a Waters®

2487 dual λ Absorbance Detector (Milford, MA, USA)" (Kovatsi *et al.*, 2012; Zauri *et al.*, 2015). Chromatographic separation was performed on a "LUNA C8 (2) 100Å (3µm, 100 x 3 mm) (Phenomenex, Torrance, CA, USA)". The mobile phase consisted of solvent A: 10 mM, phosphate buffer pH=3.7 and solvent B: acetonitrile. The following linear gradient elution was employed: 0 min: 100%A, 0–8 min: 90% A, 10% B; 8–8.5 min: 73% A, 27% B; 8.5–13.5 min: 65% A, 35% B; 13–13.5 min: 100%A; 14–20 min (column equilibration): 100% A. The column oven temperature was set at 25 °C. UV detection was carried out at 277 nm. The standards of 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) were purchased from Berry & associates Inc.

Calculation

The global level of dC, 5-mdC, 5-hmdC, 5-fdC and 5-cadC was calculated as the percentage of each individual's molar quantity divided by the total molar quantity of all the detected cytidine forms. Take dC as an example, the percentage of 5-mC was calculated using the following equations:

$$dC\% = 100 \times Q_{dC} / (Q_{dC} + Q_{5-mdC} + Q_{5-hmdC} + Q_{5-fdC} + Q_{5-cadC})$$

Where Q_{dC} , Q_{5-mdC} , Q_{5-hmdC} , Q_{5-fdC} , Q_{5-cadC} are the molar quantity of dC, 5mdC, 5hmdC, 5-fdC and 5-cadC, respectively.

Analysis of mRNA levels: quantitative RT-PCR

Total RNA extraction and cDNA synthesis

The analysis of mRNA levels was conducted in liver tissues of trout. Samples were homogenised in Trizol reagent (Invitrogen) with Precellys®24 (Bertin Technologies, Montigny-le-Bretonneux, France), and total RNA was then extracted according to the Trizol manufacturer's instructions. 1 µg of total RNA was subsequently reverse-transcribed to cDNA in duplicate using The Super-Script III RNase H-Reverse transcriptase kit (Invitrogen) with random primers (Promega, Charbonnières, France).

Quantitative RT-PCR assays

The primers used in quantitative real-time-PCR (qPCR) assays genes have been reported in previous study (Liu *et al.*, 2019, under review). qPCR assays

were performed with the Roche Lightcycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France). The reaction mix was 6 μL per sample, including 2 μL of diluted cDNA template (1:76), 0.12 μL of each primer (10 $\mu\text{mol L}^{-1}$), 3 μL of Light Cycler 480 SYBR® Green I Master mix and 0.76 μL of DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany). The qPCR protocol was initiated at 95 °C for 10 min for the initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at 95 °C; 10 s at 60 °C). Melting curves were monitored systematically (temperature gradient 0.11 °C per second from 65 to 97 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each qPCR assay included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse transcriptase- and cDNA template-free samples). The relative quantification of mRNA levels of target genes were normalised to the transcript abundance of *rsp16* in feeding trial 1, *ef1a* in feeding trial 2 and exogenous *luciferase* in injection trial as previously described using the E-method on Light Cycler software (Liu et al., under review)(Marandel et al., 2015).

SAM, SAH and aminothiols content in liver

Liver samples (around 200mg) were homogenised in 10 volume of phosphate buffer (50mM pH 6.5, EDTA 1mM) using an ultra-turrax homogeniser (IKA™ ULTRA-TURRAX T25 digital, Germany). Homogenising process was set as two times 2000rpm \times 30 s with 10 s pause. Homogenates were centrifuged at 4 °C for 15 min at 10000g. Take 2 mL of the supernatant for protein quantification using the BCA method. Take another 2 mL of supernatant for deproteinisation with Metaphosphoric acid (MPA 1.25 M). An equal volume of the MPA reagent was added to the sample and centrifuged 5 min at 2000g. The supernatant was recovered and then stored in -20 °C until HPLC-UV analysis.

Instrumental determinations of SAM/SAH and aminothiols were performed using a Waters® Alliance System (2695 separations module) equipped with a Waters® 2695 Alliance autosampler supported with a Waters® 2487 dual λ Absorbance Detector (Milford, MA, USA).

SAM and S-adenosyl-L-homocysteine (SAH) in the liver were analysed according to the modified protocol (Cook et al., 1989). Chromatographic separation was achieved on Waters® Resolve C18 column (3.9 mm \times 150 mm i.d. 5 μm). The column was operated at 40 °C. The injection volume was 50 μL and the flow rate was set at 0.7 mL/min. A binary solvent system was used, consisting of (A) 8mM octenylsuccinic anhydride (OSA) and pH 2.7 20mM phosphate buffer adjusted with trifluoroacetic acid (TFA), (B) methanol. The mobile phase was filtered through in-line 0.2 μm membrane filters. The following linear gradient elution was employed: 0–10 min: 95% A, 5% B; 20 min: 30% A, 70% B; 35–45 min (column equilibration): 95% A, 5% B. The eluate was monitored with absorbance detection at 258 nm. The standards of SAM and SAH were purchased from Sigma-Aldrich® (Germany).

Aminothiols in liver were analysed according to the modified protocol of Toyooka and Imai (Toyooka and Imai, 1984). Deproteinised liver homogenate aliquot was subjected to a derivatisation process using 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F). Briefly, 25 μL of the sample homogenate aliquot was submitted for derivatization adding 62.5 μL of AccQ·Fluor™ borate buffer and 5 μL of NaOH 1.55 N. Then 25 μL of 4.5mM ABD-F (in AccQ·Fluor™ borate buffer) was added. Derivatization with thiols residues was done at 60 °C for 20 min. The reaction was stopped by adding 12.5 μL of HCl 1N. Acidified samples were cooled at 4°C for 15 min prior to HPLC analysis.

Chromatographic separation was achieved on Waters® AccQ·Tag™ column (3.9 mm \times 150 mm i.d. 4 μm). The column was operated at 40 °C. The injection volume was 100 μL and the flow rate was set at 1 mL/min. A tertiary solvent system was used, consisting of (A) aqueous solution of AccQ·Tag™ Eluent A, (B) ultrapure water and (C) methanol. The mobile phase was filtered through in-line 0.2 μm membrane filters. The following linear gradient elution was employed: 0–2 min: 97% A, 3% C; 20 min: 96% A, 4% C; 25 min: 20% B, 80% C; 30–35 min (column equilibration): 97% A, 3% C. The eluate was monitored with fluorescence detection (excitation at 385 nm, emission at 515 nm).

AccQ·Fluor™ borate buffer and a mobile phase buffer, Eluent A were purchased from Waters (Massachusetts, USA). All of the thiols in their reduced forms (L-Cysteine (Cys), L-Homocysteine

(hCys), L-Cysteinylglycine (CysGly), γ -L-Glutamyl-L-Cysteine (GluCys), L-Glutathione (GSH) and the derivatisation agent, 4-Fluoro-7-sulfamoylbenzofurazan (ABD-F) were purchased from Sigma-Aldrich (Germany). Other reagents that used were of HPLC grade.

Statistical analysis

The results are presented as mean \pm SD (standard deviation). Normality distribution was evaluated using the Shapiro-Wilk test. When the normality of data were respected, one-way ANOVA was used to detect significant differences. If not, data were then analysed by the Kruskal-Wallis non-parametric test followed by Tukey's test as the *post hoc* analysis. Statistical analyses were done using R software (v3.1.0)/R Commander with the level of significance set as $p < 0.05$.

Results

Glycaemia level

Results from the feeding trial 1 showed that the glycaemia level was significantly higher in trout fed with the LP-HC diet (9.93 mmol/L) compared to fasted (3.42 mmol/L) trout or those fed with the HP-LC (3.54 mmol/L) diet ($p < 0.05$, **Fig. 2a**) (previously published in Marandel et al., 2015). By contrast, the glycaemia levels in trout fed with the CTR, HC and LP diet were 6.93 mmol/L, 10.51 mmol/L and 6.87 mol/L, respectively, which were

significantly higher compared to those in fasted group (4.35 mmol/L) ($p < 0.05$, **Fig. 2b**). No statistically difference was found in glycaemia level among three feeding groups ($p > 0.05$). For injection trial, results showed that the plasma glucose level in fish received glucose injection dramatically increased at 6 h post injection (19.00 mmol/L), and decreased significantly to a basal level at 24 h post injection (5.83 mmol/L) ($p < 0.05$, **Fig. 2c**). For the fish which received saline injection, the plasma glucose level was also increased at 6 h post injection (9.73 mmol/L), but was significantly lower compared to their counterparts which received glucose injection previously ($p < 0.05$, **Fig. 2c**).

Global DNA methylation in liver

Global CpG methylation analysed by LUMA

We analysed the global CpG methylation level in the liver of fish subjected to 4-day fasting followed by 4-day feeding with either HP-LC diet, LP-HC diet in feeding trial 1, or CTR diet, HC diet, and LP diet in feeding trial 2. No statistically difference was found among different groups in both feeding trials ($p > 0.05$, **Fig. 3a** and **Fig. 3b**). For injection trial, at 24 h after injection, fish previously injected with glucose solution showed significantly lower hepatic CpG methylation level (85.25%) compared to those injected with saline solution (86.39%), whilst there was no significant difference in hepatic global CpG methylation level at other time point (T0 and T6) (**Fig. 3c**).

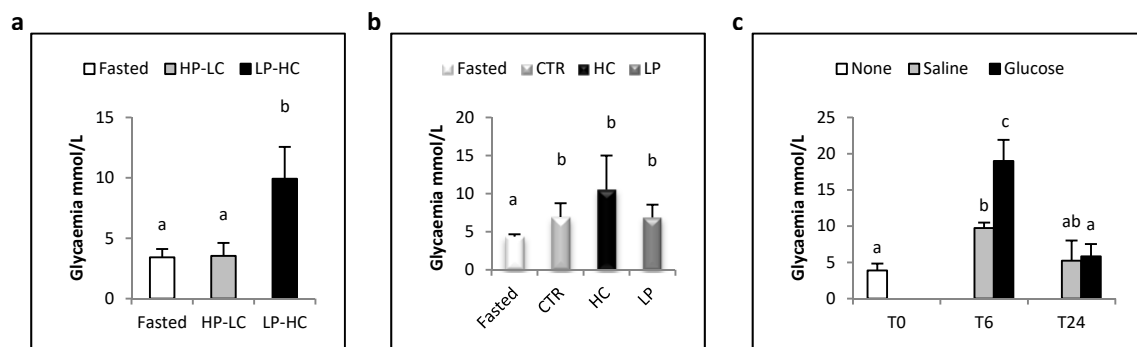


Figure 5 Glycaemia levels of trout subjected to 4-day fasting followed by 4-day feeding trial 1 (a), feeding trial 2 (b) or single intraperitoneal glucose injection trial (c). (a) Glycaemia was measured after fasting or at 6 h after last meal (n=18). HP-LC, high protein/low carbohydrate diet; LP-HC, low protein/high carbohydrate diet. (b) Glycaemia was measured after fasting (n=9) or at 6 h after last meal (n=12). CTR, control; HC, high carbohydrate; LP, low protein. (c) Glycaemia level in fasted trout without injection (T0), and in fish subjected to intraperitoneal administration of glucose (750mg/kg body weight) or saline (0.7%, pH=7.2) solution at 6h (T6) or 24h (T24) after injection. Data were presented as mean \pm SD. Bars bearing the same letter were not significant different with each other ($p > 0.05$).

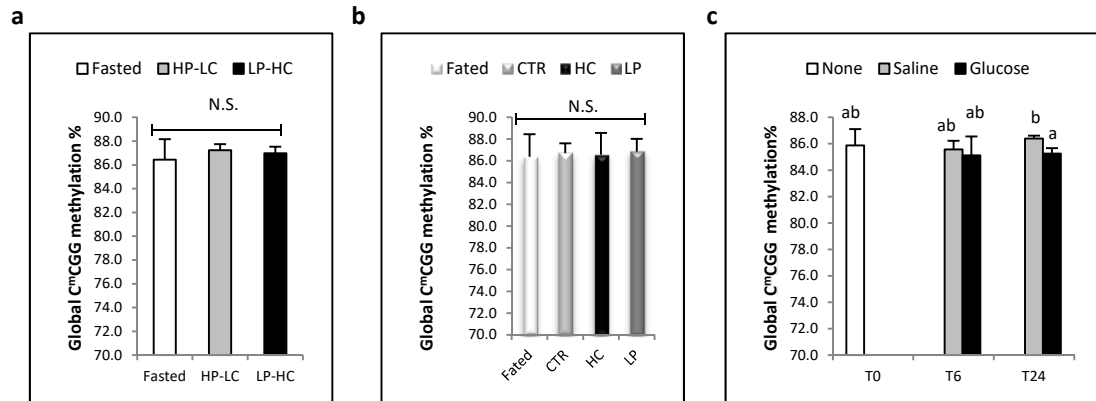


Figure 6 Global hepatic C^mCGG methylation of trout subjected to 4-day fasting followed by 4-day feeding trial 1 (a), feeding trial 2 (b) or single intraperitoneal injection trial (c). (a) Feeding trial 1. Global hepatic C^mCGG methylation was measured after fasting or at 6 h after last meal (n=3). HP-LC, high protein/low carbohydrate diet; LP-HC, low protein/high carbohydrate diet. (b) Feeding trial 2. Global hepatic C^mCGG methylation was measured after fasting or at 6 h after last meal (n=6). CTR, control; HC, high carbohydrate; LP, low protein. (c) Injection trial. Global hepatic C^mCGG methylation in fasted trout without injection (T0), and in fish subjected to intraperitoneal administration of glucose (750mg/kg body weight) or saline (0.7%, pH=7.2) solution at 6h (T6) or 24h (T24) after injection. Data were presented as mean \pm SD. Bars bearing the same letter were not significant different with each other ($p > 0.05$). N.S., no significant difference.

Global 5-mdC, 5-hmdC, 5-fdC and 5-cadC, dC level analysed by HPLC

In feeding trial 1, compared to the fasted group, the global hepatic dC and 5-mdC level was significantly decreased in trout fed with either HP-LC or LP-HC diet ($p < 0.05$, **Fig. 4a**). Besides, the LP-HC group showed the lowest dC and 5-mdC level, which was significantly lower than those in fasted and HP-LC group ($p < 0.05$, **Fig. 4a**). Conversely, the hepatic abundance of 5-cadC showed completely the reverse trends as dC and 5-mdC, with the fasted fish exhibited the lowest and the LP-HC fish showed the highest value of 5-cadC ($p < 0.05$, **Fig. 4a**). Additionally, the average 5-fdC level in the liver was in the order of HP-LC > fasted > LP-HC among treatments with statistically significant difference between HP-LC group and fasted group as well as between fasted group and LP-HC group ($p < 0.05$, **Fig. 4a**). There was no significant difference for of hepatic 5-hmdC among different treatments ($p > 0.05$, **Fig. 4a**).

In feeding trial 2, trout in CTR group exhibited the highest hepatic dC level, which was significantly higher compared to those fed with the LP diet ($p <$

0.05, **Fig. 4b**), whereas the Fasted and HC groups showed intermediate hepatic dC level. Compared to the fasted trout, the global hepatic 5-mdC level was significantly decreased in all feeding groups. Besides, trout fed both HC and LP diet had significantly lower hepatic 5-mdC level compared to those fed the CTR diet ($p < 0.05$, **Fig. 4b**). By contrast, the hepatic global 5-cadC level was higher in HC and LP group compared to fasted and CTR group, but the statistically significance was achieved only between LP group and fasted/CTR groups ($p < 0.05$, **Fig. 4b**).

Concerning injection trial, our results showed that at 24 h after injection, there was a decrease tendency in hepatic global 5-hmdC level in trout previously subjected to either glucose or saline compared to fasted trout or at 6 h after injection (**Fig. 4c**). However, statistically significant difference was observed only in saline-injected trout ($p < 0.05$), with glucose-injected ones showing intermediate level of 5-hmdC level at T 24 (**Fig. 4c**). Global dC, 5-mdC, 5-fdC and 5-cadC levels were not affected by glucose/saline injection at any time point ($p > 0.05$, **Fig. 4c**).

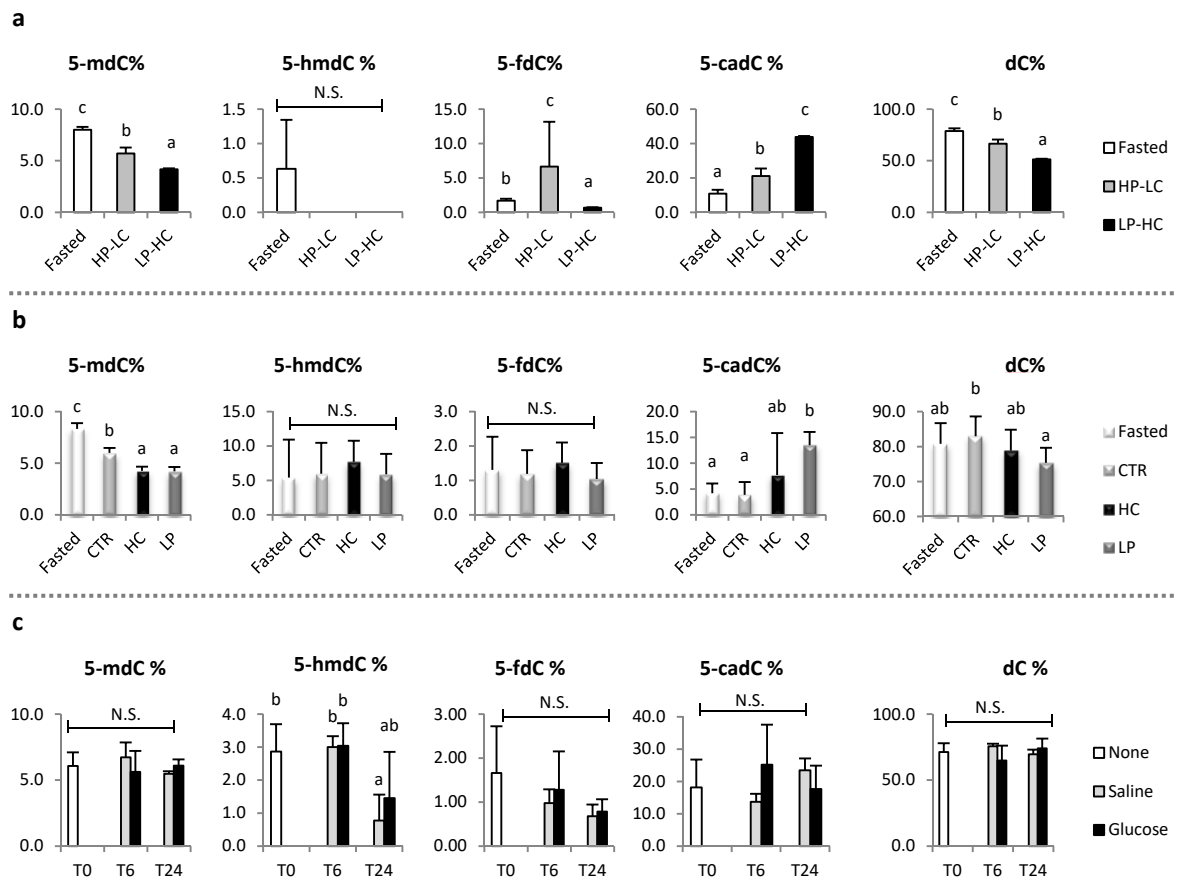


Figure 7 Global hepatic 5-mdC, 5-hmdC, 5-fdC, 5-cadC and dC levels of trout subjected to 4-day fasting followed by 4-day feeding trial 1 (a), feeding trial 2 (b) or single intraperitoneal injection trial (c). (a) Feeding trial 1. Global hepatic 5-mdC, 5-hmdC, 5-fdC, 5-cadC and dC levels were measured after fasting or at 6 h after last meal ($n=6$). HP-LC, high protein/low carbohydrate diet; LP-HC, low protein/high carbohydrate diet. (b) Feeding trial 2. Global hepatic 5-mdC, 5-hmdC, 5-fdC, 5-cadC and dC levels were measured after fasting ($n=9$) or at 6 h after last meal ($n=12$). CTR, control; HC, high carbohydrate; LP, low protein. (c) Injection trial. Global hepatic 5-mdC, 5-hmdC, 5-fdC, 5-cadC, dC levels in fasted trout without injection (T0), and in fish subjected to intraperitoneal administration of glucose (750mg/kg body weight) or saline (0.7%, pH=7.2) solution at 6 h (T6) or 24 h (T24) after injection. Data were presented as mean \pm SD. Bars bearing the same letter were not significant different with each other ($p > 0.05$). N.S., no significant difference. dC, 2'- Deoxycytidine; 5-mdC, 5-Methyl-2'-deoxycytidine; 5-hmdC, 5-Hydroxymethyl-2'-deoxycytidine; 5-fdC, 5-Formyl-2'-deoxycytidine; 5-cadC, 5-Carboethoxy-2' deoxycytidine.

The mRNA levels of genes involved in DNA methylation/demethylation pathways in the liver

Feeding trial 1

For *dnmt* genes, our results showed that the mRNA levels of *dnmt1b* and *dnmt3ab2* genes were significantly increased in two fed groups compared to the fasted group ($p < 0.05$, **Fig. 5a**), but no significant difference was found between two fed groups ($p > 0.05$, **Fig. 5a**). Besides, similar expression patterns were found for *dnmt3ab1*, *dnmt3ba1*, *dnmt3ba2* and *dnmt3bb3* genes, which

showed higher mRNA levels of these genes in two fed groups compared to the fasted group, but statistically significant differences were only observed between fasted and LP-HC groups ($p < 0.05$, **Fig. 5a**). No significant difference was found for hepatic mRNA level of the other *dnmt* genes among treatments.

For *tet* and *tdg* genes, trout fed the HP-LC or LP-HC diet showed increased mRNA levels of *tdgaa* and *tdgab* genes compared to those in fasted group ($p < 0.05$, **Fig. 5b**), whereas the other genes were not affected ($p > 0.05$, **Fig. 5b**) by the nutritional status nor the dietary composition.

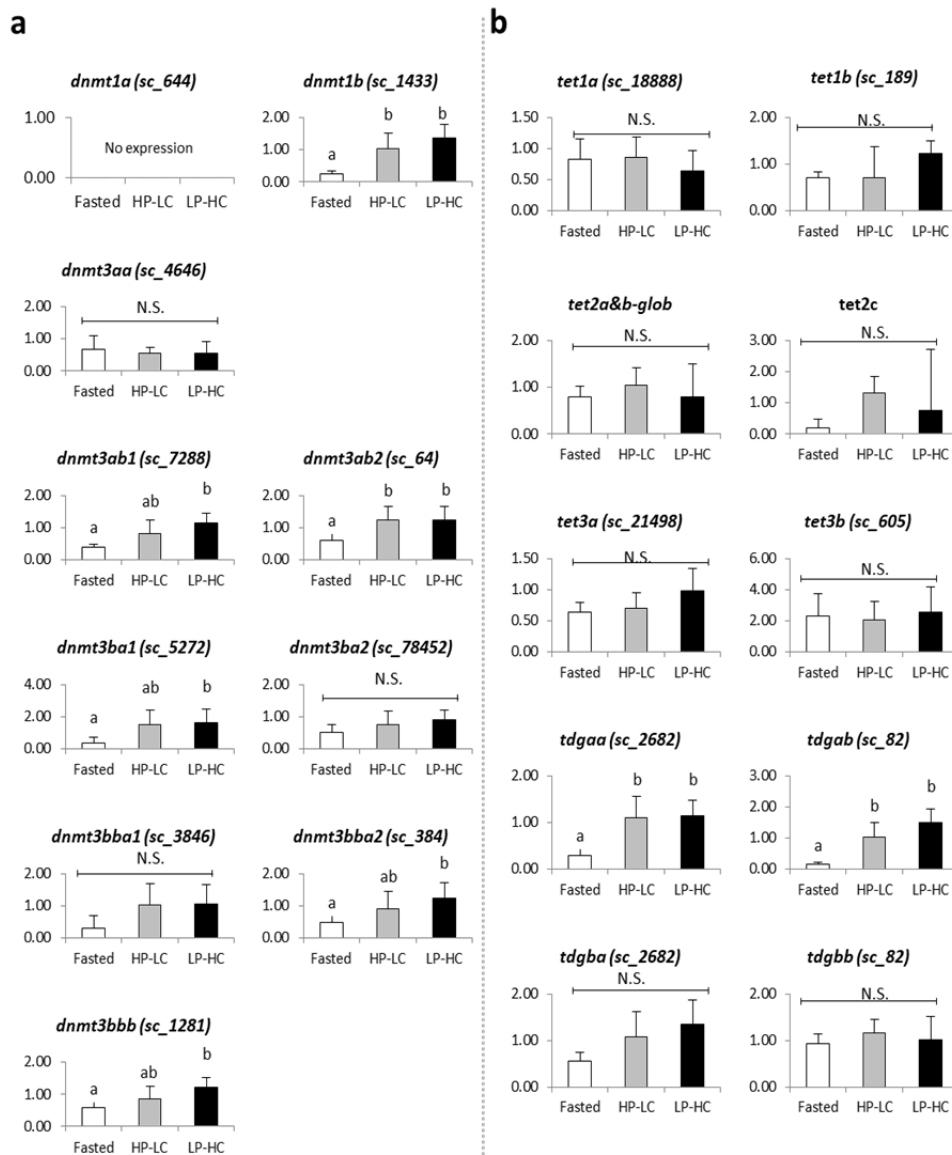


Figure 8 The mRNA levels of *dnmt* genes (a), *tet* and *tdg* genes (b) in the liver of fasted trout, and those re-fed with the HP-LC diet or the LP-HC diet. The mRNA levels of DNA methylation/demethylation related genes were measured after fasting or at 6 h after last meal. HP-LC, high protein-low carbohydrates; LP-HC, low protein-high carbohydrates. Data were presented as mean \pm SD (n = 6). Bars bearing the same letter were not significant different with each other ($p > 0.05$). N.S., no significant difference.

Feeding trial 2

Concerning *dnmt* genes, results showed that although no significant difference was found for the mRNA levels of both *dnmt1a* and *dnmt1b* among dietary groups, the mRNA levels of most of the *dnmt3* genes (except for *dnmt3bbb*) were affected by dietary treatments ($p < 0.05$, Fig. 6a). Trout in fasted group showed the highest mRNA level of *dnmt3aa*,

which was significantly higher compared to those in HC and LP groups ($p > 0.05$, Fig. 5a). Moderate *dnmt3aa* mRNA level was found in trout fed the CTR diet, which was not significantly different from those in Fasted and LP groups, but was significantly higher compared to HC group. The mRNA levels of

dnmt3ab1, *dnmt3ab2* and *dnmt3ba2* exhibited similar pattern, with the lowest transcription level observed in HC group, which was significantly lower compared to either CTR (*dnmt3ab2*) or LP (*dnmt3ba2*) group or both (*dnmt3ab1*) ($p < 0.05$, **Fig. 6a**). Besides, the mRNA levels of *dnmt3ba1*, *dnmt3bba1* and *dnmt3bba2* showed similar transcriptional pattern, with the lowest mRNA level found in fasted fish, which was significantly lower compared to those fed with the CTR diet (*dnmt3ba1*), CTR/HC diets (*dnmt3bba1*) and CTR/LP diets (*dnmt3bba2*) ($p < 0.05$, **Fig. 6a**).

For *tet* genes, compared to fasted group, trout fed the HC diet showed significantly lower mRNA levels of *tet1a*, *tet1b* and *tet2a/b* genes ($p < 0.05$, **Fig. 6b**). The mRNA levels of *tet2c*, *tet3a* and *tet3b* were not affected by dietary treatments ($p > 0.05$, **Fig. 6b**). For *tdg* genes, the mRNA levels of *tdgaa* and *tdgab* were significantly increased whereas the mRNA level of *tdgbb* was significantly decreased in all three feeding groups compared to the fasted group regardless of dietary compositions ($p < 0.05$, **Fig. 6b**). The mRNA level of *tdgba* was not affected by dietary treatments ($p > 0.05$, **Fig. 6b**).

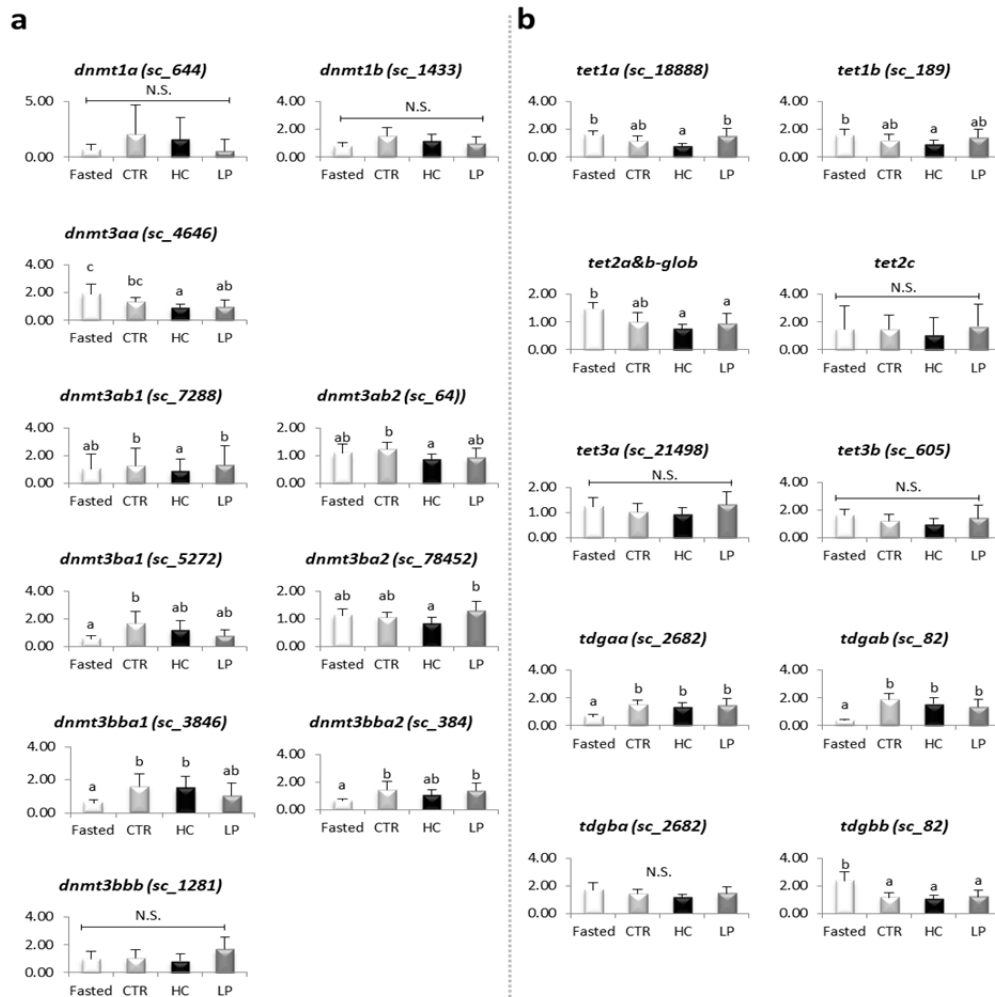


Figure 9 The mRNA levels of *dnmt* genes (a), *tet* and *tdg* genes (b) in the liver of fasted trout, and those re-fed with CTR, HC or LP diets. The mRNA levels of DNA methylation/demethylation related genes were measured after fasting (n=9) or at 6 h after last meal (n=12). CTR, control; HC, high carbohydrate; LP, low protein. Data were presented as mean \pm SD. Bars bearing the same letter were not significant different with each other ($p > 0.05$). N.S., no significant difference.

Glucose Injection trial

There was in general no significant difference in the mRNA levels of most of the DNA methylation/demethylation related genes between glucose and saline injection group at each individual time point (T6 or T24). However, we found that there were significant differences in the mRNA levels of some genes between non-injected and post injected fish (Fig. 7).

For *dnmt* genes, our results showed that compared with the fasted group, the mRNA level of *dnmt3ab1* tended to decrease in the liver of fish previously received glucose or saline injection at 6h and 24h, but significant difference was only observed in fish received glucose injection at 24 h after ($p < 0.05$, Fig. 7a). Similarly, the mRNA level of *dnmt3ba2* tended

to decrease in fish subjected to both saline and glucose injection at T6 and T24, again the significant difference was achieved only in fish subjected to saline injection at T6 ($p < 0.05$, Fig. 7a). Besides, there was a significant decrease in mRNA level of *dnmt3ab2* at 6 h post injection in both glucose and saline group, and then increased again at 24h post injection ($p < 0.05$, Fig. 6a). By contrast, the mRNA level of *dnmt3bbb* showed an opposite changing trend compared with *dnmt3ab2*, which was significantly increased at 6h post injection in both glucose and saline group and then decreased again at 24 h post injection ($p < 0.05$, Fig. 7a). Neither glucose nor saline injection affected the mRNA levels of the rest *dnmt* genes (*dnmt1* ohnologs, *dnmt3aa*, *dnmt3ba1*, *dnmt3bba* ohnologs) in the liver of trout at 6 h and 24 h post injection ($p > 0.05$, Fig. 7a).

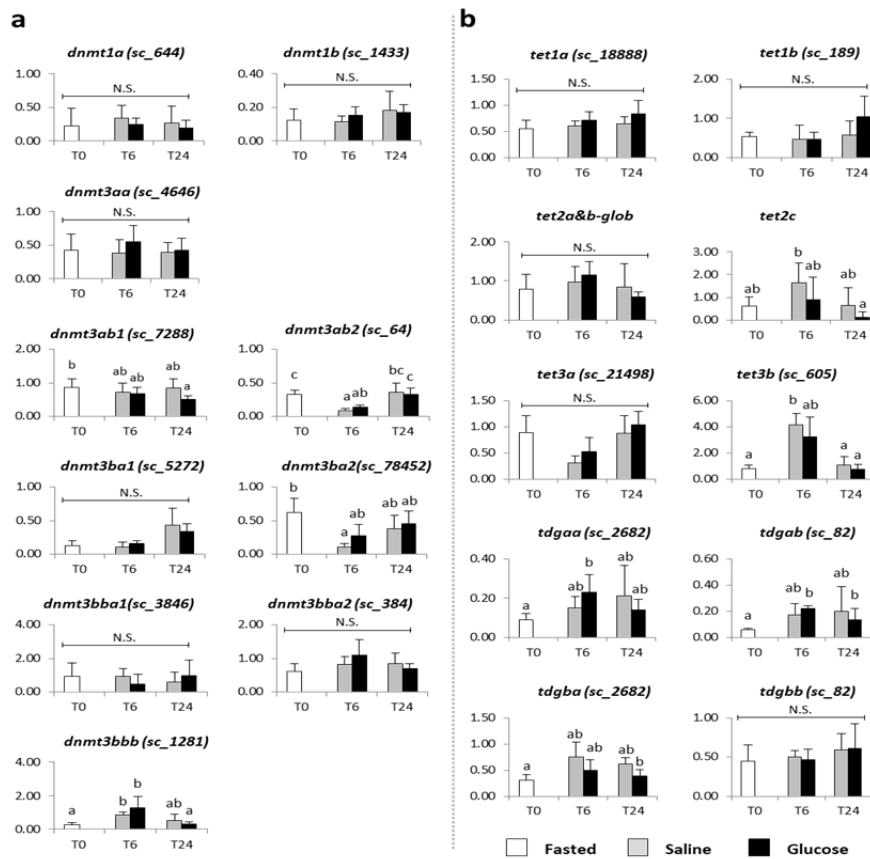


Figure 10 The mRNA levels of *dnmt* genes (a), *tet* and *tdg* genes (b) in the liver of fasted trout without injection (T0), and in fish subjected to intraperitoneal administration of glucose (750mg/kg BW) or saline (0.7%, pH=7.2) solution at 6h (T6) or 24h (T24) after injection. Data were presented as mean \pm SD (n = 4 or 5). Bars bearing the same letter were not significant different with each other ($p > 0.05$). N.S., no significant difference.

For *tet* genes, we found that the expression patterns of *tet2c* and *tet3b* were similar to that of *dnmt3bbb*, which increased in fish received glucose and saline injection at 6 h after, and decreased at 24 h after ($p < 0.05$, **Fig. 7b**). There was no effect of glucose or saline injection on the mRNA levels of *tet1* ohnologs, *tet2a/b*, *tet3a* in trout liver at 6 h and 24 h post injection ($p > 0.05$, **Fig. 7b**).

Interestingly, our results showed that the mRNA levels of *tdg* genes were significantly increased by glucose injection at T6 (*tdgaa*) or T24 (*tdgba*), or both T6 and T24 (*tdgab*) ($p < 0.05$, **Fig. 7b**). But the fish received saline injection showed moderate mRNA level of these *tdg* paralogs at 6 h and 24 h post injection, which were not significantly different with both non-injected fish and glucose-injected fish ($p > 0.05$, **Fig. 7b**). There was no effect of saline or glucose injection on the mRNA level of *tdgbb* in the liver of trout at any time point ($p > 0.05$, **Fig. 7b**).

SAM, SAH and SAM/SAH in liver

In feeding trial 1, although there was no significant effect of fasting/feeding or dietary composition on hepatic SAM and SAH content ($p > 0.05$), the SAM/SAH ratio was significantly higher in trout fed the LP-HC diet compared to the fasted or the HP-LC-fed group ($p < 0.05$, **Fig. 8a**). In feeding trial 2, the hepatic SAM level was significantly higher in all feeding groups compared to fasted group, regardless of dietary composition ($p < 0.05$, **Fig. 8b**). Similar trends were also observed for SAM/SAH ratio, but the HC group had significantly higher SAM/SAH ratio than that in LP group ($p < 0.05$, **Fig. 8b**). In injection trail, we found that at 6 h post injection, there was a significant decrease in hepatic SAM content in trout previously injected with glucose compared to the fasted group ($p < 0.05$, **Fig. 8c**). No significant changes in SAH content and SAM/SAH ratio in liver of trout was found at any time point after glucose or saline injection ($p > 0.05$, **Fig. 8c**).

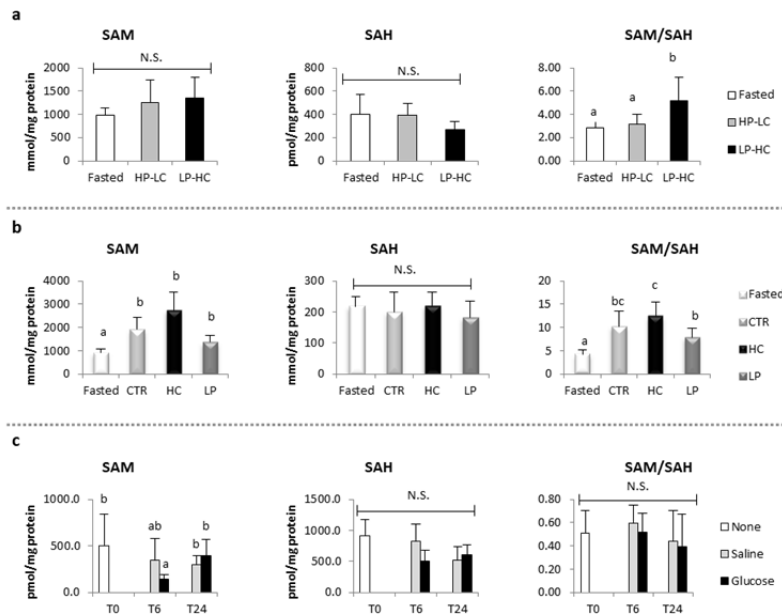


Figure 11 Hepatic abundance of SAM, SAH and their ratio in trout subjected to 4-day fasting followed by 4-day feeding trial 1 (a), feeding trial 2 (b) and single intraperitoneal injection trial (c). (a) Feeding trial 1 (n=6). HP-LC, high protein/low carbohydrate diet; LP-HC, low protein/high carbohydrate diet. (b) Feeding trial 2 (n=9 in Fasted group, and n=12 in feeding groups). CTR, control; HC, high carbohydrate; LP, low protein. (c) Injection trial. Hepatic abundance of SAM, SAH and their ratio in fasted trout (n=4) without injection (T0), and in fish subjected to intraperitoneal administration of glucose (750mg/kg body weight, n=5) or saline (0.7%, pH=7.2, n=5) solution at 6h (T6) or 24h (T24) after injection. Data were presented as mean \pm SD. Bars bearing the same letter were not significant different with each other ($p > 0.05$). N.S., no significant difference.

Aminothiols content in the liver

The hepatic levels of aminothiols L-Cysteine, L-Homocysteine, L-Cysteinyl-Glycine, γ -L-Glutamyl-L-Cysteine and L-Glutathione (metabolites belong to transsulfuration pathways) were analysed. In feeding trial 1, our results found that the abundance of cysteine was significantly decreased in trout fed with the HP-LC diet compared to those in fasted and LP-HC diet fed group ($p < 0.05$, Fig. 9a). Apart from this, trout fed the LP-HC diet had significantly higher homocysteine and GSH levels compared to the fasted fish and those fed with the HP-LC diet ($p < 0.05$, Fig. 9a). Similarly, the highest hepatic level of cysteinyl-glycine was found in trout fed the LP-HC diet, which was significantly higher than that in fasted fish ($p < 0.05$, Fig. 9a). Trout fed the HP-LC diet showed intermediary level of cysteinyl-glycine,

which was not significantly different with the other two groups ($p > 0.05$, Fig. 9a).

In feeding trial 2, we found that the hepatic cysteine level was significantly higher in HC group compared to that in the LP diet-feeding group ($p < 0.05$, Fig. 9b). The hepatic homocysteine level was significantly lower in trout fed the LP diet compared to the fasted and the HC diet-feeding groups ($p < 0.05$, Fig. 9b). Fish fed the CTR and the HC diets showed significantly higher cysteinyl-glycine level in the liver compared to those in fasted and the LP-diet-feeding trout ($p < 0.05$, Fig. 9b). The hepatic GSH level was significantly higher in the HC diet-feeding group compared to the LP diet-feeding group ($p < 0.05$, Fig. 9b). No significant difference was found for hepatic Glutamyl-Cysteine level among treatments ($p > 0.05$, Fig. 9b)

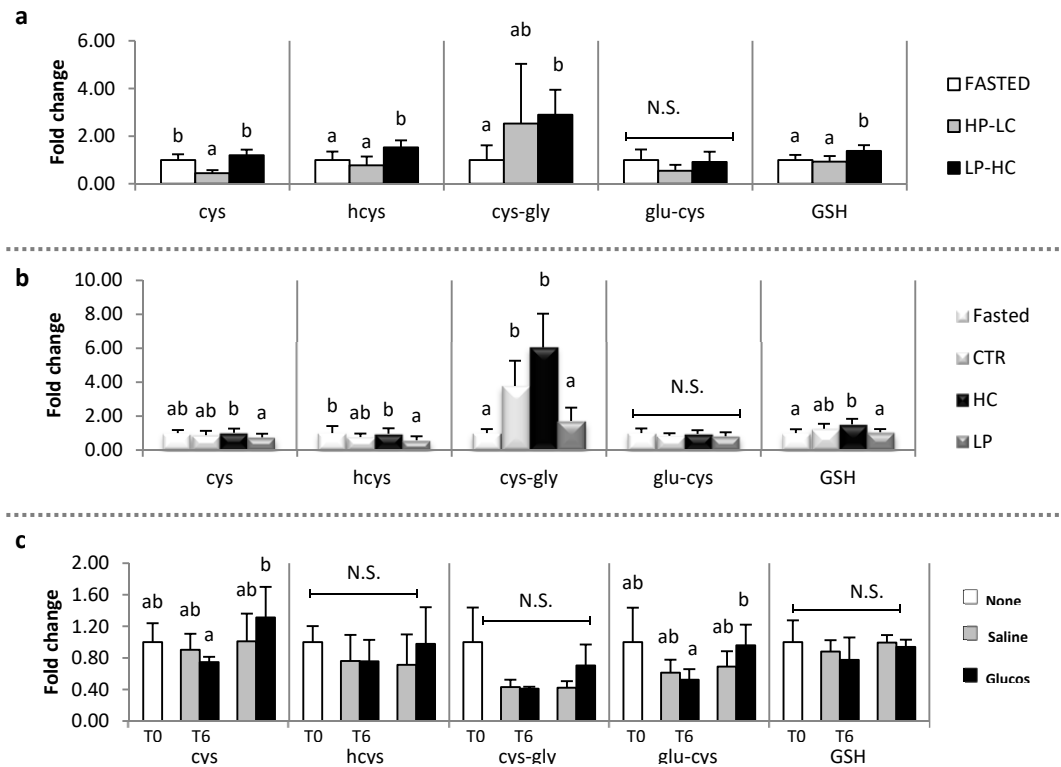


Figure 12 Hepatic abundance of aminothiols of trout subjected to 4-day fasting followed by 4-day feeding trial 1 (a), feeding trial 2 (b) and single intraperitoneal injection trial (c). (a) Feeding trial 1 (n=6). HP-LC, high protein/low carbohydrate diet; LP-HC, low protein/high carbohydrate diet. (b) Feeding trial 2 (n=9 in Fasted group, and n=12 in feeding groups). CTR, control; HC, high carbohydrate; LP, low protein. (c) Injection trial. Hepatic abundance of SAM, SAH and their ratio in fasted trout (n=4) without injection (T0), and in fish subjected to intraperitoneal administration of glucose (750mg/kg body weight, n=5) or saline (0.7%, pH=7.2, n=5) solution at 6h (T6) or 24h (T24) after injection. Data were presented as mean \pm SD. Bars bearing the same letter were not significant different with each other ($p > 0.05$). N.S., no significant difference. cys, L-Cysteine; hcys, L-Homocysteine; cys-gly, L-Cysteinyl-Glycine; glu-cys, γ -L-Glutamyl-L-Cysteine; GSH, L-Glutathione.

Concerning the effects of glucose IP injection on hepatic aminothiols abundance, our results showed that trout subjected to glucose injection had significantly lower cysteine and glutamyl-cysteine levels at T6 compared to those at T24 ($p < 0.05$, **Fig. 9c**), whereas no significant difference was found between other conditions ($p > 0.05$, **Fig. 9c**). By contrast, hepatic homocysteine, cysteinyl-glycine, and GSH levels were not affected by glucose or saline injection ($p > 0.05$, **Fig. 9c**).

Discussion

DNA methylation is an important epigenetic mechanism that can regulate gene expression and affect genome stability. The alterations of DNA methylation pattern has been reported to be profoundly related with the physiological and pathological conditions of living organisms. Site-specific DNA hypermethylation and global DNA hypomethylation is a common feature for several cancer diseases (Torano et al., 2012). Besides, global loss of DNA methylation has also been reported in diabetic animals (Luttmer et al., 2013; Williams et al., 2008).

Previous studies have found that global DNA hypomethylation phenotype can be induced by dietary manipulation in rainbow trout (Craig and Moon, 2013). Most recent studies have proven that this hepatic global DNA demethylation phenomenon can be observed right after 4-day feeding of a high carbohydrate-low protein (HC-LP) diet in this species (Marandel et al., 2016). Nonetheless, it is still unknown what could be the real factors that caused this demethylation process and what are the underlying mechanisms. Moreover, the global DNA methylation level was assessed with commercial ELISA kit in both previous studies (Craig and Moon, 2013; Marandel et al., 2016), which determines the overall methylated cytosine in both CpG and non-CpG sites. It has already been acknowledged that CpG methylation and non-CpG methylation may lead to different alterations in gene functions and more particularly in metabolism-related genes. Therefore, further studies to answer these critical questions could be of great importance to understanding the global hepatic hypomethylation phenotype in trout as well as in other animals. Such as: Where the demethylation process took place in hepatic genome of trout after feeding a HC-LP diet? Which is the real factor that triggered the global demethylation, dietary protein/carbohydrate level or hyperglycaemia? Is this

global loss of methylation occurred through an active demethylation pathway, or a passive demethylation pathway?

How the global DNA demethylation took place in the genome.

In order to have a more specific view about where the DNA demethylation process took place in trout after feeding a high carbohydrate diet, *i.e.* at CpG and/or non-CpG sites, we first tried to analyse the global CpG methylation level (by LUMA) as well as the overall 5-mdC contents at both CpG and non-CpG sites (HPLC method) in samples from feeding trial1. Our results showed no significant difference in global DNA C^mCGG methylation level among treatments. By contrast, through HPLC method, significant decrease of 5-mdC was found in the liver of fed trout compared to fasted ones. This result was also confirmed in the feeding trial 2, where fasted fish displayed higher 5-mdC levels than fish fed either the 3 different diets. Moreover, in the 2 feeding trials, global C^mCGG methylation levels measured by LUMA assay remained stable independent of the nutritional status, indicating that the decrease in 5-mdC may occur at non-CpG sites or at CpG sites out of C^mCGG sequences. These results for the first time demonstrated that the hepatic global DNA methylome could be affected by a short-term fasting-refeeding manipulation in trout.

In addition, 4-day feeding of low a protein/high carbohydrate diet significantly induced hepatic global DNA hypomethylation phenotype in trout marked by a significant lower 5-mdC level in the LP-HC diet-feeding group compared to the HP-LC diet-feeding group. Compared to the fasted fish, the decrease in hepatic 5-mdC level in trout fed the HP-LC and the LP-HC diets was due to the accumulation of 5-fdC + 5-cadC and 5-cadC alone, respectively. Fish fed with the LP-HC diet displayed a higher level of 5-cadC compared to those fed with the HP-LC diet whereas the 5-fdC profile was opposite. Firstly, these results further proved that the demethylation process took place through an active manner. Secondly, accumulating evidence has shown that the oxidised 5-mdC intermediates could also have some important regulatory functions (Kellinger et al., 2012; Song and He, 2013; Sun et al., 2014). Of interest, previous studies showed that the 5-cadC content could undergo dynamic changes during hepatic differentiation, and activate the expression of several genes involved in hepatic specification through

accumulation at their promoter regions (Lewis et al., 2017). Thus, these first results demonstrated that modifying the protein/carbohydrate content in the diet of trout and by consequence the glycaemia of fish have an impact on the hepatic DNA methylome and highlighted the putative importance of 5-cadC in dietary challenges induced hepatic global hypomethylation in trout. However, this feeding trial 1 did not allow discriminate the factor(s) responsible for the hypomethylated phenotype among the increase of dietary digestible carbohydrate content in the diet, the decrease of dietary protein content and/or the induced hyperglycaemia in LP-HC fed fish.

Trying to segregate the effect of the above factors (dietary high carbohydrates, dietary low proteins, hyperglycaemia) on the methylome, we designed a new experiment composed of a glucose injection trial and completed by an independent feeding trial (feeding trial 2) which utilised 3 dietary treatments (the CTR, HC and LP diets).

On one hand, the results of the glucose injection trial showed a significant decrease in global C^mCGG methylation level in the liver of trout at 24 h post glucose injection compared to those received a saline injection. Because there was a significantly higher glycaemia level found at T6 in trout previously injected with glucose compared to those injected with saline, it could be hypothesised that the hyperglycaemic history could induce global loss of CpG methylation in the liver with a time-lag. Besides, the glycaemia in glucose-injected fish at T6 (20 mmol/L) was actually very high when compared with the moderate hyperglycaemia level found in fish fed with the LP-HC diet in feeding trial 1 (10 mmol/L) and fish fed the HC diet in feeding trial 2 (9 mmol/L). These could lead to the hypothesis that the trigger of global CpG demethylation requires dramatic hyperglycaemic stimulus. Nonetheless, there was in general no significant difference in 5-mdC and other oxidised intermediates in trout liver at 6 h and 24 h post glucose/saline injection. There are two possible reasons to explain the lack of impact by injection treatment. First, when looking at the glycaemia level in trout received saline injection at T6, it is obvious that although this value was significantly lower compared to those injected with glucose, it was still higher compared to non-injected group. This increasing in glycaemia could be due to the handling stress of injection. Therefore, the stressful condition could counteract the effects between glucose and saline injection, which further influence the global

DNA methylation level (Blaze et al., 2018; Secco et al., 2015). Second, we set the sampling time as 6 h and 24 h after injection, which maybe too late to observe the changes in 5-mdC and other oxidised intermediates at these time points. Indeed, a previous study did in mice reported that the genome-wide 5-hmdC levels were rapidly increased in various mouse tissues within 30 min of glucose injection, and rapidly declined/recovered at 60 min post glucose injection (Yang et al., 2014). However and of importance in the context of the present study, this glucose injection trial as well as one previously described in mouse (see above) did not lead to a modification of the 5-cadC level, suggesting that hyperglycaemia has few even no influence on the methylome profile obtain in the feeding trial 1.

On the other hand, in feeding trial 2 we observed that 5-mdC levels in fish fed the HC and LP diets were significantly lower than the one monitored in the CTR group. Interestingly, and as it was shown in fish fed the LP-HC diet in the feeding trial 1, the decrease in 5-mdC level in HC and LP diets-feeding fish was positively correlated with an increase in 5-cadC level although it was more pronounced in fish fed the LP diet. Indeed, in trout fed the HC diet, the 5-cadC level was intermediary between Fasted/CTR diets-feeding groups and LP diet-feeding group.

Altogether, results from this second experiment including the glucose injection and the feeding trial 2 strongly suggested that the hypomethylation correlated to the accumulation of 5-cadC may be induced mostly by a decrease in protein content in the diet of trout. Interestingly, in HepG2 human hepatocyte cultured in a medium containing insulin but a low concentration in one of the essential amino acids, *i.e.* methionine, induced a decrease in 5-mdC compared with cells stimulated with insulin in a complete medium (Chiang et al., 2009). This result opens new trails for future studies to investigate the role of nutritional status-related hormones and more particularly insulin.

However, intermediary profiles obtained in fish fed the HC diet (feeding trial 2) may suggest that dietary carbohydrates, probably in interaction with dietary proteins, can also initiate a global hypomethylation of DNA. Again and interestingly, as for insulin, a stimulation of HepG2 human cells with glucose combined with a methionine deficiency led to a decrease in the level of 5-mdC compared to cells cultured in a complete medium containing glucose.

In addition, the cytosine modification profiles slightly differed between LP-HC (feeding trial 1) and HC (feeding trial 2) diets whereas they both contained 40% crude proteins and 30% digestible carbohydrates. Indeed, overall formula were somewhat different in feeding trial 2 (the addition of CPSP90 proteins, the addition of cellulose in CTR diet, *etc.*) in order to face the constraint of the *in vivo* experimental design (*i.e.* decreasing the protein or carbohydrate content without affecting the lipid content) which can explain differences observed in the methylation results. Finally but noteworthy, data obtained from the present study indicated that the estimation of CpG methylation given by the LUMA method seems not to be a good marker of the variation in the global methylation induced by nutrients.

Mechanisms of the global DNA demethylation

When looking at the detail mechanisms that may underline the global DNA demethylation observed in the present study, we monitored the mRNA levels of *dnmt*, *tet* and *tdg* genes, SAM and SAH contents as well as the contents of aminothiols.

Regarding results of the mRNA levels of *dnmt*, *tet* and *tdg* genes, we found barely significant difference between dietary treatments or between glucose/saline injection. Most of the effects were found between fasting and feeding or between pre-injection and post-injection. Perhaps the regulation of these key enzymes involved in DNA methylation/demethylation pathways was mainly occurred at enzymatic activity level other than at the mRNA level. However, results obtained from the feeding trial 2 intriguingly showed a similar changing pattern of the hepatic *dnmt3aa* mRNA level and the 5-mdC abundance (Fasted > CTR > HC and LP). This result was to some extent coherent with the previous studies in mammals which demonstrated that non-CpG methylation is tightly linked to the expression of DNMT3 (Fuso, 2018; Patil et al., 2014; Zhang et al., 2017), although no similar finding was observed in feeding trial 1.

Apart from the mRNA levels of DNA methylation/demethylation related genes, we also measured the SAM and SAH contents in the liver. It has been widely reported that DNA methylation process is closely linked to one-carbon metabolism because both of them require methyl as substrates (Clare et al., 2018; Masih et al., 2014). The reaction

of DNA methylation is mediated through the function of Dnmt, with S-adenosylmethionine (SAM) serves as the primary methyl donor and S-adenosylhomocysteine (SAH) as a by-product during methyltransferase reactions and also a strong inhibitor for Dnmt (Anderson et al., 2012). Indeed, in several mammalian models, the level of SAH seems to be more closely correlated with the global DNA methylation level compared to SAM (Mattocks et al., 2017). Also, in most cases researchers consider not only the SAM and SAH concentrations separately but also the ratio between them—SAM/SAH, the so called ‘methylation index’ (Waterland, 2006). A lower SAM/SAH ratio is considered as an indicator of reduced methylation potential (Caudill et al., 2001). Therefore, there is not a simple correlation between the methyl donor concentrations and the DNA methylation level. It is important to note that the perturbation in SAM: SAH ratio is not invariably reflected the methylation level of macromolecules such as DNA (Mason, 2003; Xia et al., 2015). Concerning results obtained from the present study, we found that SAM/SAH ratio was largely increased in trout fed the LP-HC diet (feeding trial 1) or the HC diet (feeding trial 2), which showed relatively lower global 5-mdC level. This modification of the SAM: SAH ratio is mainly due to an increase of SAM without any changes in SAH level. Similar results have been reported in a previous study did in another teleost species *Megalobrama amblycephala* (Xu et al., 2018) fed with a high content of dietary carbohydrates. Moreover, in HepG2 human cells, an addition of glucose in the medium of culture leads to an increase of SAM concentration by enhancing homocysteine remethylation (Chiang et al., 2009). Therefore, our data strongly suggest that the alterations in hepatic levels of SAM, SAH, and their ratio were not directly linked to the changes in global DNA methylation. We could also hypothesise that the accumulation of SAM in hypomethylated liver may also reflect its non-utilisation because of the decrease even arrest of DNA methylation process.

In addition, results obtained from the two feeding trials in the present study showed that compared to the feeding groups, the fasted groups had lower hepatic SAM content, although statistical significance was only observed in feeding trial 2. This finding is in accordance with the previous study in HepG2 human cells in which an addition of insulin in the complete medium of culture can lead to an accumulation of SAM concentration. Moreover previous studies in mammals demonstrated that

stressful conditions including fasting could lead to a reduction of SAM in liver (Bartosek et al., 1978; Schalinske and Steele, 1992). The significant decrease of hepatic SAM content in trout at 6 h post glucose injection might be also due to hyperglycaemia induced oxidative stress.

Besides, the contents of aminothiols, which reflects the clearance of homocysteine through the transsulfuration pathway to form GSH, are also important for DNA methylation (Cyr and Domann, 2011). GSH represents an important antioxidant and plays crucial roles in regulating the redox status. For instance, previous studies have demonstrated that oxidative stress can promote the homocysteine entering the transsulfuration pathway and thus leading to the depletion of SAM, which can further affect DNA methylation/demethylation process (Cyr and Domann, 2011; Kreuz and Fischle, 2016). Although results obtain in the present study showed significant alterations in hepatic contents of some of these metabolites, it is hard to correlate them to the observed changes in DNA methylation pattern. It could be hypothesised that the alterations in methyl donor metabolites are not the reasons for the hepatic global DNA demethylation in trout in the present study. Indeed, we may postulate that the dietary nutrients or glucose injection induced decreasing in hepatic global DNA methylation level could be achieved through the alterations in TCA (Kreb's Cycle) metabolites thereby affecting the activity of Tet enzymes. Tet enzymes belong to the family of Fe (II)- and 2-oxoglutarate-dependent dioxygenases. The dietary factors that can modulate 2-oxoglutarate and Fe (II) availabilities are supposed to have an impact on the function of TET. Besides, TET activities can be also inhibited by intermediates of the TCA cycle such as fumarate and succinate, which are downstream products of 2-oxoglutarate (Xiao et al., 2012).

Conclusions

To sum up, our results showed that the nutritional transition between fasted (4 days) and refed (4 days) fish induced a global DNA demethylation event which did not occur at CpG sites within C^mCGG sequences. A 4-day dietary low protein feeding can induce global DNA hypomethylation in the liver of trout. This dietary factor caused loss of 5-mdC content could occur through an active demethylation pathway, and lead to an accumulation of 5-cadC level. Closed profiles were found in the presence digestible

dietary carbohydrates, suggesting that dietary carbohydrates, probably in interaction with dietary proteins, can also trigger hepatic global DNA hypomethylation. Even if hyperglycaemia does not seem to have an impact on DNA methylation profiles observed in feeding trials, our results showed that extreme hyperglycaemia induced by glucose injection at 6 h after injection, is correlated with a hepatic global C^mCGG hypomethylation at 24 h after injection. The detail mechanisms of these demethylation processes remain to be elucidated.

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Competing interests

The authors declare that there are no conflicts of interests.

Authors contributions

L.M. and S.P. designed the study. L.M. and J. L. managed the study. J.L. performed the experiment, statistical analysis and wrote the manuscript. C. H. developed HPLC analysis and provided technical assistance. H. J. contributed to the technical assistance in LUMA assay. C. B. helped to design the diet formula. V.V. provided technical assistance in DNA extraction and LUMA assay. L.M. and S.P. contributed to the manuscript corrections.

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3.4 Part 3: Metabolic programming effects of hypoxia during early development and/or high carbohydrate dietary stimulus at first feeding on epigenetic landscapes and glucose metabolism in trout juveniles

The last part of the present thesis consists of two experiments, which aimed at investigating the metabolic programming effects of a **high carbohydrate dietary stimulus at the first-feeding** stage combined either with **i) an acute hypoxic stimulus** or **ii) a chronic hypoxic stimulus BEFORE** first-feeding stage, respectively (Fig. 3-4). Early stimuli were followed by a growth trial during 24 or 27 weeks in acute or chronic hypoxia experiment respectively, and fish were then all challenged with a diet containing 30% of digestible carbohydrates during 5 days. All the details will be described in the following chapters for each experiment (Publications n°3, n°4 and n°5).

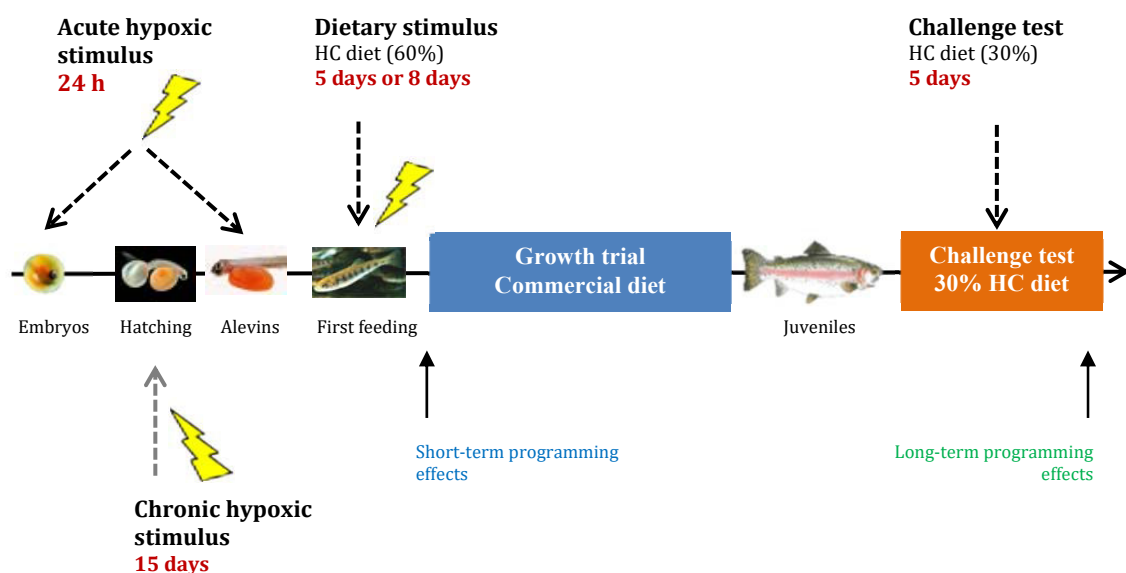


Figure 0-1 Global overview of the Part 3 of the thesis. Metabolic programming effects of an early hypoxic stimulus during embryonic development and a high carbohydrate dietary stimulus at the first-feeding stage on glucose metabolism and hepatic epigenetic landscapes in trout. HC, high carbohydrates.

3.4.1 Acute hypoxia & high-carbohydrates diets as stimuli

Preamble: overview of all the acute hypoxia experiments

In order to investigate the programming effects of 24 h acute hypoxic stimulus on glucose metabolism and the epigenetic landscape in trout, two hypoxic level [$2.5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ (about 25% dissolved oxygen) or $5.0 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ (about 50% dissolved oxygen)] were applied at two different “windows of plasticity (152 °D embryos, or 552 °D hatching alevins) in the present study (**Fig. 3-5**). (‘Windows of plasticity’ is defined as critical periods for a particular tissue or organ during development. During these periods, a suite of morphological and physiological modifications may occur in response to abnormal environmental stimuli (Burggren and Reyna, 2011)). Trout which were consistently kept in normoxic condition ($11.0 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$) served as a control. At the time of first-feeding stage (654 °D), alevins were fed 5 days with either a high carbohydrate diet (HC diet, 60% CHO, 20% glucose, 40% gelatinised starch) or a carbohydrate-free diet (NC diet, 0% CHO). Thus there were in total 10 experimental conditions (Condition 1-10) at this stage. Three samplings were done at the last hour of two hypoxic stimuli and 3 h after the last-meal after 5-day feeding. This dietary manipulation at first-feeding stage can be considered either as a challenge test for the short-term programming effects, or the nutritional stimulus for the long-term programming effects. Results obtained from the first three samplings were organised into one publication (**Publication 3**).

After the 3rd sampling, we choose to continue the programming experiment for a 24-week growth trial followed by a 5-day HC dietary challenge test (30% CHO) in trout juveniles **only** in **Condition 1, 2, 3, 4** [24 h acute hypoxic stimulus ($5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$) applied at 152 °D embryos and the normoxic control, **Fig. 3-5**]. The 4th sampling was done at 6 h after the last meal in challenge test, and the data were organised into another one publication (**Publication 4**) corresponding to the long-term programming effects.

By contrast, the other experimental conditions were stopped after the 3rd sampling (**Fig. 3-5**; see the STOP logo). The selection of conditions used to perform long-term programming effects was based on the results obtained at the first-feeding stage. We stopped all the groups that received a $5.0 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ hypoxic stimulus because the glucose metabolism-related genes were barely affected in first-feeding alevins which had been exposed to this moderate level of hypoxia. Moreover, we also stopped the groups that received $2.5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ at 552 °D (hatching alevins) because a significant increase of mortality was induced by this stimulus (to

avoid the potential selection effects). Nonetheless, regarding our results obtained at the 3rd sampling (Publication 3), it seems that 2.5 mg·L⁻¹ O₂ at 552 °D (hatching alevins) probably did not ‘select’ trout who were better metabolically adapted (at least concerning glucose metabolism) to hypoxia because the effects of hypoxia were similar to those in fish subjected to hypoxia at the embryo stage (with no differences in survival).

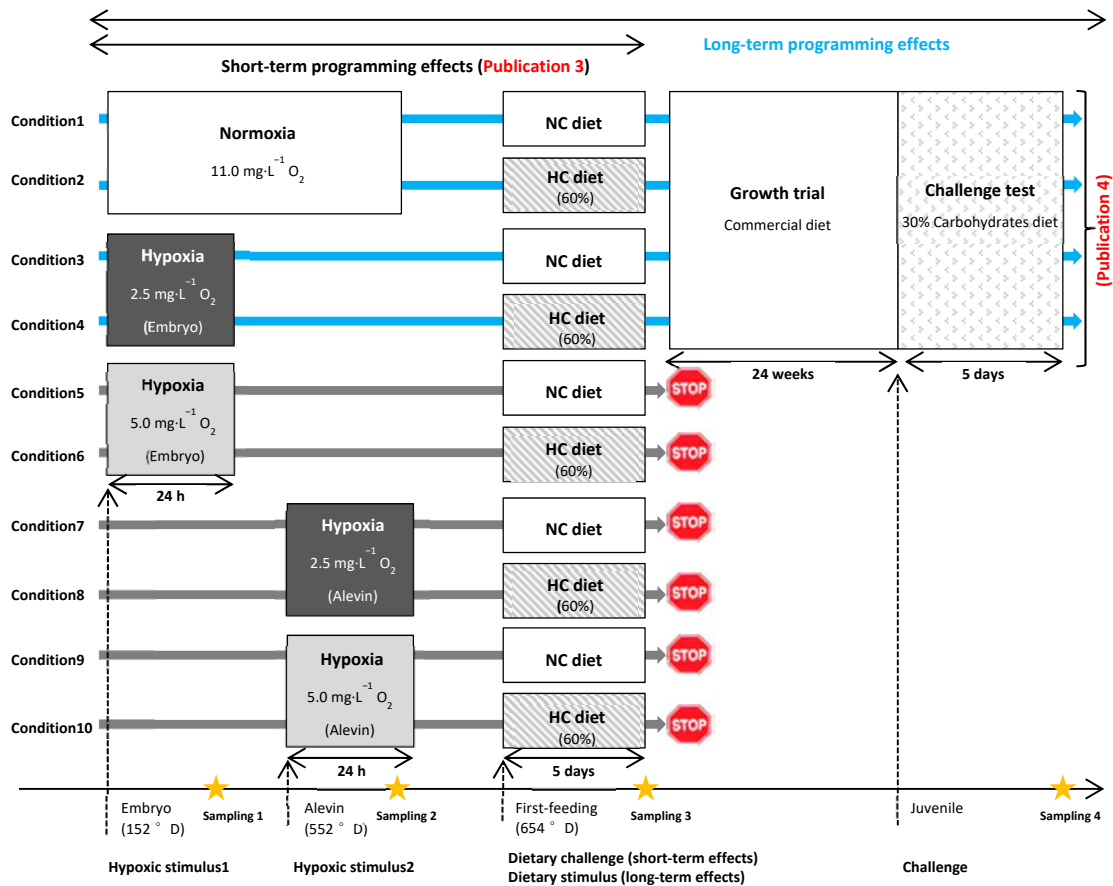


Figure 0-2 Whole experimental design of metabolic programming effects of acute hypoxic stimulus during early development and high carbohydrate dietary stimulus at first feeding on glucose metabolism and hepatic epigenetic landscape in trout. HC, high carbohydrate; NC, no carbohydrate.

3.4.1.1 Short-term effects: Exposure to an acute hypoxic stimulus during early life affects the expression of glucose metabolism-related genes at first-feeding in trout - Publication 3

Objectives

The main goal of the present study is to evaluate the short-term programming effects of an acute hypoxia exposure level [$2.5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ (about 25% dissolved oxygen) or $5.0 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ (about 50% dissolved oxygen)] during early development (embryonic stage or hatching stage) on glucose metabolism in rainbow trout at first-feeding.

Experimental design

The schematic overview of the experimental design was shown in Fig. 3-6. Details for experimental design have been described previously in section 3.4.2.

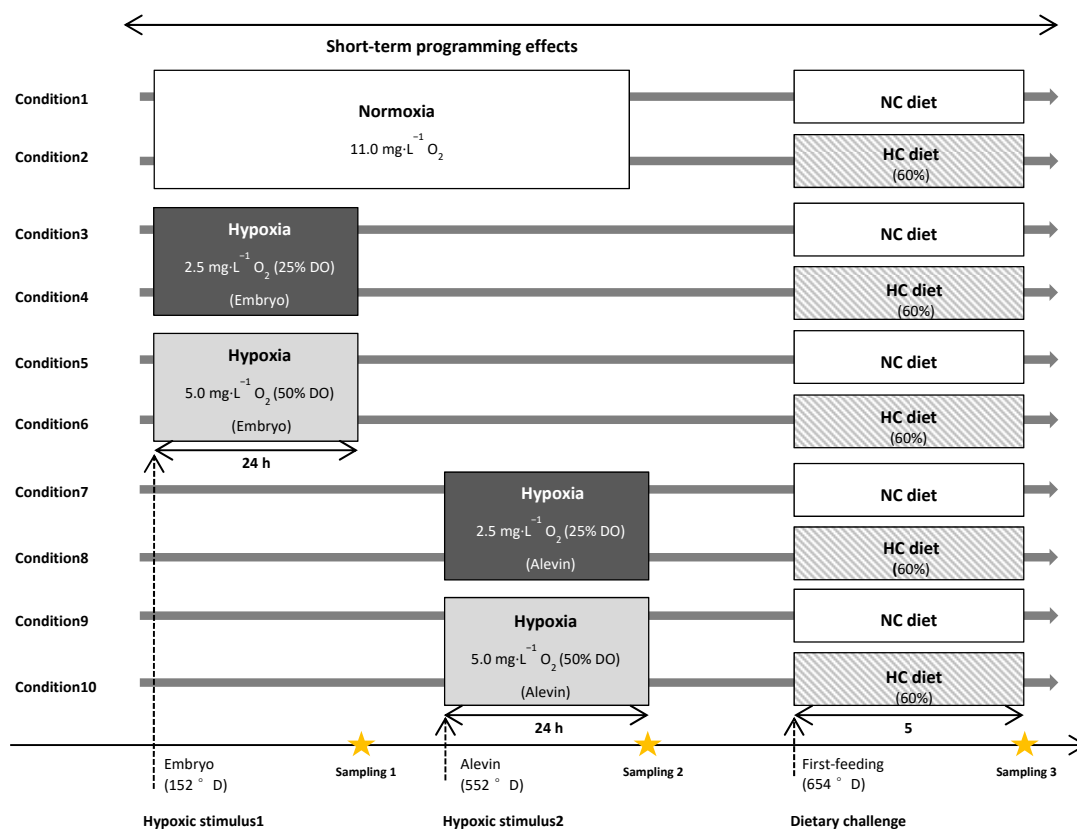


Figure 0-3 Experimental design of metabolic programming effects of acute hypoxic stimulus during early development on glucose metabolism in trout alevins at the first-feeding. DO, dissolved oxygen; HC, high carbohydrate; NC, no carbohydrate.

Highlights

1. $2.5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ (25% dissolved oxygen) hypoxic stimulus at embryonic or hatching stage induced the increase of the mRNA levels of genes involved in glycolysis and glucose transport pathway in trout alevins at first-feeding.
2. $5.0 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$ (25% dissolved oxygen) hypoxic stimulus at embryonic or hatching stage barely affect the glucose metabolism related genes in trout alevins at first-feeding.
3. Interactions were found for hypoxic history and dietary high carbohydrate feeding on the mRNA levels of *g6pcb2b* and *gckb* in trout alevins at first-feeding.


Conclusions

The present study for the first time confirmed that a non-nutritional stimulus, hypoxia, experienced during early development could induce short-term programming effect on glucose metabolism in trout alevins at the first-feeding. An acute hypoxic stimulus ($2.5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$, 25% dissolved oxygen) received either at embryo stage or at hatching alevin stage can profoundly upregulated the mRNA levels of glycolytic genes and glucose transporter genes in trout alevins at first-feeding.

Of Note: Due to the fact that, the body size of trout alevin is very small at the first-feeding stage, it is difficult to dissect specific organs such as liver. Therefore, all the analyses were done on whole-body, and hepatic DNA methylation level was not measured at this stage.

Publication 3

SCIENTIFIC REPORTS



OPEN

Exposure to an acute hypoxic stimulus during early life affects the expression of glucose metabolism-related genes at first-feeding in trout

Jingwei Liu, Elisabeth Plagnes-Juan, Inge Geurden, Stéphane Panserat & Lucie Marandel

Rainbow trout (*Oncorhynchus mykiss*) is considered a “glucose-intolerant” species. With the aim of programming trout to improve their metabolic use of dietary carbohydrates, we hypothesised that a hypoxic stimulus applied during embryogenesis could later affect glucose metabolism at the first-feeding stage. An acute hypoxic stimulus (2.5 or 5.0 mg·L⁻¹ O₂) was applied for 24 h to non-hatched embryos or early hatched alevins followed by a challenge test with a high carbohydrate diet at first-feeding. The effectiveness of the early hypoxic stimulus was confirmed by the induction of oxygen-sensitive markers such as *egln3*. At first-feeding, trout previously subjected to the 2.5 mg·L⁻¹ O₂ hypoxia displayed a strong induction of glycolytic and glucose transport genes, whereas these glucose metabolism-related genes were affected much less in trout subjected to the less severe (5.0 mg·L⁻¹ O₂) hypoxia. Our results demonstrate that an acute hypoxic stimulus during early development can affect glucose metabolism in trout at first-feeding.

Early life is a critical period of developmental plasticity. During this period, modulations of environmental parameters (e.g., nutrition and oxygen) can affect the phenotype of living organisms, physiologically modifying them in the medium- or long-term of their lifetime and even on a multigenerational scale^{1,2}. This concept is termed “programming”, more specifically, “metabolic programming” when examining the modulation of the metabolism induced by a stimulus received early in life. These “programming effects” on metabolism can be reflected as modified gene expression in critical metabolic pathways in later life^{3–6}. Indeed, the long-lasting modification in gene expression pattern is one of the most important biological mechanisms for the persistence of programming effects⁷. The positive aspect of moderate environmental impact (named a stimulus) on phenotypes is now considered to condition livestock to better cope with their future environment, bringing into play their adaptive plasticity. This research aspect has been emerging over a decade in aquaculture and particularly in rainbow trout (*Oncorhynchus mykiss*). The poor use of dietary carbohydrates in this carnivorous species has limited the development of economical plant-based diet formulas, and is a disadvantage for the sustainable development of the salmon farming industry^{8–10}.

In this context, programming seems an interesting approach and was explored to try to modify glucose metabolism in trout for the better utilisation of dietary carbohydrates^{11,12}. Recent studies demonstrated for instance that a 5-day high-carbohydrate dietary stimulus at first-feeding resulted in a long-term modification of glucose metabolism-related gene expressions in the muscle and of the intestinal microbiota profile in trout juveniles¹². However, no persistent programming effects were noted in the liver in juvenile fish, despite the liver’s pivotal role in glucose metabolism, which could be related to the experimental conditions used for programming. In addition to the importance of the type of the stimulus applied, another decisive point in the concept of programming is that the susceptibility of organisms may be limited to a critical period (a window of metabolic plasticity) during which an environmental stimulus may impact durably on metabolism¹³. In trout juveniles two of the five genes encoding the glucose-6-phosphatase (G6pc), the enzyme catalysing the last step of gluconeogenesis, were shown

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Stimulus stage	Hypoxia level	2 days after hypoxic stimulus	At hatching	Before first-feeding	
		Survival	Survival	Survival	Malformation
Embryos (152 °D)	2.5 mg·L ⁻¹ O ₂	99.1 ± 0.4%	96.3 ± 0.8%	76.3 ± 2.0%	5.6 ± 2.2%
	5.0 mg·L ⁻¹ O ₂	95.4 ± 0.5%	95.5 ± 1.1%	76.7 ± 2.7%	5.6 ± 2.0%
	Normoxia	99.2 ± 0.4%	94.9 ± 0.7%	75.0 ± 2.8%	4.5 ± 1.6%
Alevins (552 °D)	2.5 mg·L ⁻¹ O ₂	41.9 ± 7.2% ^a		41.4 ± 6.8% ^a	1.3 ± 1.3% ^a
	5.0 mg·L ⁻¹ O ₂	65.1 ± 7.2% ^b		64.6 ± 7.0% ^b	0.9 ± 0.6% ^a
	Normoxia	78.5 ± 3.8% ^c		75.0 ± 2.8% ^c	4.5 ± 1.6% ^b

Table 1. Survival of embryos and alevins after hypoxic stimulus, at hatching and before first-feeding. Data are represented by means (n = 6), data bearing the same superscripts within a column were not significantly different ($p > 0.05$, Pearson's Chi-squared test).

to be atypically up-regulated by dietary carbohydrates^{14,15}, and this phenotype was demonstrated to take place as soon as the first-feeding stages¹⁵. These results suggest that applying a stimulus at first-feeding, as Geurden *et al.* did¹², may be too late to programme glucose metabolism (or at least gluconeogenesis) in trout for the long-term. Moreover, these results established the premise that first-feeding can be considered the earliest period at which the preliminary outcome of metabolic programming can be assessed.

Here we hypothesised that applying a stimulus before first-feeding, *i.e.*, during embryogenesis, may be more efficient for programming glucose metabolism in rainbow trout. Fish embryos and larvae depend exclusively on their endogenous yolk reserves until the first-feeding. Therefore, a non-nutritional stimulus (*i.e.*, temperature or hypoxia) appears to be an ideal choice, because embryos of the oviparous trout are directly exposed to environmental variations. In the present study, hypoxia was chosen as a stimulus for several reasons. Oxygen is closely linked to glucose metabolism in aerobic organisms¹⁶. Hypoxia can trigger a shift in metabolism from mitochondrial oxidative phosphorylation to an anaerobic glycolytic pathway in mammals¹⁷. Similarly, hypoxia is known to modulate glucose metabolism in several fish species, including rainbow trout^{18–26}. Although no data relating to programming assays with hypoxia in trout are available, previous studies of mammals have found that intra-uterine hypoxia could inhibit gluconeogenesis and alter plasma glucose content in offspring^{6,27}, demonstrating that hypoxia has a long-lasting programming effect on glucose metabolism. Moreover, such a stimulus is easy to apply on a large scale to individuals compared to microinjections performed directly in the yolk as previously tested in zebrafish²⁸. We thus hypothesise that an early exposure to hypoxia in trout may modulate glucose metabolism and that such a modification could be recorded as a “physiological memory” and reveal itself later in life when fish are challenged with a high carbohydrate diet. The stimulus duration was set as a 24 h acute hypoxic stimulus as trout are considered as an oxygen-sensitive species²⁹, that are susceptible to chronic hypoxia with possible adverse consequences on growth performance^{30,31}.

Another important factor in a nutritional programming strategy is the time point at which the stimulus is applied. A previous study in trout conducted during ontogenesis revealed that mRNA levels of genes involved in glucose metabolism increased in embryos during the development of the primitive liver (approximately 160 °D, degree day)¹⁵. Hence, a stimulus applied just before this stage could be a promising time point to programme glucose metabolism. However, before hatching, the presence of the chorion protects the embryo from external environmental changes³² and may function as a barrier for oxygen diffusion^{31,33}. It is therefore uncertain whether an acute 24 h hypoxic stimulus applied at this stage can affect the oxygen availability inside the chorion. Hence, an additional stage was selected for applying the stimulus: between hatching and first-feeding (552 °D).

In summary, the present study proposes to investigate the short-term effects of an early acute hypoxic stimulus applied at two different developmental stages on glucose metabolism in trout by challenging with a high carbohydrate diet at the first-feeding. As transcriptional modification is the first step to mediate metabolic programming, we mainly focus on the programming effects at the transcriptional level, *i.e.*, alteration in mRNA levels of critical genes involved in glycolysis, gluconeogenesis and the glucose transport pathway.

Results

Survival of embryos and alevins. In the present study, the hypoxic stimulus applied at the embryo stage (152 °D) did not affect the survival and malformation monitored later in life (2 days after the hypoxic stimulus, at hatching and at first-feeding, Table 1). However, there was a decrease in the survival rates in fish subjected to the hypoxic stimulus at the alevin stage (552 °D) both after the hypoxic stimulus and before first-feeding. The mortality increased significantly with the elevation of the hypoxia level ($p < 0.05$). In addition, fish which were subjected to hypoxia at 552 °D displayed a significant decrease in the malformation ($p < 0.05$).

In silico analysis of hypoxia-sensitive and glucose metabolism-related genes. By analysing the rainbow trout genome³⁴, we identified for the first time several genes sharing high sequence homology with the hypoxia-sensitive and some of the glucose metabolism-related mammalian orthologues. One gene is related to *Pdk1* (pyruvate dehydrogenase kinase 1, which is responsible for the inactivation of the enzyme pyruvate dehydrogenase, leading to the blockade of pyruvate conversion into acetyl-coA). Two genes are related to each of the following mammalian genes: *Hif1α* (Hypoxia inducible factor 1 alpha, the master transcriptional factor involved

in hypoxic responses), *Ldha* (Lactate dehydrogenase A, catalyses the conversion of L-lactate and NAD to pyruvate and NADH in the final step of anaerobic glycolysis), *Slc16a3* (solute carrier family 16 (monocarboxylic acid transporters), member 3, which mediates lactic acid transport across plasma membranes), *Slc2a2* (solute carrier family 2 (facilitated glucose transporter), member 2, also called *Glut2*), *Slc2a4* (solute carrier family 2 (facilitated glucose transporter), member 4, also called *Glut4*) and *Egln3* (egl-9 family hypoxia-inducible factor 3, cellular oxygen sensor, which catalyses the degradation process of Hif1 α protein, but is also a downstream gene of Hif1). Finally, four genes each are related to *Slc2a1* (solute carrier family 2 (facilitated glucose transporter), member 1, also called *Glut1*), *Pfkm* (phosphofructokinase, muscle, which catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate in glycolysis) and *Pkm* (pyruvate kinase, muscle, which catalyses the transformation of phosphoenolpyruvate to pyruvate in glycolysis) genes. A syntenic analysis and when necessary a phylogenetic analysis, using full-length vertebrate protein sequences were then performed to clarify the identity of these rainbow trout sequences. These results are presented in the Supplementary File; final annotations of the different genes can be found in Supplementary Table S1. The *in silico* analyses were performed in previous studies^{14,15} for *Gck* (glucokinase, responsible for the phosphorylation glucose to glucose-6-phosphate), *Pfkl* (phosphofructokinase, liver, which catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate in glycolysis), *Pkl* (pyruvate kinase, liver, which catalyses the transform of phosphoenolpyruvate to pyruvate in glycolysis), *Pck* (phosphoenolpyruvate carboxykinase, cytosolic *Pck1* and mitochondrial *Pck2*, which catalyses the conversion of oxaloacetate to phosphoenolpyruvate), *Fbp* (fructose-1,6-bisphosphatase, which catalyses the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate), and *G6pc* (glucose-6-phosphatase, which catalyses the hydrolysis of D-glucose 6-phosphate to D-glucose).

Expression analysis of targeted hypoxia-sensitive genes at the end of the acute hypoxic stimulus in both embryos and alevins.

To ensure the effectiveness of hypoxia, qPCR was performed to determine the mRNA levels of several hypoxia-related genes in the whole body of embryos and alevins at the end of the 24 h hypoxic stimulus. Compared with the normoxia group, trout that were exposed to the 24 h hypoxic stimulus displayed significantly higher mRNA levels of *egln3* orthologous genes ($p < 0.001$), in both stages at which the stimulus was applied and both levels of hypoxia (Fig. 1a). Similar results were found for one *glut1* gene, *glut1ba*, which displayed an increase in its mRNA level in trout subjected to hypoxia at the embryo stage at both hypoxic levels (2.5 mg·L⁻¹ O₂ or 5.0 mg·L⁻¹ O₂, $p < 0.001$). Additionally, trout exposed to a 5.0 mg·L⁻¹ O₂ hypoxic stimulus at the alevin stage displayed a higher mRNA level of *glut1ba* compared with control fish (Fig. 1b, $p < 0.05$). In addition, at the embryo stage, the mRNA level of *slc16a3a* was significantly higher in trout exposed to the 2.5 mg·L⁻¹ O₂ hypoxic stimulus compared with those in the normoxic condition (Fig. 1c, $p < 0.05$). No significant differences were found in the mRNA levels of *hif1 α* , *glut2* and *glut4* orthologues, *pdk1*, *ldha* duplicated genes and *slc16a3b* gene among treatments.

Expression analysis of targeted hypoxia-sensitive *egln3* orthologs at first-feeding.

The mRNA levels of *egln3a* and *egln3b* were measured in trout at first-feeding (Table 2). There was no significant difference in *egln3b* mRNA levels in trout regardless of the treatment. In trout subjected to 5.0 mg·L⁻¹ O₂ hypoxia, the *egln3a* mRNA level remained stable irrespective of the diet they received. However, in trout exposed to the 2.5 mg·L⁻¹ O₂ hypoxic stimulus at both stages, the *egln3a* mRNA level was significantly higher in trout fed the HC (high carbohydrate) diet than those fed the NC (no carbohydrate) diet at first feeding ($p < 0.05$). Additionally, trout subjected to 2.5 mg·L⁻¹ O₂ hypoxic stimulus at the alevin stage exhibited significantly higher *egln3a* mRNA level than normoxic groups (Table 2, $p < 0.05$).

Expression analysis of glucose metabolism-related genes at first-feeding.

Genes involved in glycolysis pathway. At the time of first-feeding, mRNA levels of the glycolytic genes *gcka*, *gckb* and *pfkmba* were increased in trout fed the HC diet (Table 3, $p < 0.05$), regardless of the stage (embryo vs alevin) or the level of hypoxic stimulus. Compared with the normoxic group, the mRNA levels of most of the glycolytic genes (*pfkla*, *pfklb*, *pfkmaa*, *pfkmbb*, *pkmaa*, *pkmb*, *pkmba* and *pkmbb*) were significantly increased in trout subjected to a high level of hypoxia (2.5 mg·L⁻¹ O₂) at the embryo stage or the alevin stage ($p < 0.05$). However, the mRNA levels of *gckb* and *pfkmb* were only increased in trout subjected to the 2.5 mg·L⁻¹ O₂ hypoxic stimulus at the alevin stage ($p < 0.05$), but not at the embryo stage. By contrast, the low level of hypoxia (5.0 mg·L⁻¹ O₂) applied during the early stimulus failed to affect the mRNA levels of glycolytic genes. Interestingly, an interaction between the diet received at first-feeding (*i.e.*, HC or NC diet) and the oxygen level during the early stimulus (*i.e.*, normoxia vs hypoxia) was observed for *gckb* ($p = 0.04$) in trout subjected to the 2.5 mg·L⁻¹ O₂ hypoxic stimulus at the alevin stage (Table 3).

Genes involved in gluconeogenesis pathway. When fed the HC diet, trout exhibited a significant decrease in the mRNA levels of *pck1* and *fbp1b1*, and a significant increase in the mRNA level of *g6pcb2a* (Table 4, $p \leq 0.01$). For the effect of hypoxia, trout subjected to the 2.5 mg·L⁻¹ O₂ hypoxic stimulus at either the embryo stage or the alevin stage displayed higher mRNA levels of *fbp1a* ($p < 0.05$) and *g6pcb2b* ($p \leq 0.01$) than the normoxic group (Table 4). By contrast, the mRNA level of *g6pcb1a* was decreased in trout subjected to the 5.0 mg·L⁻¹ O₂ hypoxic stimulus at the embryo stage (Table 4, $p = 0.05$). Our results also highlighted a significant interaction between the diet received at first-feeding (*i.e.*, HC or NC diet) and the level of oxygen during the early stimulus (*i.e.*, normoxia vs hypoxia) for *g6pcb2b* in trout exposed to the 2.5 mg·L⁻¹ O₂ hypoxic stimulus at the embryo stage (Table 4, $p \leq 0.01$).

Genes involved in the glucose transport pathway. At first-feeding, the diet had no significant effect on the mRNA levels of glucose transport genes, with the exception of a significant increase in the *glut1ba* mRNA level ($p \leq 0.01$)

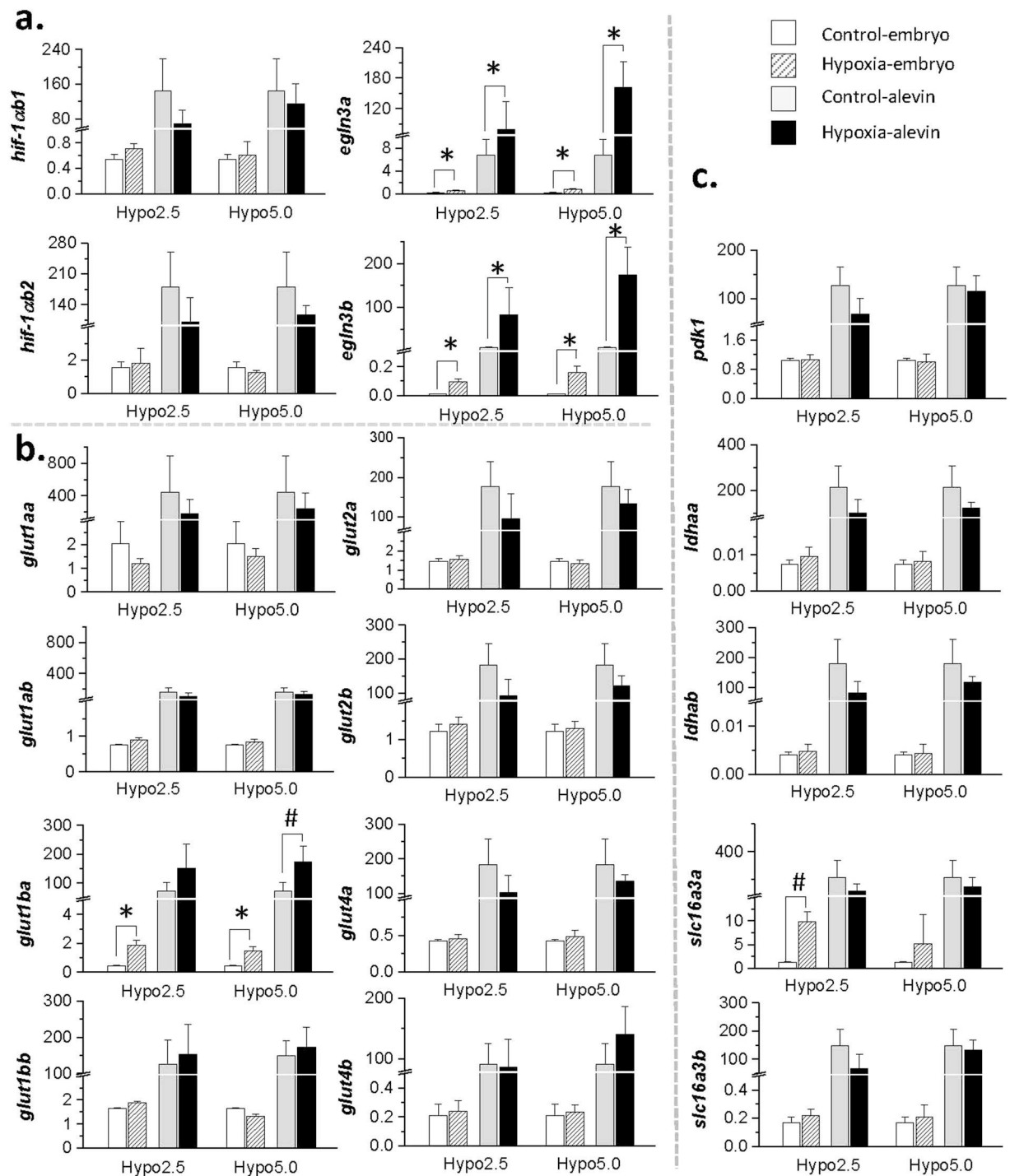


Figure 1. Molecular confirmation of the effect of the hypoxic stimulus on targeted oxygen-sensitive genes in non-hatched embryos and early hatched alevins. The relative abundance of mRNA of (a) hypoxia-responsive genes, (b) glucose transport genes and (c) pyruvate conversion and lactate transport genes in embryos (kept under normoxia, white bars; exposed to hypoxia, slash bars) and in alevins (kept under normoxia, grey bars; exposed to hypoxia, black bars). Data are presented as the mean \pm SD ($n = 6$), significant differences were evaluated by nonparametric Kruskal-Wallis test followed by a Tukey's test (* $p < 0.05$; * $p < 0.001$).

in trout subjected to the $2.5 \text{ mg-L}^{-1} \text{ O}_2$ hypoxic stimulus at the alevin stage (Table 5). However, compared with the normoxic group, the mRNA levels of *glut1aa*, *glu1ba* and *glut1bb* were significantly increased in trout subjected to the $2.5 \text{ mg-L}^{-1} \text{ O}_2$ hypoxic stimulus, regardless of the stage at which hypoxia was applied ($p \leq 0.01$). Meanwhile, the mRNA level of *glut1ab* was significantly higher in trout subjected to $2.5 \text{ mg-L}^{-1} \text{ O}_2$ hypoxia at the alevin stage but not at the embryo stage (Table 5, ($p < 0.05$)). By contrast, the *glut4a* mRNA level was increased in first-feeding trout subjected to this hypoxic stimulus at the embryo stage ($p < 0.05$) but not at the alevin stage (Table 5). No

Target gene	Hypoxia stage	Hypoxia level: 2.5 mg·L ⁻¹							Target gene	Hypoxia stage	Hypoxia level: 5.0 mg·L ⁻¹						
		N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA					N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA		
		Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O			Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O
<i>egln3a</i>	Embryo	1.96 ± 1.66	3.89 ± 4.17	2.89 ± 1.67	6.90 ± 1.19	0.01	0.07	0.32	<i>egln3a</i>	Embryo	2.87 ± 2.07	4.20 ± 4.10	1.80 ± 1.97	3.30 ± 3.08	0.25	0.42	0.94
	Alevin			3.82 ± 1.42	8.05 ± 3.07	0.01	0.02	0.33		Alevin			2.67 ± 2.53	4.74 ± 4.87	0.26	0.91	0.80
<i>egln3b</i>	Embryo	2.79 ± 2.90	3.51 ± 3.57	3.64 ± 1.72	5.02 ± 1.22	0.32	0.27	0.75	<i>egln3b</i>	Embryo	4.07 ± 3.44	3.74 ± 3.44	2.76 ± 2.29	3.19 ± 2.46	0.97	0.45	0.76
	Alevin			3.84 ± 0.60	5.52 ± 1.90	0.26	0.15	0.64		Alevin			3.22 ± 2.42	4.18 ± 3.83	0.82	0.88	0.64

Tables 2. Effects of early hypoxia and high-carbohydrate diet at first-feeding on mRNA level of *egln3a* and *egln3b*. N-NC, N-HC, H-NC and H-HC stands for fish subjected to normoxia and no carbohydrate diet, normoxia and high carbohydrate diet, hypoxia and no carbohydrate diet and hypoxia and high carbohydrate diet, respectively. Data were presented as mean ± SD (n = 6), statistical differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold).

Target gene	Hypoxia stage	Hypoxia level: 2.5 mg·L ⁻¹							Target gene	Hypoxia stage	Hypoxia level: 5.0 mg·L ⁻¹						
		N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA					N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA		
		Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O			Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O
<i>gcka</i>	Embryo	0.02 ± 0.01	4.19 ± 2.88	0.03 ± 0.01	4.40 ± 2.50	0.00	0.88	0.90	<i>gcka</i>	Embryo	0.02 ± 0.02	5.97 ± 4.97	0.04 ± 0.03	2.47 ± 1.55	0.00	0.12	0.11
	Alevin			0.02 ± 0.00	5.60 ± 2.05	0.00	0.34	0.34		Alevin	0.02 ± 0.02	5.37 ± 4.82	0.00	0.84	0.83		
<i>gckb</i>	Embryo	0.03 ± 0.02	5.14 ± 4.01	0.17 ± 0.15	7.77 ± 5.04	0.00	0.31	0.36	<i>gckb</i>	Embryo	0.10 ± 0.08	5.83 ± 4.71	0.10 ± 0.08	3.77 ± 2.72	0.00	0.37	0.37
	Alevin			0.07 ± 0.01	11.03 ± 5.19	0.00	0.04	0.04		Alevin			0.09 ± 0.07	5.09 ± 4.60	0.00	0.78	0.79
<i>pfkla</i>	Embryo	2.54 ± 1.69	3.33 ± 2.69	5.49 ± 3.03	4.52 ± 1.46	0.92	0.04	0.36	<i>pfkla</i>	Embryo	3.41 ± 2.41	3.69 ± 2.98	2.61 ± 1.71	2.32 ± 1.59	0.99	0.25	0.76
	Alevin			5.11 ± 0.97	5.01 ± 1.31	0.65	0.01	0.55		Alevin			3.16 ± 2.24	3.16 ± 2.69	0.90	0.72	0.90
<i>pfklb</i>	Embryo	2.95 ± 1.84	4.15 ± 3.14	6.22 ± 2.65	5.35 ± 0.66	0.86	0.03	0.28	<i>pfklb</i>	Embryo	3.17 ± 2.18	3.63 ± 2.77	3.16 ± 2.56	2.27 ± 1.60	0.82	0.48	0.48
	Alevin			5.25 ± 0.47	5.43 ± 0.59	0.38	0.03	0.50		Alevin			3.08 ± 2.12	3.07 ± 2.43	0.82	0.74	0.81
<i>pfkmaa</i>	Embryo	1.53 ± 1.14	3.12 ± 2.26	4.01 ± 1.95	4.84 ± 1.11	0.10	0.01	0.59	<i>pfkmaa</i>	Embryo	2.13 ± 1.43	3.39 ± 2.47	1.83 ± 1.25	2.97 ± 2.25	0.14	0.65	0.94
	Alevin			3.91 ± 0.72	4.70 ± 0.97	0.05	0.00	0.50		Alevin			2.25 ± 1.53	2.85 ± 2.31	0.27	0.80	0.69
<i>pfkmab</i>	Embryo	1.95 ± 1.56	3.16 ± 2.40	4.06 ± 2.26	4.05 ± 1.18	0.45	0.07	0.44	<i>pfkmab</i>	Embryo	2.67 ± 2.04	3.31 ± 2.33	2.07 ± 1.40	2.43 ± 1.52	0.52	0.34	0.86
	Alevin			3.99 ± 0.76	4.51 ± 0.92	0.19	0.01	0.59		Alevin			2.35 ± 1.48	3.01 ± 2.34	0.45	0.72	0.99
<i>pfkmba</i>	Embryo	1.33 ± 0.75	6.77 ± 5.33	4.03 ± 0.69	7.16 ± 5.87	0.02	0.35	0.49	<i>pfkmba</i>	Embryo	2.65 ± 1.87	7.94 ± 6.06	2.34 ± 1.74	4.86 ± 3.83	0.02	0.29	0.38
	Alevin			3.51 ± 1.06	6.82 ± 5.74	0.01	0.50	0.52		Alevin			3.38 ± 2.04	6.68 ± 5.92	0.03	0.88	0.59
<i>pfkmbb</i>	Embryo	2.40 ± 1.75	3.63 ± 2.69	5.53 ± 2.17	4.44 ± 0.95	0.93	0.03	0.17	<i>pfkmbb</i>	Embryo	4.34 ± 3.24	4.59 ± 3.52	3.20 ± 2.28	4.38 ± 3.05	0.58	0.59	0.71
	Alevin			4.84 ± 0.84	5.73 ± 1.51	0.17	0.01	0.82		Alevin			3.63 ± 2.45	4.46 ± 3.47	0.68	0.75	0.83
<i>pkmaa</i>	Embryo	2.30 ± 1.68	2.93 ± 2.13	5.66 ± 1.87	3.89 ± 1.02	0.43	0.01	0.10	<i>pkmaa</i>	Embryo	4.26 ± 3.00	3.93 ± 2.94	3.03 ± 2.15	3.29 ± 2.71	0.97	0.41	0.79
	Alevin			4.94 ± 0.55	4.96 ± 1.30	0.61	0.00	0.63		Alevin			3.49 ± 2.55	3.55 ± 2.94	0.91	0.63	0.87
<i>pkmab</i>	Embryo	3.48 ± 2.52	4.13 ± 3.30	7.48 ± 3.23	5.25 ± 1.21	0.48	0.03	0.21	<i>pkmab</i>	Embryo	4.74 ± 3.66	4.15 ± 3.22	3.77 ± 2.86	2.58 ± 1.62	0.47	0.30	0.81
	Alevin			6.96 ± 0.91	5.36 ± 0.90	0.60	0.02	0.22		Alevin			3.89 ± 2.59	3.35 ± 2.74	0.66	0.52	0.98
<i>pkmba</i>	Embryo	2.33 ± 1.78	3.19 ± 2.47	5.66 ± 1.85	4.39 ± 1.29	0.79	0.01	0.18	<i>pkmba</i>	Embryo	2.89 ± 2.07	3.28 ± 2.54	2.67 ± 1.98	2.3 ± 1.69	0.99	0.49	0.66
	Alevin			4.98 ± 0.45	4.24 ± 0.75	0.93	0.01	0.23		Alevin			2.75 ± 1.67	2.9 ± 2.25	0.77	0.77	0.89
<i>pkmbb</i>	Embryo	2.53 ± 1.71	3.66 ± 2.81	5.67 ± 2.45	4.99 ± 1.26	0.80	0.02	0.31	<i>pkmbb</i>	Embryo	3.45 ± 2.48	3.74 ± 2.87	3.07 ± 2.23	2.83 ± 2.01	0.98	0.52	0.79
	Alevin			5.58 ± 0.56	4.49 ± 0.79	0.98	0.01	0.13		Alevin			2.96 ± 1.69	3.44 ± 2.69	0.71	0.70	0.93
<i>pklr</i>	Embryo	3.78 ± 2.80	4.05 ± 2.93	7.61 ± 4.47	4.25 ± 1.11	0.23	0.12	0.16	<i>pklr</i>	Embryo	3.92 ± 2.84	3.67 ± 2.63	4.05 ± 3.38	2.69 ± 2.07	0.48	0.71	0.63
	Alevin			5.67 ± 1.14	4.74 ± 1.11	0.72	0.16	0.51		Alevin			3.72 ± 2.51	3.18 ± 2.68	0.72	0.76	0.90

Table 3. Effects of early hypoxia and high-carbohydrate diet at first-feeding on gene expressions involved in glycolysis pathway. N-NC, N-HC, H-NC and H-HC stands for fish subjected to normoxia and no carbohydrate diet, normoxia and high carbohydrate diet, hypoxia and no carbohydrate diet and hypoxia and high carbohydrate diet, respectively. Data were presented as mean ± SD (n = 6), statistical differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold).

effect of the 5.0 mg·L⁻¹ O₂ hypoxic stimulus or of an interaction between the diet received at first-feeding (*i.e.*, HC or NC) and the level of oxygen during the early stimulus (*i.e.*, normoxia vs hypoxia) was found on the mRNA levels of genes involved in glucose transport (Table 5).

Discussion

Rainbow trout belongs to a high trophic level, defining it as a carnivorous fish that is metabolically adapted for the high catabolism of proteins and the low use of dietary carbohydrates^{35,36}. The latter physiological feature is considered a constraint in the search for alternative feedstuffs to minimise the dependence on fish meal by increasing the carbohydrate content in the diet of trout. In this context, early nutritional programming has emerged as a new nutritional strategy to improve the use of dietary carbohydrates in carnivorous fish while avoiding the need to set up large-scale genetic breeding programmes or to genetically modify the animals^{11,12}. The exploration of this strategy is only in its beginnings in trout^{11,12} and requires deeper investigations to be optimised. The present study

Target gene	Hypoxia stage	Hypoxia level: 2.5 mg·L ⁻¹							Target gene	Hypoxia stage	Hypoxia level: 5.0 mg·L ⁻¹						
		N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA					N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA		
		Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O			Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O
<i>pck1</i>	Embryo	4.38 ± 2.63	1.46 ± 1.65	7.24 ± 6.04	1.25 ± 0.68	0.00	0.35	0.28	<i>pck1</i>	Embryo	5.54 ± 3.32	1.54 ± 1.87	3.60 ± 1.80	0.43 ± 0.23	0.00	0.09	0.64
	Alevin			6.73 ± 3.32	1.33 ± 0.79	0.00	0.25	0.20		Alevin			5.22 ± 5.05	1.03 ± 0.85	0.01	0.76	0.94
<i>pck2</i>	Embryo	2.13 ± 1.80	1.82 ± 1.29	3.73 ± 1.45	2.55 ± 0.79	0.20	0.05	0.45	<i>pck2</i>	Embryo	2.72 ± 1.60	2.56 ± 2.04	2.66 ± 2.76	2.52 ± 2.01	0.86	0.96	0.99
	Alevin			2.62 ± 0.40	2.62 ± 1.27	0.77	0.24	0.77		Alevin			3.82 ± 2.43	2.59 ± 2.38	0.43	0.52	0.55
<i>fbp1a</i>	Embryo	3.23 ± 2.48	3.04 ± 2.23	6.31 ± 2.59	4.17 ± 0.51	0.19	0.02	0.27	<i>fbp1a</i>	Embryo	4.07 ± 2.90	3.55 ± 2.59	3.53 ± 2.75	1.98 ± 1.15	0.31	0.30	0.61
	Alevin			5.83 ± 1.14	3.83 ± 0.57	0.15	0.03	0.23		Alevin			4.08 ± 2.82	2.91 ± 2.31	0.45	0.78	0.77
<i>fbp1b1</i>	Embryo	4.56 ± 3.30	2.06 ± 1.24	9.27 ± 5.58	3.14 ± 0.90	0.01	0.07	0.25	<i>fbp1b1</i>	Embryo	5.77 ± 4.37	2.11 ± 1.35	4.78 ± 3.82	1.94 ± 1.65	0.02	0.65	0.75
	Alevin			6.04 ± 1.64	3.31 ± 1.05	0.00	0.11	0.89		Alevin			4.73 ± 3.10	2.20 ± 1.96	0.02	0.70	0.64
<i>fbp1b2</i>	Embryo	2.96 ± 2.38	2.43 ± 1.71	4.93 ± 3.17	2.79 ± 0.51	0.15	0.20	0.38	<i>fbp1b2</i>	Embryo	3.83 ± 2.95	3.07 ± 2.19	3.48 ± 3.11	2.28 ± 1.53	0.35	0.59	0.83
	Alevin			4.25 ± 1.03	3.14 ± 0.61	0.22	0.14	0.66		Alevin			3.42 ± 2.27	2.51 ± 1.88	0.40	0.62	0.94
<i>g6pca</i>	Embryo	3.55 ± 3.00	3.05 ± 2.25	7.95 ± 5.58	4.02 ± 1.80	0.13	0.07	0.24	<i>g6pca</i>	Embryo	4.05 ± 3.02	2.81 ± 1.95	4.19 ± 3.17	2.20 ± 1.44	0.13	0.82	0.72
	Alevin			2.39 ± 1.78	4.54 ± 6.05	0.31	0.05	0.61		Alevin			3.77 ± 2.55	2.73 ± 2.43	0.28	0.87	0.92
<i>g6pcb1a</i>	Embryo	6.4 ± 6.81	8.61 ± 9.41	6.9 ± 2.26	6.38 ± 5.06	0.75	0.75	0.61	<i>g6pcb1a</i>	Embryo	6.60 ± 7.50	6.93 ± 7.21	2.77 ± 2.13	1.56 ± 1.38	0.84	0.05	0.73
	Alevin			8.96 ± 6.80	4.68 ± 2.91	0.72	0.81	0.26		Alevin			4.58 ± 3.69	5.58 ± 7.47	0.81	0.54	0.90
<i>g6pcb1b</i>	Embryo	2.98 ± 2.10	2.94 ± 1.87	6.24 ± 4.39	3.77 ± 0.86	0.26	0.07	0.27	<i>g6pcb1b</i>	Embryo	3.24 ± 2.22	2.77 ± 1.70	2.68 ± 2.18	2.42 ± 1.77	0.66	0.58	0.90
	Alevin			4.41 ± 1.00	3.94 ± 1.54	0.72	0.09	0.76		Alevin			2.96 ± 1.72	3.00 ± 2.68	0.81	0.97	0.77
<i>g6pcb2a</i>	Embryo	0.71 ± 0.90	3.23 ± 1.95	1.75 ± 1.15	3.32 ± 2.23	0.01	0.41	0.49	<i>g6pcb2a</i>	Embryo	0.54 ± 0.28	3.54 ± 2.08	1.10 ± 1.30	4.54 ± 5.72	0.02	0.55	0.86
	Alevin			1.22 ± 0.36	3.43 ± 2.32	0.00	0.59	0.81		Alevin			0.91 ± 0.71	4.99 ± 6.45	0.02	0.52	0.70
<i>g6pcb2b</i>	Embryo	1.75 ± 0.95	3.2 ± 2.20	5.30 ± 1.67	3.70 ± 1.38	0.91	0.01	0.03	<i>g6pcb2b</i>	Embryo	2.92 ± 1.94	3.97 ± 2.75	3.13 ± 2.47	3.02 ± 2.29	0.64	0.71	0.56
	Alevin			4.27 ± 0.45	4.52 ± 1.41	0.16	0.00	0.31		Alevin			2.92 ± 1.86	3.54 ± 2.77	0.40	0.83	0.83

Table 4. Effects of early hypoxia and high-carbohydrate diet at first-feeding on gene expressions involved in gluconeogenesis pathway. N-NC, N-HC, H-NC and H-HC stands for fish subjected to normoxia and no carbohydrate diet, normoxia and high carbohydrate diet, hypoxia and no carbohydrate diet and hypoxia and high carbohydrate diet, respectively. Data were presented as mean ± SD (n = 6), statistical differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold).

Target gene	Hypoxia stage	Hypoxia level: 2.5 mg·L ⁻¹							Target gene	Hypoxia stage	Hypoxia level: 5.0 mg·L ⁻¹						
		N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA					N-NC	N-HC	H-NC	H-HC	p of Two-way ANOVA		
		Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O			Means ± SD	Means ± SD	Means ± SD	Means ± SD	Diet	Oxygen	D*O
<i>glut1aa</i>	Embryo	2.12 ± 2.04	0.45 ± 0.84	5.54 ± 5.89	10.54 ± 6.80	0.39	0.00	0.09	<i>glut1aa</i>	Embryo	3.10 ± 4.26	0.36 ± 0.77	3.94 ± 4.59	4.31 ± 7.53	0.56	0.25	0.45
	Alevin			9.55 ± 5.03	6.86 ± 3.35	0.11	0.00	0.70		Alevin			4.88 ± 6.11	1.82 ± 3.46	0.10	0.35	0.93
<i>glut1ab</i>	Embryo	2.60 ± 2.08	3.54 ± 3.02	5.65 ± 2.37	4.37 ± 1.35	0.86	0.05	0.25	<i>glut1ab</i>	Embryo	3.97 ± 2.98	3.89 ± 3.18	2.99 ± 2.04	3.07 ± 2.57	1.00	0.43	0.94
	Alevin			5.21 ± 0.69	4.63 ± 0.96	0.82	0.03	0.34		Alevin			3.87 ± 2.86	2.79 ± 2.18	0.62	0.61	0.67
<i>glut1ba</i>	Embryo	2.38 ± 1.57	4.64 ± 3.57	6.24 ± 2.20	7.36 ± 1.70	0.10	0.00	0.57	<i>glut1ba</i>	Embryo	3.66 ± 2.78	4.99 ± 3.75	3.09 ± 2.35	3.46 ± 2.34	0.47	0.38	0.68
	Alevin			5.26 ± 0.78	7.70 ± 1.41	0.01	0.00	0.92		Alevin			3.29 ± 2.23	3.93 ± 2.87	0.42	0.56	0.78
<i>glut1bb</i>	Embryo	2.45 ± 1.56	4.03 ± 2.92	5.43 ± 2.13	5.53 ± 0.86	0.32	0.01	0.38	<i>glut1bb</i>	Embryo	3.55 ± 2.47	4.52 ± 3.17	3.27 ± 2.41	3.23 ± 2.32	0.67	0.47	0.64
	Alevin			5.15 ± 1.08	5.44 ± 0.70	0.21	0.01	0.38		Alevin			3.35 ± 2.32	3.80 ± 2.86	0.53	0.68	0.82
<i>glut2a</i>	Embryo	2.59 ± 1.90	3.40 ± 2.61	5.05 ± 2.86	4.29 ± 1.69	0.98	0.09	0.42	<i>glut2a</i>	Embryo	3.13 ± 2.34	4.11 ± 3.11	3.23 ± 2.62	2.29 ± 1.59	0.99	0.40	0.36
	Alevin			3.28 ± 0.96	3.86 ± 0.99	0.35	0.43	0.87		Alevin			2.76 ± 2.32	3.25 ± 2.60	0.50	0.57	0.82
<i>glut2b</i>	Embryo	2.22 ± 1.68	3.32 ± 2.28	6.06 ± 4.54	3.78 ± 0.93	0.60	0.07	0.14	<i>glut2b</i>	Embryo	2.80 ± 2.14	3.40 ± 2.26	2.83 ± 2.41	2.14 ± 1.23	0.96	0.48	0.45
	Alevin			3.86 ± 1.05	4.01 ± 1.55	0.38	0.11	0.60		Alevin			2.86 ± 2.03	3.08 ± 2.63	0.66	0.89	0.84
<i>glut4a</i>	Embryo	2.59 ± 1.75	3.76 ± 2.72	5.45 ± 1.69	4.77 ± 1.47	0.77	0.03	0.27	<i>glut4a</i>	Embryo	3.48 ± 2.37	3.77 ± 2.64	2.63 ± 1.78	2.64 ± 1.79	0.87	0.28	0.88
	Alevin			5.03 ± 0.99	4.20 ± 0.71	0.82	0.06	0.17		Alevin			2.99 ± 1.95	2.88 ± 2.08	0.93	0.47	0.83
<i>glut4b</i>	Embryo	3.15 ± 2.36	4.43 ± 3.13	5.49 ± 1.26	5.60 ± 1.26	0.44	0.06	0.51	<i>glut4b</i>	Embryo	3.57 ± 2.51	4.29 ± 3.13	2.48 ± 1.92	2.84 ± 2.03	0.59	0.22	0.86
	Alevin			5.76 ± 1.10	4.96 ± 0.57	0.78	0.08	0.23		Alevin			2.84 ± 1.90	3.00 ± 2.21	0.67	0.33	0.78

Table 5. Effects of early hypoxia and high-carbohydrate diet at first-feeding on gene expressions involved in glucose transport pathway. N-NC, N-HC, H-NC and H-HC stands for fish subjected to normoxia and no carbohydrate diet, normoxia and high carbohydrate diet, hypoxia and no carbohydrate diet and hypoxia and high carbohydrate diet, respectively. Data were presented as mean ± SD (n = 6), statistical differences were evaluated by two-way ANOVA ($p < 0.05$, values in bold).

is the first to test the programming effect of a non-nutritional stimulus, hypoxia applied during early developmental stages on glucose metabolic pathways in trout at first-feeding. A 24 h acute hypoxic stimulus (2.5 or 5.0 mg·L⁻¹ O₂) was applied to two different stages: non-hatched embryos (152 °D) and early hatched alevins (552 °D), which were long before the first-feeding (654 °D).

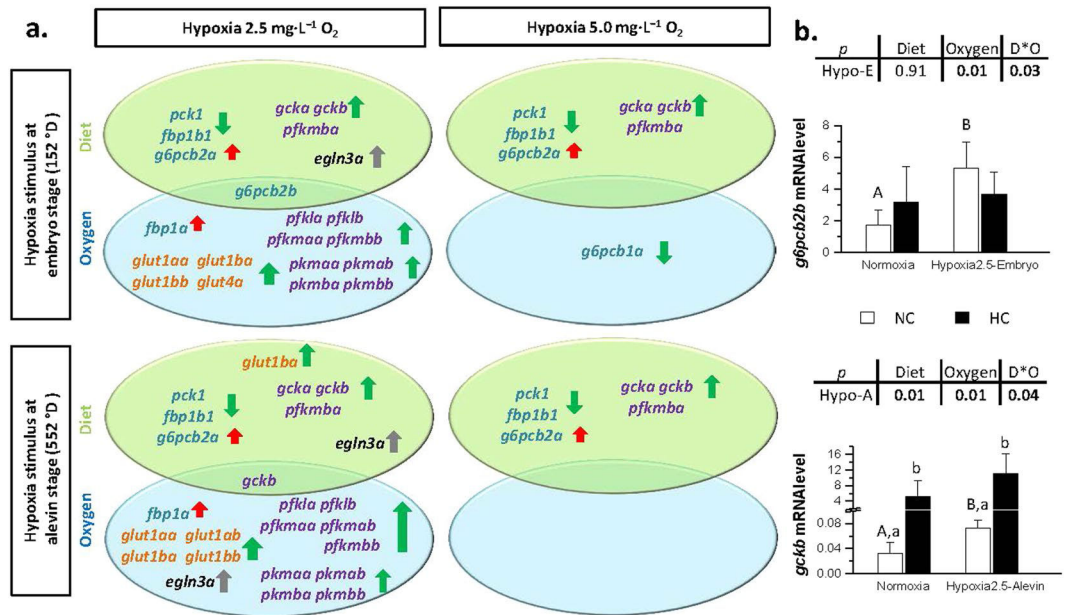


Figure 2. An overview of the effects of oxygen and diet on the mRNA levels of glucose metabolism-related genes and *egln3* genes in trout at first-feeding. **(a)** mRNA levels of genes significantly affected by high carbohydrate diet (green oval) or by the hypoxic stimulus (blue oval) at first-feeding. Genes written in blue, purple and orange belong to gluconeogenic, glycolytic and glucose transport pathway, respectively. Arrows represent up- or down- modulation of the mRNA level, with green colour in favour of glucose expenditures and red colour in favour of glucose production. **(b)** Effect of interactions between the early hypoxic stimulus and the high carbohydrate diet on mRNA levels of *g6pcb2b* and *gckb* in trout at first-feeding. White and black bars stand for trout fed the no carbohydrate (NC) or the high carbohydrate (HC) diet at first-feeding, respectively. Hypo-E and Hypo-A stands for trout subjected to hypoxic stimulus at embryo or alevin stage, respectively. Data are presented as the mean \pm SD ($n = 6$), and significant differences were evaluated by two-way ANOVA followed by a subsequent one-way ANOVA analysis ($p < 0.05$). Capital and lowercase letters indicate the significant effect ($p < 0.05$) of early hypoxic stimulus or high carbohydrate diet within their corresponding groups, respectively.

To test whether the hypoxic stimulus had a physiological impact on the fish, we first analysed the mRNA level of genes that are known to mediate the hypoxic response of cells. We observed a dramatic increase in mRNA levels of *egln3a* and *egln3b* genes in hypoxia-treated groups compared with the normoxic controls. Genes encoding the Egl-9 homologue 3 protein are considered as cellular oxygen sensors whose transcription is induced by hypoxia via the hypoxia-inducible factor 1 (Hif1)-mediated pathway^{37,38}. The induction of the expression of these duplicated genes confirmed the physiological response of both embryos and alevins to the hypoxic stimulus. However, no significant difference among treatments was found in the mRNA levels of Hif1 encoding genes, the master regulators of the cellular hypoxia response. This result was not completely surprising because *hif1* is reported to be regulated predominantly at the post-translational level³⁹.

Limited oxygen availability is also known to drive a switch in metabolism from mitochondrial aerobic oxidative phosphorylation to an anaerobic glycolytic pathway¹⁶, resulting in increased lactate production^{23,26} and glucose uptake^{21,40,41}. In the present study, the higher mRNA level of the glucose transporter *glut1ba* as well as the up-regulation of one of the duplicated proton-lactate symporter encoding genes (*slc16a3a*) in fish subjected to hypoxia suggests the formation of a functional cellular response to hypoxia at the metabolic level. Indeed, similar to *egln3a* and *egln3b*, *glut1* and *slc16a3* were also reported to be downstream target genes of Hif1^{22,42}. Together, our results confirm that the hypoxic stimulus was being sensed by both embryos and alevins at the cellular level.

We then investigated the combined effects of early hypoxic stimulus and a high carbohydrate diet on the mRNA levels of glucose-metabolism related genes in trout alevins at first-feeding. A summary of these effects is given in Fig. 2. Note that, the gene expression assays were conducted in the whole body of embryos or alevins because of their small size limited their dissection.

The effects of a high dietary carbohydrate supply at first-feeding on glucose metabolism-related genes were similar to a previous report by Marandel *et al.*¹⁵ in trout. These effects include the increase in mRNA levels of the glycolytic genes *gcka*, *gckb*, the inhibition of the gluconeogenic genes *pck1* and *fbp1b1*, and also an atypical up-regulation of *g6pcb2a* (Fig. 2a). In addition, our results showed an increase in the mRNA level of *pfkmba* in trout fed the HC diet at first-feeding, one of the four *pfkm* paralogous genes involved in the muscular glycolysis pathway. It is the first time that different paralogous genes of *pfkm* have been identified and separately amplified. This increase in the *pfkm* mRNA level in trout fed a high carbohydrate diet was never detected in studies published before the sequencing of the trout genome³⁴. These studies investigated only one gene called 6PFK-M^{12,43}. Such a difference in mRNA level response between the four paralogous genes suggests a neo- or a

sub-functionalisation of *pfkmba* in relation to dietary glucose. Regarding the glucose transport genes in general, there was no significant effect of the diet, confirming the results obtained in first-feeding alevins in a previous nutritional programming study¹².

Our results showed a clear programming effect of the early acute hypoxic stimulus on glucose metabolism-related genes at first-feeding, either directly or interacting with the dietary carbohydrate content received at first-feeding (Fig. 2a).

With respect to the effects of hypoxia, we observed that the moderate hypoxic stimulus (5.0 mg·L⁻¹ O₂) did not induce a durable modification of the glucose metabolism in trout at the time of first-feeding (except for a downregulation of *g6pcb1a* in trout exposed to this level of hypoxia at the embryo stage). By contrast, the more severe level of hypoxia (2.5 mg·L⁻¹ O₂) increased the mRNA levels of genes involved in glycolysis (*pfkla*, *pfklb*, *pfkmaa*, *pfkmbb*, *pkmaa*, *pkmab*, *pkmba*, *pkmbb*), gluconeogenesis (*fbp1a*, *g6pcb2b*) and glucose transport (*glut1aa*, *glut1ba*, *glut1bb*), irrespective of the time at which the stimulus was applied. The overall higher mRNA levels of glycolytic genes and of *glut1* paralogous genes in the hypoxic treatment group is in line with previous observations in vertebrates, including fish^{44–46}. It is well established that under hypoxic conditions, the uptake of glucose and the glycolytic flux are cooperatively induced to ensure enough ATP production to compensate for the lower efficiency of glycolysis compared with oxidative phosphorylation. These compensations are mainly achieved by upregulating genes encoding for glucose transporters (especially *glut1* and *glut3*)^{21, 47} and glycolytic enzymes^{16, 24, 25} in a Hif-1 dependent manner. Meanwhile, the increase in the mRNA levels of gluconeogenic *fbp1b1* and *g6pcb2b* in fish subjected to 2.5 mg·L⁻¹ O₂ hypoxia seems paradoxical, but such a phenomenon has also been reported in previous studies^{48–50}. A few genes were also differentially affected depending on the developmental stage at which they received the hypoxic stimulus (*i.e.*, embryo or alevin). Indeed, *gckb*, *pfkmab*, and *glut1ba* mRNA levels were altered by the hypoxic stimulus when applied in alevins, whereas the *glut4a* mRNA level was increased only in trout subjected to hypoxia at the embryo stage.

Finally, it should be noted that the higher mortality rate observed before first-feeding in the fish subjected to the 2.5 mg·L⁻¹ O₂ hypoxia at the alevin stage did not seem to have selected animals who were better metabolically adapted (at least concerning glucose metabolism) to hypoxia because the effects of hypoxia were similar to those in fish subjected to hypoxia at the embryo stage (with no differences in survival). Both stages (152 °D and 552 °D) thus appeared to be good “windows of metabolic plasticity”. Indeed, an early acute hypoxic stimulus (2.5 mg·L⁻¹ O₂) applied at the embryo stage or the alevin stage succeeded in inducing similar programming effects on glucose metabolism in rainbow trout at first-feeding, at least on the mRNA level.

Interestingly, at the time of first-feeding, the mRNA level of *egln3a* remained significantly higher in trout which had experienced 2.5 mg·L⁻¹ O₂ hypoxia at the alevin stage (552 °D) despite their normoxic rearing conditions. Similar results have been found in European sea bass, in which an early hypoxia exposure during the larval stage induced a long-term overexpression of *egln3* in juveniles⁵¹. The authors hypothesised that transcriptional imprinting occurs during early exposure to hypoxia and should lead to the enhancement of the Hif-1 α stabilisation and thus the modulation of *egln3*, known to be regulated in a Hif-dependent manner^{37, 52, 53}. Moreover, the mRNA level of *egln3a* was significantly affected by the diet at first-feeding in fish exposed to the early 2.5 mg·L⁻¹ O₂ hypoxic stimulus, either at the embryo stage or the alevin stage, as well as the normoxic group. This upregulation may also be attributed to the stabilisation of Hif-1 α , but this time possibly mediated by the FIH-1 activity, which was induced by the excessive ROS produced in a high glucose environment⁵⁴.

Even more noteworthy, interactive effects of the early hypoxic stimulus and the diet received at first-feeding were observed on the mRNA level of *g6pcb2b* and *gckb* in trout exposed to 2.5 mg·L⁻¹ O₂ hypoxia at the embryo stage and the alevin stage, respectively. Indeed, in the normoxic group, *g6pcb2b* and *gckb* mRNA levels were both up-regulated in trout fed the HC diet; a completely reverse pattern for *g6pcb2b* or an intensified effect for *gckb* were observed in fish exposed to 2.5 mg·L⁻¹ O₂ hypoxia at the embryo and alevin stages, respectively (Fig. 2b). These results are in favour of an inhibition of the last step of gluconeogenesis and an increase in the first step of glycolysis, indicating that an early hypoxic stimulus may modify the glucose utilisation at first-feeding in trout to some extent by affecting *g6pcb2b* expression, considered to be involved in the glucose-intolerant phenotype in trout juveniles¹⁴.

In summary, the present study confirms the possibility of modulating glucose metabolism-related genes in trout by using a nutritional programming strategy during early development with a non-nutritional stimulus: hypoxia. Further investigations are now required to explore whether there is a long-term programming effect of an early hypoxic stimulus alone or combined with a high carbohydrate diet at first-feeding on glucose metabolism in trout at later life stages.

Methods

Ethical issues and approval. Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (Décret 2001-464, 29 May 2001 and Directive 2010/63/EU, respectively). This protocol and the project as a whole were approved by the French National Consultative Ethics Committee (reference numbers 2015112018112159 and 201511201756973).

Experimental design and diets. *Diets.* Two isolipidic and isoenergetic experimental diets containing either no carbohydrates (NC, 0% carbohydrates) or a high content of digestible carbohydrates (HC, ~60% carbohydrates) were prepared in our own facilities (INRA, Donzacq, France) as extruded pellets (see Supplementary Table S2). Glucose and gelatinised starch were included as the carbohydrate sources, fish meal was used as protein source, and dietary lipids were provided by fish oil and fish meal. The large increase in dietary carbohydrate content in the HC diet was compensated for by a decrease in protein content.

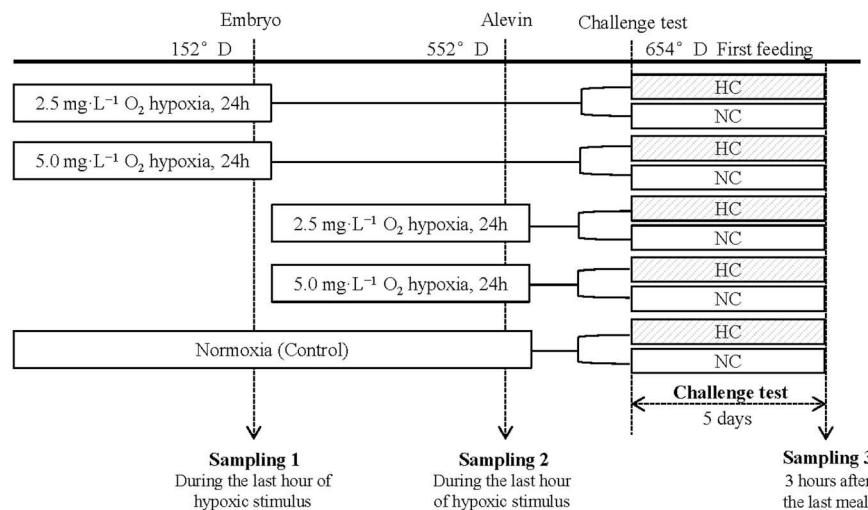


Figure 3. Experimental design. Two levels of hypoxic stimulus (2.5 or 5.0 $\text{mg}\cdot\text{L}^{-1}$ O_2) were applied to rainbow trout for 24 h at the embryo (152°D) or alevin stages (552°D). The fish were then fed either a high carbohydrate (HC) or a no carbohydrate (NC) diet at first-feeding. The normoxic group serves as the control.

Fish and Experimental design. A pool of oocytes was fertilised with neomale (the fish with a male phenotype but genetically female) sperm and reared in 30 separate tanks at 8°C in the INRA experimental facilities, Lees-Athas, France. A 24 h-acute hypoxic stimulus (2.5 $\text{mg}\cdot\text{L}^{-1}$ or 5.0 $\text{mg}\cdot\text{L}^{-1}$ dissolved oxygen) was applied to embryos at the primitive liver setting-up stage (stage 21 based on the Vernier table) at 152°D or on alevins at 552°D just after hatching and before the first-feeding⁵⁵. Two different levels of hypoxia were used at each stage: 2.5 $\text{mg}\cdot\text{L}^{-1}$ or 5.0 $\text{mg}\cdot\text{L}^{-1}$ of dissolved oxygen. Levels of hypoxia were obtained by bubbling nitrogen in the water in which the tanks containing the fish were immersed. Levels of hypoxia were checked every two hours using an oximeter (Hach, Germany). After the stimulus, fish were put back in normoxic conditions. Fish kept continuously in a normoxic conditions (11.0 $\text{mg}\cdot\text{L}^{-1}$ dissolved oxygen) were used as a control. Samples were taken during the last hour of the hypoxic stimulus (Fig. 3, 30 embryos per tank in sampling 1 and 1 alevin per tank in sampling 2). For 5 days from the first-feeding meal, each group of fish was fed with either the HC or NC diets and 2 fish per tank were sampled 3 h after the last meal (Fig. 3, sampling 3; corresponding to the postprandial peak in juveniles). The 30 tanks consisted of 10 treatments in total, with 6 replicated tanks per treatment in sampling 1 and sampling 2 and triplicated tanks per treatment in sampling 3 (Fig. 3). Embryos were directly snap-frozen in liquid nitrogen, whereas alevins were killed by terminal anaesthetisation by bathing in benzocaine (30 $\text{mg}\cdot\text{L}^{-1}$) prior to sampling and subsequently frozen in liquid nitrogen. Samples were stored at -80°C until further analysis.

Analytical methods. The chemical composition of the diets was analysed according to the following procedures: dry matter was determined after drying to constant weight at 105°C ; crude protein ($\text{N} \times 6.25$) was determined by the Kjeldahl method after acid digestion; crude lipid was determined by petroleum ether extraction (Soxtherm); gross energy was measured in an adiabatic bomb calorimeter (IKA, Heitersheim Griebelmer, Germany); ash was estimated through incinerating in a muffle furnace for 6 h at 600°C .

In silico analysis. Orthologous genes of mammalian *Hif1 α* , *Pkm*, *Pdk1*, *Ldha*, *Slc16a3*, *Slc2a1* (also called *Glut1*), *Slc2a2* (also called *Glut2*), *Slc2a4* (also called *Glut4*), *Pfkm* and *Egln3* in the rainbow trout genome³⁴ were identified in the *Oncorhynchus mykiss* genome browser (Genoscope: <http://www.genoscope.cns.fr/trout/>) and extracted from the SIGENAE database (<http://www.sigenae.org>) using the BLAST tool (all accession numbers are given in Supplementary Table S1). Sequences from other species were collected from the Ensembl Genome database (Ensembl release 84, March 2016: <http://www.ensembl.org>). Genomicus software, v01.01 (<http://www.genomicus.biologie.ens.fr/genomicus-trout-01.01/cgi-bin/search.pl>) was used to confirm the identities of the genes cited above by syntenic analysis (see Supplementary File). When the syntenic analysis was not sufficient to confirm the identity/annotation of the genes a phylogenetic analysis was performed using the MEGA package V6 software⁵⁶ as previously described¹⁴. The phylogenetic tree, based on deduced full-length amino acid sequences, was produced using the neighbour-joining (NJ) method and confirmed by the minimum evolution method (see Supplementary File). The reliability of the inferred trees was estimated using the bootstrap method with 1000 replications. New gene annotations were allocated according to ZFIN Nomenclature guidelines (<http://zfin.org/>, Supplementary Table S1).

Total RNA extraction and cDNA synthesis. Total RNA extraction was conducted on the whole body of embryos or alevins. Samples were homogenised in Trizol reagent (Invitrogen) with Precellys[®]24 (Bertin Technologies, Montigny-le-Bretonneux, France) according to the process described previously¹⁵. Luciferase control RNA (Promega), 10 pg per 1.9 mg of embryo/alevin was added to each sample to allow for data normalisation as previously described^{57,58}. Total RNA was then extracted according to the Trizol manufacturer's instructions and

1 µg of total RNA was subsequently reverse-transcribed to cDNA using The Super-Script III RNase H-Reverse transcriptase kit (Invitrogen) with random primers (Promega, Charbonnières, France).

Quantitative real-time-PCR. The primer sets used for analysis are listed in Supplementary Table S1. The newly designed primer sets used in quantitative real-time-PCR (qPCR) assays for hypoxic response and glucose metabolism-related genes were validated on a pool of cDNA and the amplified products were sequenced systematically. The primers that amplified *gck*, *pfkl*, *pkf* genes and gluconeogenic genes, which had already been published in previous studies^{14,15}, are not shown in Supplementary Table S1. qPCR assays were performed with the Roche Lightcycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France). The reaction mix was 6 µl per sample, including 2 µl of diluted cDNA template (1:25), 0.12 µl of each primer (10 µmol l⁻¹), 3 µl of Light Cycler 480 SYBR[®] Green I Master mix and 0.76 µl of DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany). The qPCR protocol was initiated at 95 °C for 10 min for the initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at 95 °C; 10 s at 60 °C). Melting curves were monitored systematically (temperature gradient 0.11 °C per second from 65 to 97 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each qPCR assay included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse transcriptase- and cDNA template-free samples). Data were subsequently normalised to the exogenous luciferase transcript abundance in samples diluted at 1:25 using the E method (Light Cycler software) as previously described⁵⁸.

Statistical analysis. Zootechnical data (*i.e.*, survival and malformation) were analysed by a Pearson's Chi-squared test. For qPCR results of hypoxic responsive genes (*i.e.*, results obtained from sampling 1 and sampling 2, Fig. 3), normality of distributions was assessed by Shapiro-Wilk test. Data were then analysed by the Kruskal-Wallis non-parametric test followed by Tukey's test as the *post hoc* analysis. qPCR results concerning the *egl3* genes and glucose metabolism-related genes (from sampling 3, Tables 2, 3, 4 and 5) were analysed by a two-way ANOVA with oxygen and diet as independent variables. When oxygen x diet interaction was significant, means were compared using one-way ANOVA. Differences were considered statistically significant at $p < 0.05$. Data were analysed using the R software (v3.1.0)/R Commander Package.

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Author Contributions

L.M. and S.P. designed the study. L.M. and J.L. managed the study and performed *in silico* analysis. I.G. established the diet formulae. J.L. performed qPCR and statistical analysis and wrote the manuscript. E.P.J. provided technical assistance in molecular biology assays. L.M. and S.P. contributed to the manuscript corrections. I.G. revised the finalized manuscript.

Additional Information

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3.4.1.2 Long-term effects: Long-term programming effect of embryonic hypoxia exposure and high-carbohydrate diet at first feeding on glucose metabolism in juvenile rainbow trout - Publication 4

Objectives

The present study aimed at investigating the long-term programming effects of an acute hypoxia exposure ($2.5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$, 25% dissolved oxygen) during embryonic stage alone or combined with a high-carbohydrate dietary stimulus at first-feeding on glucose metabolism and global methylated cytosine level in liver and muscle of juvenile rainbow trout

Experimental design

The schematic overview of the experimental design was shown in **Fig. 3-7**. Details for experimental design have been described previously in **section 3.4.1**.

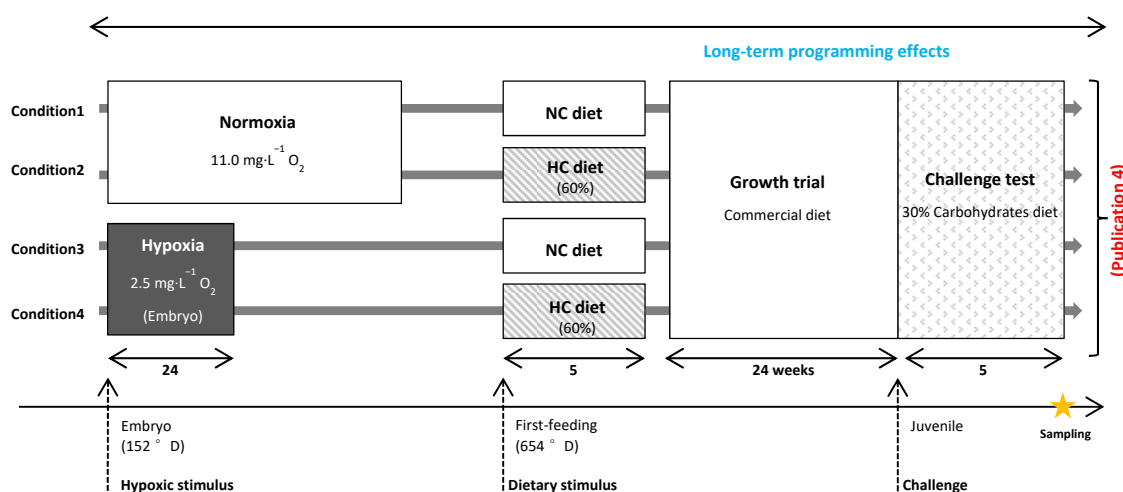


Figure 0-4 Experimental design of metabolic programming effects of acute hypoxic stimulus during early development alone or combined with a high carbohydrate dietary stimulus at the first-feeding stage on glucose metabolism in trout juveniles after 5-day challenge. HC, high carbohydrate; NC, no carbohydrate.

Highlights

1. After 24-week growth trial, trout with the HC dietary history showed decrease final body weight.

2. The mRNA levels of gluconeogenic genes in liver and glucose transporter genes in both liver and muscle were increased in juveniles with hypoxia exposure history.
3. The mRNA levels of glycolytic genes were decreased in juveniles with HC dietary history.
4. Global DNA methylation level was neither affected by hypoxic history nor HC dietary history compared to their respective control.

Conclusions

An acute hypoxic stimulus during embryogenesis alone or combined with a hyperglucidic stimulus at first-feeding can modify growth performance and the mRNA level of glucose metabolism –related genes in juvenile trout. In particular, hypoxia applied at embryo stage tends to have more effect than a high carbohydrate stimulus at first-feeding by increasing in overall hepatic gluconeogenesis, and hepatic and muscular glucose transporters. But the hepatic global DNA methylation level was not affected by oxygen or dietary history.

Publication 4

RESEARCH ARTICLE

Long-term programming effect of embryonic hypoxia exposure and high-carbohydrate diet at first feeding on glucose metabolism in juvenile rainbow trout

Jingwei Liu, Karine Dias, Elisabeth Plagnes-Juan, Vincent Veron, Stéphane Panserat and Lucie Marandel*

ABSTRACT

Environmental conditions experienced during early life play an important role in the long-term metabolic status of individuals. The present study investigated whether hypoxia exposure [for 24 h: 2.5 mg O₂ l⁻¹ (20% dissolved O₂)] during the embryonic stage alone (hypoxic history) or combined with a 5-day high-carbohydrate (60%) diet stimulus at first feeding (HC dietary history) can affect glucose metabolism later in life, i.e. in juvenile fish. After 19 weeks of growth, we observed a decrease in final body mass in fish with an HC dietary history. Feed efficiency was significantly affected by both hypoxic and HC dietary histories. After a short challenge test (5 days) performed with a 30% carbohydrate diet in juvenile trout, our results also showed that, in trout that experienced hypoxic history, mRNA levels of gluconeogenic genes in liver and glucose transport genes in both liver and muscle were significantly increased at the juvenile stage. Besides, mRNA levels of glycolytic genes were decreased in fish with an HC dietary history. Both hypoxic and dietary histories barely affected plasma metabolites or global epigenetic modifications in juvenile fish after the challenge test. In conclusion, our results demonstrated that an acute hypoxic stimulus during early development alone or combined with a hyperglucidic stimulus at first feeding can modify growth performance and glucose metabolism at the molecular level in juvenile trout.

KEY WORDS: Teleost, Hyperglycaemia, Metabolic programming, Growth, Gene expression, Epigenetics

INTRODUCTION

It is widely recognised that environmental modifications (nutritional or non-nutritional) experienced at critical periods of an organism's life (for instance during early development) can cause long-term changes in metabolism and physiology, which is termed developmental programming, or metabolic programming when modifying metabolism (Lucas, 1998). Such adaptations often manifest through persistent modifications in gene expression patterns or metabolic signalling pathways, and may persist later in life in the absence of the environmental stimulus that initiated them (George et al., 2012; Kongsted et al., 2014; Patel and Srinivasan, 2002).

Metabolic programming studies have attracted broad attention in aquaculture in recent years and shed light on how modulations in early nutrition may confer adaptive advantages to organisms to better


cope with their future nutritional environment (Balasubramanian et al., 2016; Fang et al., 2014; Panserat et al., 2017; Rocha et al., 2015; Vagner et al., 2009). In particular, in order to better understand and improve the glucose-intolerant phenotype in rainbow trout (*Oncorhynchus mykiss*), researchers have examined metabolic programming strategy using a strict nutritional stimulus in this species (Geurden et al., 2007, 2014). Promising results were obtained in some recent studies in trout. For instance, early nutritional stimulus at the first-feeding stage, such as high carbohydrate (HC) intake, was shown to induce persistent modifications in the mRNA levels of glucose-metabolism-related genes at the juvenile stage (Geurden et al., 2014). However, such a long-term programming effect was only noted in the muscle, and no persistent programming effect was observed in the liver of juvenile fish, despite the central role of this organ in glucose metabolism. These facts highlighted the necessity to optimise and review the programming conditions.

With this aim, a short-term metabolic programming assay was conducted with a strict non-nutritional stimulus, i.e. through embryonic hypoxia exposure (Liu et al., 2017). Indeed, hypoxia is a favourable stimulus candidate when investigating glucose-metabolism programming because hypoxia is known to affect hepatic glucose utilisation (Osumek et al., 2014; Zhong and Mostoslavsky, 2010). Moreover, such a stimulus can be easily applied before first feeding. Finally, effects of hypoxia are at least in part mediated by epigenetic mechanisms (Shmakova et al., 2014; Zhong and Mostoslavsky, 2010) such as DNA methylation or histone modifications (H3K4me3, H3K9me3, H3K36me3 and H3K9ac). Epigenetic modifications are considered an important mechanism mediating the long-term metabolic programming effect because they are sensitive to environmental changes and they constitute, by definition, the horizontal cell memory that is transmissible from one cell generation to another (Feil and Fraga, 2012; Szyf, 2009). In addition, the epigenetic modifications at the global level are known to affect chromatin stability, thereby leading to activation or repression of gene transcription (Klose and Bird, 2006; Kurdistani et al., 2004). Abnormal global DNA methylation or histone modification patterns are associated with metabolic disorders; thus, they were considered important biomarkers for health (Robertson, 2005; Seligson et al., 2005).

Considering hypoxia as a promising stimulus in the context of metabolic programming in trout, Liu and collaborators (2017) demonstrated that an acute hypoxic exposure during the embryonic stage affected glucose-metabolism-related gene expression in whole embryos at first feeding (Liu et al., 2017). For instance, when fed an HC diet at first feeding, significant increases in mRNA levels of genes involved in glycolytic and glucose transport pathways were noted in fish previously subjected to hypoxia. However, questions remain to be elucidated: (i) can such programming effects of an early hypoxic stimulus persist at the juvenile stage in trout; (ii) can a

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hypoxic stimulus applied during the embryonic stage combined with a nutritional stimulus at first feeding more profoundly affect glucose metabolism in trout later in life than a single stimulus; and (iii) if so, do these early stimuli modify the global epigenome in the long term?

The main objective of the present study was to investigate the above questions in juvenile trout that encountered an acute hypoxic stimulus (20% dissolved O₂ for 24 h) at the embryonic stage (152 degree days) alone (hypoxic history) or combined with an HC-diet stimulus at first feeding (HC dietary history) when challenged again with a 5-day high carbohydrate diet (30%) at the juvenile stage. The growth performance, main plasma metabolites, mRNA levels of glucose-metabolism-related genes and global epigenetic modifications were analysed in juvenile trout in the present study.

MATERIALS AND METHODS

Ethical issues and approval

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (Décret 2001-464, 29 May 2001 and Directive 2010/63/EU, respectively). This protocol and the project as a whole were approved by the French National Consultative Ethics Committee (reference numbers 2015112018112159 and 201511201756973).

Experimental design

Embryonic hypoxia and first-feeding stimuli were previously described and analysed by Liu and collaborators (Liu et al., 2017). Briefly, rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] embryos at 152 degree days [corresponding to setting up of the primitive liver (Vernier, 1969)] were initially subjected to a 24-h acute hypoxic stimulus [20% dissolved O₂ (2.5 mg l⁻¹); 8°C] or maintained at normoxic conditions (11.0 mg l⁻¹ dissolved oxygen; 8°C) (Fig. 1, phase 1, stimulus 1). This first stimulus will be referred to as ‘oxygen history’ hereafter: either hypoxic history or normoxic history, respectively. Hypoxia was obtained and maintained by bubbling nitrogen in the water as previously described by Liu and collaborators (Liu et al., 2017). After the stimulus, embryos were kept in normoxic conditions. After hatching, fish alevins were reared in a flow-through rearing system supplied with natural spring

water (18°C) under a natural photoperiod. At first feeding, fish were fed either an HC (~60% carbohydrate) diet or a no carbohydrate (NC; 0% carbohydrate) diet for 5 days (Fig. 1, phase 1, stimulus 2, and Table S1). This second stimulus will be referred as ‘dietary history’ hereafter. Each group was conducted in triplicate.

After the second stimulus (first feeding), all groups (initial average wet body mass=0.1 g) were subsequently subjected to a 24-week growth trial (Fig. 1, phase 2) with a commercial diet (Skretting, Fontaine-lès-Vervins, France) followed by a 5-day challenge test (Fig. 1, phase 3) with a challenge diet containing 30% carbohydrate (Table S1). Fish were fed eight times per day at the beginning and the feeding frequency gradually reduced to two times per day (from 18 weeks and thereafter). Each meal, the fish were fed to apparent satiation. Dry feed intake was calculated as the amount of feed supplied minus the amount of unconsumed feed. Residual feed pellets were counted and the corresponding mass was deducted from feed intake. The parameters for growth performance were monitored every 3 weeks and the following variables were calculated: (1) survival (%)=100×final fish number/initial fish number; (2) specific growth rate (SGR; % day⁻¹)=100×(lnM₂–lnM₁)/d; (3) feed intake (% day⁻¹)=100×{dry feed intake (g)/[(M₂+M₁)/2]}/d; and (4) feed efficiency (FE)=(M₂+M₃–M₁)/dry feed intake, where M₁ and M₂ are the initial and final average wet body mass, respectively, M₃ is the mass for dead fish (Li et al., 2010; Silva-Carrillo et al., 2012; Thodesen et al., 1999), and d is the experimental period in days.

At the end of the 5-day challenge test, fish were first anaesthetized in a benzocaine bath at 30 mg l⁻¹ and then killed in a benzocaine bath at 60 mg l⁻¹ 6 h after the last meal. Blood was collected from the caudal vasculature of nine fish per condition (three fish per tank) and centrifuged (3000 g, 5 min). The recovered plasma was frozen and stored at –20°C until analysis. Liver and white muscle tissues of six fish per condition (two fish per tank) were dissected and immediately frozen in liquid nitrogen, and stored at –80°C for further analysis.

Chemical composition of the diets

The approximate composition of the diets was analysed as follows: dry matter was determined after drying to constant mass at 105°C; crude protein (N×6.25) was determined by the Kjeldahl method

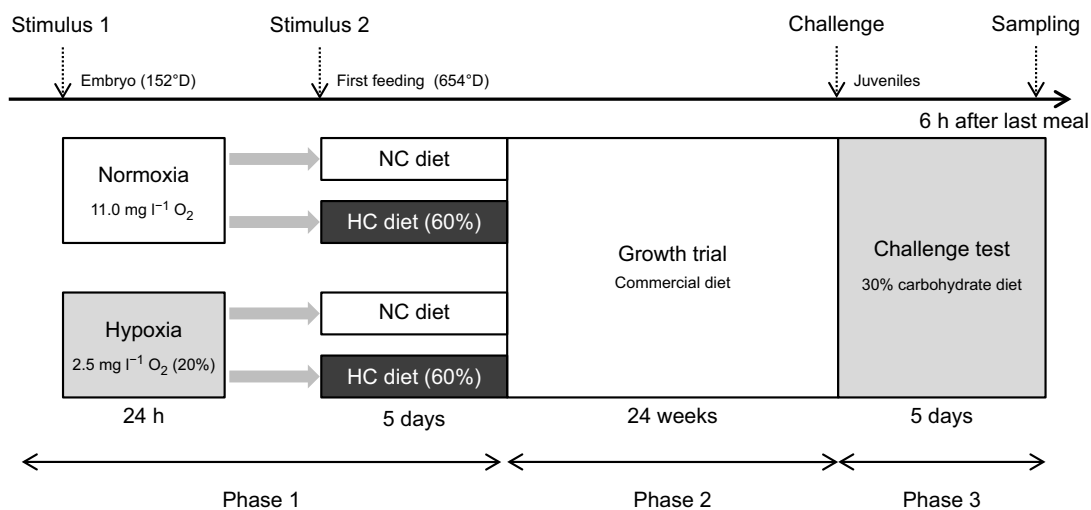


Fig. 1. Experimental design. A 24-h hypoxic stimulus was applied at the embryonic stage [152 degree day (152°D)] and a 5-day dietary stimulus (60% carbohydrate) was performed at first-feeding stage (654°D) in rainbow trout. After a 24-week growth trial with a commercial diet, fish were subjected to a 5-day challenge test with a 30% carbohydrate diet and sampled 6 h after the last meal. NC, no carbohydrate; HC, high carbohydrate.

after acid digestion; crude lipid was determined by petroleum ether extraction (Soxtherm); gross energy was measured in an adiabatic bomb calorimeter (IKA, Heitersheim Griebelmer, Germany); ash was estimated through incinerating in a muffle furnace for 6 h at 600°C.

Plasma metabolites

Plasma glucose, triglycerides, lactate and NEFA (non-esterified fatty acid) were analysed with Glucose RTU (BioMerieux, Marcy-l'Étoile, France), PAP 150 (BioMerieux), Lactate PAP (BioMerieux) and NEFA C (Wako Chemicals GmbH, Neuss, Germany) kits, respectively, according to the recommendations of each manufacturer.

Analysis of mRNA levels: quantitative real-time PCR

Total RNA extraction and cDNA synthesis

The analysis of mRNA levels was conducted in liver and white muscle tissues of juvenile fish. Samples were homogenised in Trizol reagent (Invitrogen, Carlsbad, CA, USA) with Precellys[®]24 (Bertin Technologies, Montigny-le-Bretonneux, France), and total RNA was then extracted according to the Trizol manufacturer's instructions. Total RNA (1 µg) was subsequently reverse-transcribed to cDNA in duplicate using the SuperScript III RNase H-Reverse Transcriptase kit (Invitrogen) with random primers (Promega, Charbonnières-les-Bains, France).

Quantitative real-time PCR assays

The primers used in quantitative real-time PCR (qPCR) assays for glucose-metabolism-related genes have previously been published, and the primer sequences used in the present study can be found in the previous studies (Marandel et al., 2015, 2016b; Liu et al., 2017). qPCR assays were performed with the Roche Lightcycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France). The reaction mix was 6 µl per sample, including 2 µl of diluted cDNA template (1:76), 0.12 µl of each primer (10 µmol l⁻¹), 3 µl of Light Cyler 480 SYBR[®] Green I Master mix and 0.76 µl of DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany). The qPCR protocol was initiated at 95°C for 10 min for the initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a two-step amplification programme (15 s at 95°C; 10 s at 60°C). Melting curves were monitored systematically (temperature gradient 0.11°C s⁻¹ from 65 to 97°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each qPCR assay included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse-transcriptase- and cDNA-template-free samples). The relative quantification of mRNA levels of target genes were normalised to the transcript abundance of reference genes (β -actin for liver: forward 5'-GATGGCCAGAAAGACAGCTA-3', reverse 5'-TCGTCCAGTTGG-TGACGAT-3'; *efl α* for muscle: forward 5'-TCCTCTTGGTCTG-TTTCGCTG-3', reverse 5'-ACCCGAGGGACATCCTGTG-3') using the E-method on Light Cyler software.

DNA extraction and global DNA methylation analysis

DNA extraction was performed on liver and muscle tissues of six fish per experimental treatment. Ten mg liver or muscle samples were subjected to 37°C overnight digestion under agitation (250 rpm) in 1 ml TNES buffer (pH=8; 125 mmol l⁻¹ NaCl, 10 mmol l⁻¹ EDTA, 0.5% SDS, 4 mol l⁻¹ urea, 10 mmol l⁻¹ Tris-HCl) supplemented with 100 µg proteinase K (P6556, Sigma-Aldrich, St Louis, MO, USA). One ml phenol–chloroform–isoamyl-alcohol (25:24:1) was added to each sample and mixed gently for 15 min followed by a

10,000 g centrifuge at room temperature for 15 min. A total of 100 µl 5 mol l⁻¹ NaCl and 1 ml 100% ice-cold ethanol was added to the supernatant and centrifuged for 15 min at 10,000 g, 4°C. The pellets were washed in 1 ml 75% ice-cold ethanol and centrifuged for 15 min at 10,000 g, 4°C again. Finally, the pellets were dried and re-suspended in DNase-free water and treated with 2.4 µg RNase (A7973, Promega) for 1 h at 37°C. DNA was quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and the quality (high molecular weight) was verified on 1% agarose gel. DNA global methylation pattern [5-methylcytosine (5-mC)] was assessed using the MethylFlash Methylated DNA Quantification kit (EpiGentek, Farmingdale, NY, USA). Each analysis was performed in duplicate with 100 ng liver DNA or 200 ng muscle DNA samples following the manufacturer's instructions.

Histone extraction and global histone modification analysis

Histone extraction was performed on liver and muscle tissues of six fish per experimental treatment. One hundred mg tissue was homogenised using Precellys[®]24 (Bertin Technologies) in 2 ml tubes containing 1 ml of TEB buffer [1×PBS, 0.5% Triton X-100, 5 mmol l⁻¹ NaBu and one tablet of protease inhibitor (Roche, cat. no. 04693116001)] and four 2.8 mm ceramic beads. The grinding programme was set as 2×10 s (15 s off) at 5000 rpm for liver, and 2×20 s (15 s off) at 5500 rpm for muscle samples. The homogenates were kept on ice for 20 min, transferred to a new 1.5 ml tube and centrifuged for 10 min at 2000 rpm, 4°C. Pellets were then re-suspended in 0.5 N HCl containing 10% glycerol (volume between 100 µl and 1200 µl depending on the size of the pellet) and incubated on ice for 30 min (vortex every 10 min). Samples were then centrifuged for 5 min at 12,000 rpm, 4°C. Three volumes of iced acetone were then added to the supernatant and precipitation was performed at -20°C overnight. Samples were centrifuged at 12,000 rpm, 4°C for 5 min and pellets were washed three times with 100% ice-cold acetone. Pellets were re-suspended in distilled water and incubated at 60°C for 1 h. Total protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany). Lysate (5 µg and 14 µg of total protein for liver and muscle samples, respectively) were subjected to SDS-PAGE and western blotting using the appropriate antibody. Samples were loaded on AnykD[™] Criterion[™] TGX[™] Precast Midi Protein Gel for the analysis of H3K4me3, H3K9me3, H3K36me3 and H3K9ac marks. Western blotting was performed with the Trans-Blot[®] Turbo[™] Transfer Starter System (Bio-Rad Laboratories) using the pre-programmed protocol Mixed MW (Turbo) 5–150 kDa and 7 min for transfer. Anti-H3K4me3 (C15410003), anti-H3K9me3 (C15410056), anti-H3K36me3 (C15410192) and anti-H3K9ac (C15410004) were purchased from Diagenode (Liège, Belgium); anti-H3 (ab1791) was purchased from Abcam (Cambridge, UK).

Statistical analysis

The results are presented as means±s.d. The effects of oxygen, diet and oxygen×diet on different parameters were tested using R software (v3.1.0)/R Commander by two-way ANOVA with oxygen and diet as independent variables. When oxygen×diet interaction was significant, means of all treatments were compared by a Tukey's *post hoc* analysis.

RESULTS

Survival and growth performance of trout from stimuli to challenge

In general, there were few significant differences in survival and growth performance among different groups during the 24-week

Table 1. Effects of embryonic hypoxia and high carbohydrate diet stimuli on growth performance

Index	NC dietary history		HC dietary history		P-value		
	Normoxic history	Hypoxic history	Normoxic history	Hypoxic history	Oxygen history	Dietary history	Interaction
Weeks 0–18 of growth trial							
Survival (%)	78.89±4.28	80.28±3.85	79.44±4.19	81.94±1.73	0.385	0.614	0.799
Final body mass (g)	33.45±0.62	33.87±2.30	32.90±1.34	31.41±0.69	0.532	0.101	0.274
0- to 18-week SGR (% day ⁻¹)	4.61±0.01	4.62±0.05	4.60±0.03	4.56±0.02	0.498	0.066	0.318
0- to 18-week feed intake (% day ⁻¹)	1.10±0.01	1.08±0.02	1.09±0.02	1.09±0.01	0.148	1.000	0.540
0- to 18-week feed efficiency	1.43±0.02	1.46±0.04	1.44±0.03	1.46±0.02	0.145	0.823	0.657
Weeks 19–24 of growth trial							
Survival (%)	78.89±4.28	80.28±3.85	79.44±4.19	81.94±1.73	0.385	0.614	0.799
Final body mass (g)	100.77±0.96	102.93±0.28	90.87±2.95	93.46±7.92	0.362	0.004**	0.932
19- to 24-week SGR (% day ⁻¹)	2.69±0.04	2.71±0.16	2.48±0.09	2.65±0.16	0.208	0.098	0.345
19- to 24-week feed intake (% day ⁻¹)	2.19±0.03	2.16±0.12	2.14±0.09	2.18±0.05	0.855	0.855	0.411
19- to 24-week feed efficiency	1.11±0.01	1.13±0.01	1.09±0.01	1.11±0.01	0.012*	0.012*	0.608

Data are presented as means±s.d. (triplicate tank per condition, $n=3$); statistical differences were evaluated by two-way ANOVA followed by Tukey's *post hoc* analysis. Statistical significance is shown in bold: * $P\leq 0.05$, ** $P\leq 0.01$. NC, no carbohydrate; HC, high carbohydrate; SGR, specific growth rate.

growth trial (Fig. 1, phase 2; Table 1). However, during the last 6 weeks (19–24 weeks), juveniles with the HC dietary history showed significantly lower final body mass and feed efficiency compared with those receiving the NC diet at first feeding. Additionally, fish with a hypoxic history showed a significantly higher feed efficiency compared with those kept under normoxia during early development.

Plasma metabolites of juvenile trout at the end of the 5-day challenge test

The major plasma metabolites (Table 2) related to hypoxia and carbohydrate intake (glucose, triglycerides, lactate and NEFA) were measured in juveniles at the end of the 5-day challenge test (6 h after the last meal; Fig. 1, phase 3). There was no significant effect of oxygen or dietary history on plasma metabolites.

mRNA levels of glucose-metabolism-related genes in liver and muscle of juvenile trout at the end of the 5-day challenge test

We then analysed mRNA levels of glucose-metabolism-related genes in both liver and muscle of juvenile trout after the 5-day challenge test (6 h after the last meal; Fig. 1, phase 3). No interaction effect between oxygen and dietary histories were observed on the mRNA levels of all target genes in juvenile fish.

Regarding the effect of dietary history in liver tissue (Table 3), *gcka* (glucokinase a) and *gckb* (glucokinase b) mRNA levels were significantly lower in fish that received the HC-diet stimulus at first feeding compared with those fed the NC diet. Concerning the effect of oxygen history, fish subjected to a hypoxic stimulus during embryogenesis displayed significantly higher mRNA levels of gluconeogenic *fbp1a* (fructose 1,6-bisphosphatase-1a), *g6pcb1b*

(glucose 6-phosphatase-b1 b) and *g6pcb2a* (glucose 6-phosphatase-b2 a) compared with their normoxic controls. Similarly higher mRNA levels of glucose transporters *glut1ba* (glucose transporter-1b a) and *glut2b* (glucose transporter-2b) were found in juvenile trout with the hypoxic history than in those kept under normoxia during early life.

In muscle (Table 4), no significant effect of dietary history was observed in juvenile fish except for the mRNA level of *pfkmbb* [phosphofructokinase (muscle)-bb], which was significantly lower in fish with the HC dietary history than in fish with the NC dietary history. With respect to the effect of oxygen history (Fig. 1, phase 1, stimulus 1), significantly higher mRNA levels of glucose transport genes *glut4a* (glucose transporter-4a) and *glut4b* (glucose transporter-4b) and glycolytic *pkmaa* [pyruvate kinase (muscle)-aa] were observed in juvenile fish with hypoxic history compared with their controls.

Global DNA methylation and global histone modifications in liver and muscle of juvenile trout at the end of the 5-day challenge test

The 5-methyl-cytosine methylation status was analysed in liver and muscle tissues of juvenile trout to test the global DNA methylation level (Table 5). No significant differences in global DNA methylation level in either liver or muscle of juvenile trout were observed regardless of the oxygen or dietary history.

Global levels of selected histone modifications (H3K4me3, H3K9me3, H3K36me3 and H3K4ac) were also measured in both liver and muscle tissues of juvenile trout (Fig. 2). In liver (Fig. 2A), a significant interaction between the effects of oxygen and dietary histories (Fig. 1, phase 1, stimuli 1 and 2, respectively) was observed on H3K9me3 level even though no significant independent effect of oxygen or nutritional history was monitored. By contrast, the global

Table 2. Effects of embryonic hypoxia and HC-diet stimuli on postprandial plasma metabolite levels of juvenile trout subjected to a 5-day challenge test

Plasma metabolite	NC dietary history		HC dietary history		P-value		
	Normoxic history	Hypoxic history	Normoxic history	Hypoxic history	Oxygen history	Dietary history	Interaction
Glucose	8.31±1.75	6.62±2.28	7.41±1.14	7.10±2.29	0.128	0.745	0.288
Triglycerides	5.11±2.03	4.73±2.03	4.62±1.61	4.13±3.66	0.617	0.532	0.948
Lactate	5.32±1.93	6.28±2.37	5.39±1.28	6.29±2.81	0.209	0.963	0.969
NEFA	0.16±0.05	0.16±0.03	0.13±0.07	0.17±0.04	0.263	0.706	0.324

Data are mmol l⁻¹ and are presented as means±s.d. ($n=9$ fish); statistical differences were evaluated by two-way ANOVA followed by Tukey's *post hoc* analysis. NEFA, non-esterified fatty acid.

Table 3. Effects of embryonic hypoxia and HC-diet stimuli on the mRNA level of glucose-metabolism-related genes in liver of juvenile trout subjected to a 5-day challenge test

Target gene	NC dietary history		HC dietary history		P-value		
	Normoxic history	Hypoxic history	Normoxic history	Hypoxic history	Oxygen history	Dietary history	Interaction
<i>gcka</i>	1.24±0.36	1.26±0.59	0.66±0.59	0.66±0.56	0.949	0.014*	0.961
<i>gckb</i>	1.20±0.54	1.41±0.49	0.67±0.67	0.64±0.58	0.716	0.012*	0.600
<i>pfkla</i>	0.93±0.26	1.18±0.40	0.86±0.46	0.90±0.26	0.315	0.243	0.453
<i>pfklb</i>	0.93±0.22	1.22±0.48	1.06±0.43	0.98±0.26	0.479	0.712	0.215
<i>pklr</i>	0.87±0.15	0.82±0.28	0.90±0.29	0.84±0.32	0.641	0.830	0.994
<i>pck1</i>	0.58±0.41	1.49±1.29	0.84±1.08	0.62±0.46	0.353	0.420	0.141
<i>pck2</i>	0.52±0.16	0.85±0.32	0.77±0.35	0.79±0.35	0.881	0.336	0.249
<i>fbp1a</i>	0.84±0.59	1.43±0.75	0.62±0.38	1.38±0.79	0.019*	0.599	0.743
<i>fbp1b1</i>	0.86±0.23	1.01±0.33	1.17±0.49	1.20±0.53	0.618	0.160	0.745
<i>fbp1b2</i>	0.81±0.54	1.20±0.74	1.11±0.45	1.04±0.41	0.487	0.739	0.313
<i>g6pca</i>	0.88±0.13	1.12±0.50	1.11±0.37	1.04±0.42	0.619	0.619	0.335
<i>g6pcb1a</i>	0.68±0.52	0.76±1.04	1.65±2.85	0.97±0.86	0.651	0.377	0.569
<i>g6pcb1b</i>	0.67±0.14	1.11±0.84	0.87±0.38	1.75±1.21	0.047*	0.200	0.489
<i>g6pc b2a</i>	1.11±0.83	1.94±1.61	0.59±0.42	1.44±0.47	0.044*	0.208	0.971
<i>g6pcb2b</i>	0.98±1.15	2.42±1.74	1.00±1.58	1.47±1.73	0.150	0.474	0.454
<i>glut1ba</i>	0.62±0.12	0.97±0.36	0.75±0.14	1.02±0.41	0.018*	0.442	0.743
<i>glut1bb</i>	0.87±0.51	0.94±0.32	1.02±0.44	1.11±0.38	0.638	0.341	0.962
<i>glut2a</i>	0.81±0.12	0.91±0.36	0.87±0.20	0.98±0.24	0.300	0.524	1.000
<i>glut2b</i>	0.83±0.16	1.07±0.20	0.86±0.15	1.07±0.29	0.016*	0.832	0.847

Data show the relative quantification of mRNA levels of target genes normalised to the transcript abundance of β -actin and are presented as means \pm s.d. ($n=6$ fish); statistical differences were evaluated by two-way ANOVA followed by Tukey's *post hoc* analysis. Statistical significance is shown in bold: * $P\leq 0.05$. *fbp*, fructose 1,6-bisphosphatase; *g6pc*, glucose 6-phosphatase; *gck*, glucokinase; *glut1* (*slc2a1*), solute carrier family 2 (facilitated glucose transporter), member 1; *glut2* (*slc2a2*), solute carrier family 2 (facilitated glucose transporter), member 2; *pklr*, pyruvate kinase (liver and red blood cell); *pck*, phosphoenol pyruvate carboxykinase (cytosolic *pck1* and mitochondrial *pck2*); *pfkl*, phosphofructokinase (liver).

level of all these four histone modifications remained stable in the muscle of juvenile fish (Fig. 2B), whatever the historical stimuli conditions.

DISCUSSION

Previous studies in mammals extensively documented that early-life history may profoundly affect the metabolic adaptability and risk of developing disease in adults, making metabolic programming an important topic in scientific research (Dunn and Bale, 2011; Hochberg et al., 2010; Lucas, 1998; Romani-Perez et al., 2016; Taylor and Poston, 2007; Vickers et al., 2000). This type of study was introduced into aquaculture a decade ago, raising the prospect of

tailoring the metabolic capacities in fish through the manipulation of early nutrition (Fang et al., 2014; Geurden et al., 2007, 2014; Gong et al., 2015; Izquierdo et al., 2015; Lazzarotto et al., 2015; Rocha et al., 2015). Recently, we reported that an acute non-nutritional hypoxic stimulus during early developmental stages can affect glucose metabolism in rainbow trout alevins in the short term (Liu et al., 2017). With the aim of investigating the long-term effects in juvenile fish induced by such an early hypoxic stimulus, the present study further tested the effects of an acute hypoxic stimulus alone or combined with an HC diet at first feeding on the growth performance, levels of plasma metabolites, mRNA level of glucose-metabolism-related genes and epigenetic modifications in juvenile trout.

Table 4. Effects of embryonic hypoxia and HC-diet stimuli on the mRNA level of glucose-metabolism-related genes in muscle of juvenile trout subjected to a 5-day challenge test

Target gene	NC dietary history		HC dietary history		P-value		
	Normoxic history	Hypoxic history	Normoxic history	Hypoxic history	Oxygen history	Dietary history	Interaction
<i>pfkmaa</i>	0.97±0.36	1.10±0.30	1.02±0.23	1.22±0.37	0.230	0.562	0.788
<i>pfkmab</i>	0.73±0.42	1.12±0.30	1.05±0.35	1.09±0.39	0.176	0.365	0.251
<i>pfkmba</i>	0.84±0.41	1.23±0.43	1.06±0.18	1.17±0.57	0.161	0.640	0.436
<i>pfkmbb</i>	0.92±0.29	1.15±0.20	0.69±0.21	0.86±0.28	0.064	0.018*	0.733
<i>pkmaa</i>	0.90±0.22	1.30±0.60	0.87±0.21	1.07±0.13	0.048*	0.366	0.493
<i>pkmab</i>	0.86±0.36	1.21±0.23	1.06±0.18	1.12±0.26	0.075	0.593	0.200
<i>pkmba</i>	0.83±0.33	1.13±0.23	1.01±0.17	1.02±0.24	0.148	0.715	0.180
<i>pkmbb</i>	0.79±0.34	1.09±0.26	0.98±0.17	0.93±0.16	0.238	0.856	0.104
<i>glut1ba</i>	0.71±0.26	0.99±0.35	0.89±0.43	0.95±0.42	0.279	0.670	0.494
<i>glut1bb</i>	0.73±0.26	1.10±0.17	1.04±0.61	1.05±0.46	0.277	0.450	0.294
<i>glut4a</i>	0.75±0.30	1.14±0.37	0.60±0.19	1.04±0.47	0.008**	0.400	0.852
<i>glut4b</i>	0.71±0.22	1.20±0.23	0.95±0.32	1.05±0.38	0.025*	0.673	0.119
<i>slc16a3a</i>	0.87±0.61	1.18±0.61	0.98±0.28	0.80±0.44	0.744	0.531	0.243
<i>slc16a3b</i>	0.72±0.56	1.01±0.53	0.59±0.30	1.17±1.03	0.119	0.961	0.588

Data show the relative quantification of mRNA levels of target genes normalised to the transcript abundance of *ef1 α* and are presented as means \pm s.d. ($n=6$ fish); statistical differences were evaluated by two-way ANOVA followed by a Tukey's *post hoc* test. Statistical significance is shown in bold: * $P\leq 0.05$, ** $P\leq 0.01$. *glut1* (*slc2a1*), solute carrier family 2 (facilitated glucose transporter), member 1; *glut4* (*slc2a4*), solute carrier family 2 (facilitated glucose transporter), member 4; *pfkm*, phosphofructokinase (muscle); *pkm*, pyruvate kinase (muscle); *slc16a3*, solute carrier family 16 (monocarboxylate transporter), member 3.

Table 5. Effects of embryonic hypoxia and HC-diet stimuli on global DNA methylation of juvenile trout subjected to a 5-day challenge test

Tissue	NC dietary history		HC dietary history		P-value		
	Normoxic history	Hypoxic history	Normoxic history	Hypoxic history	Oxygen history	Dietary history	Interaction
Liver	1.62±0.48	1.81±0.70	1.92±0.57	1.66±0.60	0.895	0.747	0.373
Muscle	0.44±0.27	0.39±0.12	0.36±0.23	0.48±0.19	0.676	0.909	0.356

Data show the levels of 5-methylcytosine (5-mC%: 5-mC/total DNA) in DNA and are presented as means±s.d. ($n=6$ fish individuals); statistical differences were evaluated by two-way ANOVA followed by Tukey's *post hoc* analysis.

HC dietary history decreases growth performance and affects glycolysis-related genes in juvenile trout

In the present study, long-term programming effects of an HC stimulus applied at first feeding was tested in juvenile trout after the 24-week growth trial. Significantly lower body mass and feed efficiency were observed at the end of the growth trial in fish with an HC dietary history compared with those fed the NC diet at first feeding. Results indicated that an early HC dietary stimulus may lead to some detrimental effects in long-term growth performance in trout. However, such effects were not reported in the study previously performed by Geurden and collaborators (2014), in which a 5-day HC dietary stimulus at first feeding did not affect growth performance in juvenile trout. This difference can possibly be explained by the different durations of the growth trial and of the challenge period in these two studies. In the present study, the inferior growth performance of fish with the HC dietary history was not observed until the end of the growth trial (19–24 weeks; 133–168 days). In comparison, the corresponding pre-challenge period in the study of Geurden and collaborators was done over 105 days, which may be too short to monitor a significant difference in growth.

Durable adaptive changes in gene expression patterns are one of the most important biological mechanisms that mediate the persistence of programming effects (Lucas, 1998). At the molecular level, results obtained from the present study showed that dietary history significantly affected mRNA levels of some glycolytic genes in juvenile trout. Indeed, the HC dietary stimulus at first feeding seems to decrease glycolysis in juvenile trout, i.e. mRNA levels of hepatic *gcka* and *gckb* and muscular *pfkmbb* were lower in fish with an HC dietary history than those with an NC dietary history. These findings were opposite to what was reported for the short-term effect (i.e. at first feeding) (Liu et al., 2017). Indeed, at first feeding, *gcka* and *gckb* mRNA levels were increased in fish that received the HC dietary stimulus compared with the control fish (Liu et al., 2017). Additionally, the lower mRNA level of *pfkmbb* in the muscle of juvenile fish with the HC dietary history was not detected previously in the short term. All these results revealed the presumable existence of indirect programming regulations (i.e. through altered hormonal levels) of the long-term expression of metabolic genes by early dietary stimulus. In comparison, the decrease in glycolytic genes in juvenile fish fed an HC diet at first feeding was consistent with the previous study performed by Geurden and collaborators (2014), which showed a decrease in *hk1* (hexokinase 1) and *pkm* mRNA levels in the muscle of juvenile trout with an HC dietary history (Geurden et al., 2014).

It should be noted that, similar to previous studies in mammals (Bellinger and Langley-Evans, 2005; de Maredsous et al., 2016; Devaskar and Thamocharan, 2007), the present study balanced the dietary carbohydrate proportion against protein content to keep the same energy level in diets. Therefore, cautions should be taken when considering the exact causal factor for the long-term effect of dietary history: high dietary carbohydrates, low dietary protein, or both.

Hypoxic history enhances the mRNA level of gluconeogenic and glucose-transport-related genes in juvenile trout

Apart from perinatal nutrition, fetal hypoxia is one of the most extensively studied environmental factors able to induce long-term metabolic changes in mammals (Myatt, 2006; Seckl and Holmes, 2007). Several studies in both mammals and teleost species showed that hypoxia exposure during early life can affect growth and glucose metabolism in adulthood (Camm et al., 2011; Osunek et al., 2014; Vanderplancke et al., 2015). In the present study, a subtle but significant increase in feed efficiency in weeks 19–24 was found in juveniles with a hypoxic history compared with those with a normoxic history. Such an increase in feed efficiency by early hypoxic stimulus has never been reported in trout, although the detailed mechanism behind this long-term effect is still not clear.

Concerning the long-term effect of oxygen history on glucose metabolism in juvenile fish challenged with an HC diet, our results highlighted a significant activating effect of hypoxic history on mRNA levels of genes involved in gluconeogenesis (*fbp1a*, *g6pcb1b*, *g6pcb2a*) and glucose transport (*glut1ba* and *glut2b* in liver, and *glut4a*, *glut4b* in muscle) pathways. Meanwhile, the hypoxic stimulus rarely induced long-term modifications in mRNA levels of glycolytic genes, except for *pkmaa* in the muscle of juvenile fish.

These results were quite in accordance with the modifications induced by hypoxic history in the short term described in the previous study (Liu et al., 2017). Indeed, mRNA levels of *fbp1a*, *glut1ba*, *glut4a* and *pkmaa* in fish with a hypoxic history were consistently higher compared with those with a normoxic history in both the short (first-feeding alevins) (Liu et al., 2017) and long (juveniles) term. The persistent alteration in the expression pattern of these genes demonstrated that an acute hypoxic stimulus applied during early development can quickly modify the transcription of glucose-metabolism-related genes in trout, and these modifications may remain until the juvenile stage.

It is to be noted that, both in the short-term (Liu et al., 2017) and long-term experiments, we observed a strong and consistent activating effect of hypoxic history on glucose-transport genes in trout. These results were in line with a previous study in mammals that showed that prenatal hypoxia can cause persistent alterations in the expression of glucose-transport genes in adult rat offspring (Camm et al., 2011). However, contradictory results were obtained in a previous study in European sea bass, which reported that the mRNA level of *glut2* was increased after a moderate hypoxic episode during the larval stage but, concerning the long-term effect, decreased in the liver of juvenile fish (Vanderplancke et al., 2015). The differences between this study and the present study can be attributed to the different hypoxic intensity and duration, and also the different physiological responses to hypoxia within these two species. Nevertheless, the increased mRNA levels of glucose-transport genes in both liver and muscle of juvenile fish with a hypoxic history in the present study may to some extent prove a better functioning peripheral glucose disposal. Although, after the

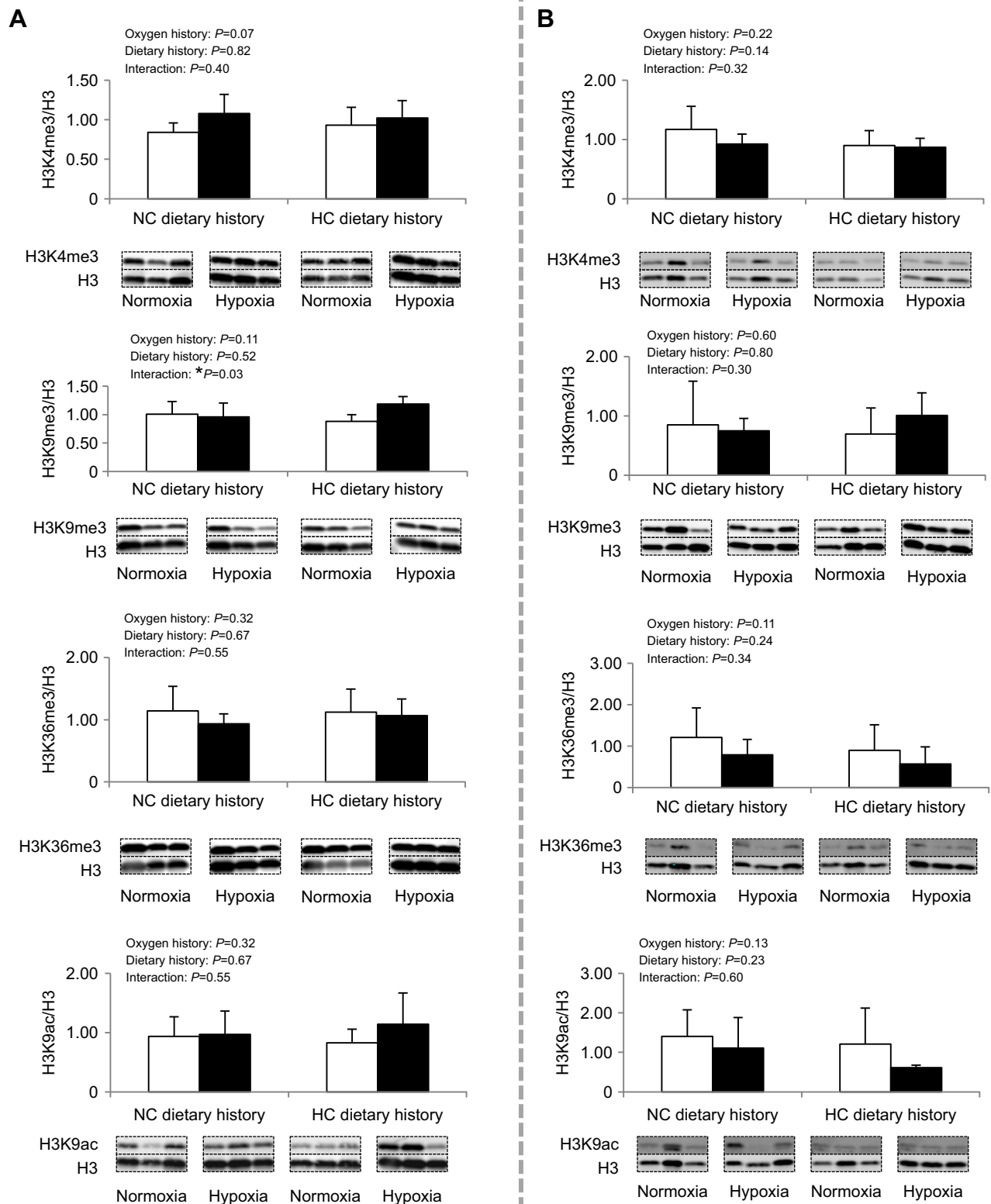


Fig. 2. Effects of embryonic hypoxia and an HC diet at first feeding on global histone modifications in liver and muscle of trout juveniles. (A) Liver. (B) Muscle. White bars and black bars represent fish subjected to normoxia or hypoxia at the embryonic stage, respectively. Data are presented as means \pm s.d. ($n=6$); statistical differences were evaluated by two-way ANOVA. When oxygen \times diet interaction was significant (*), means of all treatments were compared by Tukey's *post hoc* analysis.

challenge test, the glycaemic level was similar and ranged between 6.6 and 8.3 mmol l⁻¹ among different groups, which corresponded to the HC-diet-induced hyperglycaemia (>4 mmol l⁻¹) in rainbow

trout, it could possibly be explained by the concomitant enhanced gluconeogenesis (*fbp1a*, *g6pcb1b* and *g6pcb2a*) in the liver of fish that experienced hypoxia during development. Globally, we

hypothesised that glucose transport activated by hypoxic history might provide some clues to ameliorate the glucose-intolerant phenotype in trout, which was known to be related to low glucose transport in muscle of this species (Blasco et al., 1996; Kamalam et al., 2017; Polakof et al., 2012; West et al., 1993).

Oxygen and dietary histories affected global epigenetic modifications in juvenile trout

Remodelling of the epigenetic landscape is an important mechanism relating to the persistent metabolic adaptations by early environmental stimuli. DNA methylation and histone modifications are the most comprehensively studied forms of epigenetic markers, which are known to affect gene transcription (Klose and Bird, 2006; Kurdistani et al., 2004). Previous studies showed that both dietary carbohydrate levels and a hypoxic stimulus can alter global DNA methylation and histone modification (Craig and Moon, 2013; Marandel et al., 2016a; Chen et al., 2006; Hancock et al., 2015; Honma et al., 2007; Perez-Perri et al., 2011; Tausendschön et al., 2011; Watson et al., 2010). However, results obtained in the present study showed that the global DNA methylation level and histone modifications were barely affected by oxygen and dietary histories in juvenile fish after the challenge test, except for an interactive effect of oxygen and dietary histories on the global H3K9me3 level observed in the liver of juvenile fish. Despite this interaction, no statistically significant difference was found within groups.

Conclusions

In summary, our study showed that an acute hypoxic stimulus during embryonic development alone or an HC-diet stimulus at first feeding can affect growth performance in the long term in rainbow trout. Both stimuli also have a long-term programming effect on the mRNA levels of glucose-metabolism-related genes in juvenile trout.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.P., L.M.; Methodology: K.D., E.P.-J., V.V.; Formal analysis: J.L., K.D., E.P.-J., V.V.; Investigation: J.L., K.D., E.P.-J., V.V.; Data curation: J.L.; Writing - original draft: J.L.; Writing - review & editing: S.P., L.M.; Visualization: J.L.; Supervision: S.P., L.M.; Project administration: S.P., L.M.; Funding acquisition: S.P., L.M.

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Supplementary information

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3.4.2 Chronic hypoxia & high-carbohydrate diets as stimuli: Programming of the glucose metabolism in rainbow trout juveniles after chronic hypoxia at hatching stage combined with a high dietary carbohydrate: Protein ratios intake at first-feeding - Publication 5

Objectives

The present study aimed at investigating the impact of a chronic hypoxic stimulus (15 days, 50% dissolved O₂, 5 mgL⁻¹) at hatching alone or combined with a 60% high carbohydrates (HC) dietary stimulus (8 days) at first-feeding on growth performance and glucose metabolism of juvenile trout.

Experimental design

After hatching (312°D), trout alevins were subjected to either a 15-day chronic hypoxic stimulus (50% of O₂, between 4 and 5mg. L⁻¹ **Fig. 3-8**) or kept under normoxic (between 10-11 mg. L⁻¹, dissolved oxygen) conditions (**Fig. 3-8**, Stimulus 1). Hypoxia was obtained and maintained by supplied hypoxia tanks with N₂-bubbled water. Oxygen levels were continuously measured (Hach, Germany) and check several times per day in all tanks. During the hypoxic stimulus, the mortality of fish was noted every day to calculate the survival rate. Besides, 2 alevins per tank were sampled at the last hour of this hypoxic stimulus to ensure the efficiency of the hypoxic stimulus (**Fig. 3-8**, Sampling 1).

At the time of first-feeding (536°D), fish were fed with either a high carbohydrate (HC, 60%) diet or a no carbohydrate (NC) diet) during 8 days (**Fig. 3-8**, Stimulus 2). 2 fish per tank were sampled 3h after last meal (**Fig. 3-8**, Sampling 2). After this second stimulus, all fish were growth during 27 weeks (**Fig. 3-8**, Growth trial) with a commercial diet (Skrettring, France). At the end of the growth trial, juvenile fish were subjected to a 5-days challenge test with a 30% carbohydrate diet (challenge diet) The last sampling was conducted 6h after the last meal (**Fig. 3-8**, Sampling 3, 9 fish per condition).

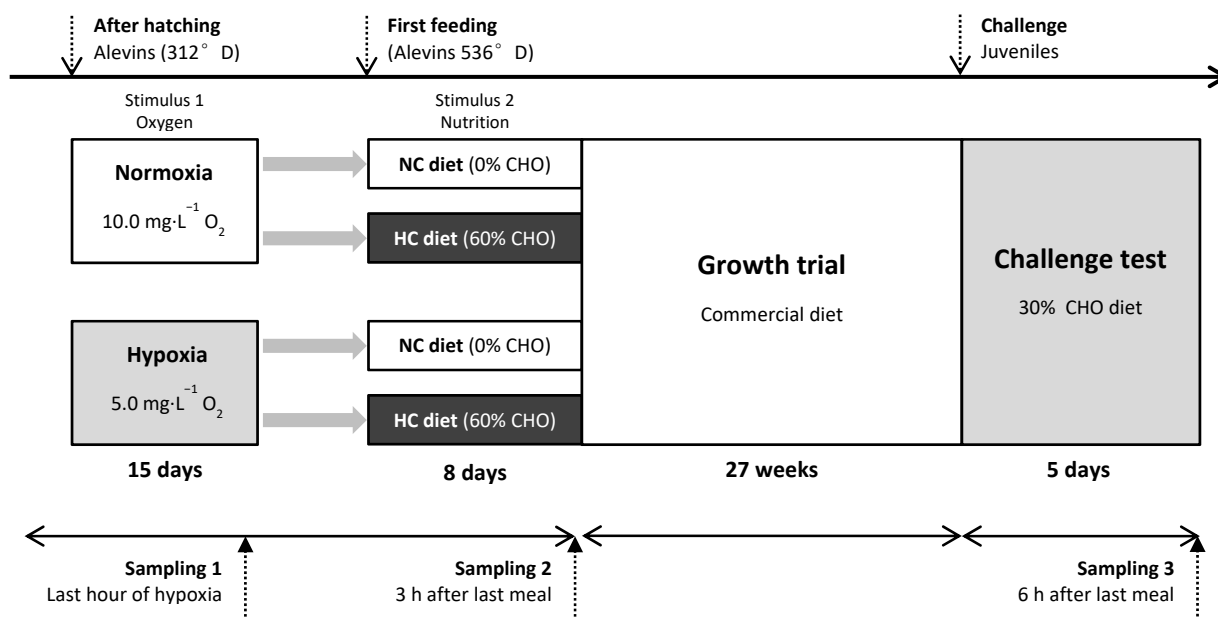


Figure 0-5 Experimental design of metabolic programming effects of chronic hypoxic stimulus after hatching alone or combined with a high carbohydrate dietary stimulus at the first-feeding stage on glucose metabolism in trout juveniles after 5-day challenge test. HC, high carbohydrate; NC, no carbohydrate.

Highlights

1. Chronic hypoxia applied during early life affected the mRNA levels of glucose transporter genes by decreasing them in both liver and muscle of juvenile trout
2. Plasma triglycerides level was decreased in juvenile trout exposed to chronic hypoxia during early life
3. The mRNA level of glycolic gene *pfkmaa* was upregulated in the muscle of juvenile trout with HC dietary history at first-feeding

Conclusions

A chronic hypoxic stimulus applied at hatching alone or combined with a high carbohydrates stimulus at first-feeding can lead to a decrease in plasma triglycerides level and a decrease in glucose-transporter mRNA levels in both liver and muscle in juvenile trout.

Of Note: Because there were relatively few glucose metabolism-related genes affected in their transcriptional expression by different oxygen/dietary histories at juvenile stage, further analyses of DNA methylation status were not performed in this experiment.

Publication 5



Programming of the glucose metabolism in rainbow trout juveniles after chronic hypoxia at hatching stage combined with a high dietary carbohydrate: Protein ratios intake at first-feeding



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ABSTRACT

It is well known that change in environmental parameters experienced during early life can have profound effect on the metabolism of individuals later in life. The purpose of the present study was to evaluate the impact of a chronic hypoxic stimulus (15 days, 50% dissolved O₂, 5.0 mg·L⁻¹) applied at hatching (oxygen history) alone or combined with a 60% high carbohydrate (HC) dietary stimulus (8 days) applied at first-feeding (dietary history) on growth performance and glucose metabolism of juvenile trout. The effectiveness of the hypoxic and the HC dietary stimuli were verified by monitoring the modulation in mRNA levels of oxygen-sensitive and dietary carbohydrate-sensitive genes, respectively. At juvenile stage (28 weeks after first-feeding), neither dietary history nor oxygen history had long-term effect on growth performance. These juveniles were then subjected to a 5 days challenge test with a 30% carbohydrate diet. Our data showed that mRNA levels of some glucose transport-related genes in liver and muscle, and plasma triglycerides were down-regulated in fish which were exposed to hypoxia during early life. Regarding to the effect of dietary history, the mRNA level of muscular *pfkmaa* was observably increased in fish previously subjected to the HC dietary stimulus. In summary, the present study indicated that a chronic hypoxic stimulus applied at hatching alone or combined with a high carbohydrate stimulus at first-feeding can affect plasma triglycerides level and glucose metabolism-related genes in juvenile trout.

1. Introduction

Environmental alterations (nutritional or non-nutritional) that are experienced during early life can lead to profound changes in metabolism and physiology of living organisms, which is termed metabolic programming (when modifying metabolism) (Lucas, 1998). Long-lasting modification in gene expression patterns is one of the most important biological mechanisms described in such case of adaptations

and which may persist later in life in the absence of the environmental stimulus that initiated them (George et al., 2012; Kongsted et al., 2014; Lucas, 1998; Patel and Srinivasan, 2002).

In aquaculture, the concept of metabolic programming has attracted more and more attention in recent years mostly in the context of the replacement of fish meal by alternative ingredients (Fang et al., 2014; Geurden et al., 2007; Geurden et al., 2014; Gong et al., 2015; Rocha et al., 2014, 2015) and more particularly by dietary carbohydrate.

Abbreviations: *gck*, glucokinase; *pfkm*, phosphofructokinase (muscle); *pfkl*, phosphofructokinase (liver); *pkm*, pyruvate kinase (muscle); *pklr*, pyruvate kinase (liver and red blood cell); *pck*, phosphoenol pyruvate carboxykinases (cytosolic *pck1* and mitochondrial *pck2*); *fbp*, fructose 1, 6-bisphosphatase; *g6pc*, glucose 6-phosphatase; *glut1/slc2a1*, solute carrier family 2 (facilitated glucose transporter), member 1; *glut2/slc2a2*, solute carrier family 2 (facilitated glucose transporter), member 2; *glut4/slc2a4*, solute carrier family 2 (facilitated glucose transporter), member 4; *slc16a3*, solute carrier family 16 (monocarboxylate transporter), member 3; *egl-9*, egl-9 family hypoxia-inducible factor 3; *hif1a*, hypoxia inducible factor 1 alpha; *pdhE1*, pyruvate dehydrogenase kinase 1; *ldha*, lactate dehydrogenase A; NEFA, non-esterified fatty acid

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Indeed, dietary carbohydrates are promising candidates for partial replacement of fish meal due to their economical production in Europe and their “sparing effect” for protein through saving this precious nutrient for growth purpose instead of utilising as an energy source. However, carnivorous fish, such as the first species produced in Europe, the rainbow trout (*Oncorhynchus mykiss*), is considered as a “glucose-intolerant” species presenting a postprandial hyperglycaemia and growth retardation when fed, in the long term, a diet containing more than 20% carbohydrates (Polakof et al., 2012; Wilson, 1994). In this context, previous studies on rainbow trout have proved the possibility of using metabolic programming strategy to modify the glucose metabolism in this fish (Geurden et al., 2007; Geurden et al., 2014; Liu et al., 2017a; Liu et al., 2017b) and tried to overcome the metabolic (nutritional) bottlenecks of poor use of dietary glucose as an energy source. First investigations were only based on a strict nutritional stimulus (Geurden et al., 2007; Geurden et al., 2014) which had no significant programming effect on the glucose metabolism of the most important metabolic organ, the liver. The programming conditions were then reviewed by Liu and collaborators (Liu et al., 2017a; Liu et al., 2017b) including an early non-nutritional stimulus based on an acute exposure to hypoxia, an environmental parameter known to affect hepatic glucose metabolism in aerobic organisms including fish (Osumek et al., 2014; Palmer and Clegg, 2014; Rissanen et al., 2006; Sappal et al., 2016; Zhong and Mostoslavsky, 2010). Indeed, under hypoxic conditions, Hif1 α sub-unit integrity is maintained by the inhibition of prolyl hydroxylase enzymes (which co-factor is O₂), and able to form a complex with Hif1 β which in turn act as a transcription factor to regulate glucose-metabolism related genes. Although some glucose metabolism related genes were affected in the long-term in these studies, the effect of acute hypoxia remained however moderate and did not modify physiological parameters such as glycaemia or growth. In this latter work, the selection for the stimulus focused on acute hypoxia because rainbow trout is considered as an oxygen sensitive teleost species (Gesser, 1977; Miller et al., 2008). However, under hypoxia, a complex of physiological and biochemical alterations take place and vary depending on the hypoxia level and the duration of the exposure (Bristow and Hill, 2008; Deldicque and Francaux, 2013; Liu et al., 2017b). Although there is limited information of long-term programming effect of early chronic hypoxia exposure on glucose metabolism in rainbow trout, a previous study in sea bass, an oxygen-sensitive teleost (Pichavant et al., 2003; Terova et al., 2009), demonstrated that chronic hypoxic exposure induced lasting alterations in the mRNA level of glucose metabolism-related genes in juveniles (Vanderplancke et al., 2015). In this sense, it can be hypothesised that chronic hypoxia exposure during early development may lead to long-term modification in glucose metabolism in trout juveniles.

In this context, the present study was thus designed to investigate

the programming effect of a moderate chronic hypoxic stimulus (50% of O₂, 5.0 mg·L⁻¹, during 15 days) applied at hatching stage (312°D) alone or combined with a 8-days high carbohydrate dietary stimulus applied at first-feeding (536°D) on growth performance, plasmatic metabolites and glucose metabolism in trout juveniles.

2. Materials and method

2.1. Ethical issues and approval

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (Décret 2001–464, 29 May 2001 and Directive 2010/63/EU, respectively). This protocol and the project as a whole were approved by the French National Consultative Ethics Committee (reference numbers 2,016,110,211,489,713, 2,015,112,018,112,159 and 201,511,201,756,973).

2.2. Experimental design and sampling

Fish were obtained from spawns in La Peima, INRA facilities, France. The experiment design was shown in Fig. 1. From fertilisation to just before first-feeding (536°D) fish were reared at 12 °C. After hatching (312°D), trout alevins were subjected to either a 15-days chronic hypoxic stimulus (50% of O₂, between 4 and 5 mg·L⁻¹ Fig. 1) or kept under normoxic (between 10 and 11 mg·L⁻¹, dissolved oxygen) conditions (Fig. 1, Stimulus 1). Each condition was conducted in triplicate tanks. Hypoxia was obtained and maintained by supplied hypoxia tanks with N₂-bubbled water. Oxygen levels were continuously measured in 2 tanks (1 normoxia and 1 hypoxia) (Hach, Germany) and check several times per day in all tanks. This first stimulus will be referred as “oxygen history” thereafter. During the hypoxic stimulus, the mortality of fish was noted every day to calculate the survival rate. Besides, 2 alevins per tank were sampled (anaesthetised in a 0.024 mL·L⁻¹ isoeugenol bath and then killed by a bath at 0.1 mL·L⁻¹) at the last hour of this hypoxic stimulus to ensure the efficiency of the hypoxic stimulus (Fig. 1, Sampling 1). Just before first-feeding, alevins were transferred to the INRA experimental facilities of Lees-Athas, France, and reared at 8 °C (with a period of adaptation). At the time of first-feeding (536°D), fish were fed with either a high carbohydrate (HC, 60%) diet or a no carbohydrate (NC) diet (Table 1) during 8 days (Fig. 1, Stimulus 2). This stimulus was referred as “dietary history” thereafter. Each treatment was performed in triplicate of tanks and 2 fish per tank were sampled 3 h after last meal (Fig. 1, Sampling 2). After this second stimulus, all fish were growth during 27 weeks (Fig. 1, Growth trial) with a commercial diet (Skrettring, France). Nine weeks after first feeding, fish were transferred to INRA experimental facilities

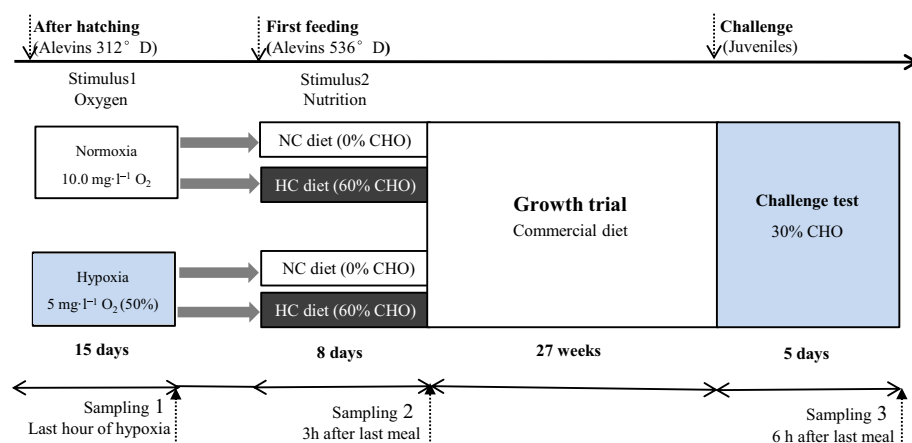


Fig. 1. - Experimental design. Hypoxic stimulus (50% of O₂, 5.0 mg·L⁻¹) was applied to rainbow trout for 15 days at the alevin stage (312°D) then the fish were fed either a high carbohydrate (HC) or a no carbohydrate (NC) diet at first-feeding (536°D). After a 27 weeks growth trial, fish were subjected to a 5 days challenge test with 30% carbohydrate diet.

Table 1

Formulation and proximate composition of the experimental diets used in this experiment at first-feeding (536 °D) and challenge test.

Ingredient g/100 g diet	NC	HC	Growth trial ^b			Challenge
			0.8 mm	1.5 mm	3 mm	
Fish meal ¹	91.20	22.80				56.40
Fish oil ²	4.80	13.20				9.60
Starch ³	0.00	40.00				30.00
Glucose ⁴	0.00	20.00				0.00
Vitamin mix ⁵	1.00	1.00				1.00
Mineral mix ⁶	1.00	1.00				1.00
Alginate ⁷	2.00	2.00				2.00
Proximate composition						
Dry matter (DM,% diet)	94.76	91.63	86.75	91.70	92.13	92.00
Crude protein (% DM)	64.94	21.19	63.84	56.23	46.82	41.90
Crude lipids (% DM)	13.20	14.45	18.65	19.55	17.79	12.90
Gross energy (kJ g ⁻¹ DM)	21.27	21.31	24.99	24.37	23.15	21.10
Ash (% DM)	18.12	5.62	9.52	7.34	6.89	11.30
Carbohydrates(% DM)	0.26	48.80	10.96	15.45	7.80	26.80

NC, no carbohydrate diet; HC, high carbohydrate diet; Challenge, challenge diet. Growth trial; growth trial diet (mean \pm SD calculated on the whole period for different batch of diet).

¹ Sopropeche (reference Ip94), Boulogne-sur-Mer, France.

² Sopropeche (reference AmSud), Boulogne-sur-Mer, France.

³ Gelatinized corn starch (Roquette, Lestrem, France).

⁴ D-(+)-glucose (Sigma-Aldrich, G7021).

⁵ Supplying (kg⁻¹ diet): 60 IU DL- α -tocopherol acetate, 5 mg sodium menadione bisulphate, 15,000 IU retinyl acetate, 3000 IU DL-cholecalciferol, 15 mg thiamine, 30 mg riboflavin, 15 mg pyridoxine, 0.05 mg vitamin B12, 175 mg nicotinic acid, 500 mg folic acid, 1000 mg inositol, 2.5 mg biotin, 50 mg calcium pantothenate and 2000 mg choline chloride.

⁶ Supplying (kg⁻¹ diet): 2.15 g calcium carbonate (40% Ca), 1.24 g magnesium oxide (60% Mg), 0.2 g ferric citrate, 0.4 mg potassium iodide (75% I), 0.4 g zinc sulphate (36% Zn), 0.3 g copper sulphate (25% Cu), 0.3 g manganese sulphate (33% Mn), 5 g dibasic calcium phosphate (20% Ca, 18% P), 2 mg cobalt sulphate, 3 mg sodium selenite (30% Se), 0.9 g potassium chloride and 0.4 g sodium chloride.

⁷ Louis François, Marne-la-Vallée, France.

⁸ Commercial diet, Skretting, France. Exact list of ingredients not available.

of Donzacq (France) and reared in a flow-through rearing system supplied with natural spring water at 17 °C. At the end of the growth trial, juvenile fish were subjected to a 5-days challenge test with a 30% carbohydrate diet (challenge diet, Table 1). The last sampling was conducted 6 h after the last meal (Fig. 1, sampling 3, 9 fish per condition = 3 fish \times 3 tanks). During this period, water quality was checked every 2 months and remained stable (NH₄: 0.06 \pm 0.01 mg/L; NO₂: 0.018 \pm 0.005; NO₃: 15.25 \pm 0.5; total phosphore: 0.015 \pm 0.06; pH: 7.62 \pm 0.09).

During the growth trail period (Fig. 1), fish were fed to apparent satiation by hand. Fish were fed 8 times per day at the beginning and the feeding frequency gradually reduced to 2 times per day (from 18 weeks and thereafter). Collected the residual feed pellets for correcting the feed intake. The growth parameters were tested every 3 weeks and calculated as follow:

Survival rate (%) = 100 \times final fish number/initial fish number

Specific growth rate (SGR, %d⁻¹) = 100 \times (LnW2–LnW1)/d

Feed intake (%/d) = 100 \times dry feed intake (g)/[(W2 + W1)/2]/d

Feed efficiency (FE) = (W2 + W3–W1)/dry feed intake

Where W1 and W2 are the initial and final average wet body weight, W3 is the weight for dead fish (Li et al., 2010; Silva-Carrillo et al., 2012; Thodesen et al., 1999), and d is the experimental period in days.

For the sampling, fish were first anaesthetised by a benzocaine bath at 30 mg·L⁻¹ and then killed by a benzocaine bath at 60 mg·L⁻¹. For sampling 3, blood samples were obtained from the caudal vessels by heparinized syringes and centrifuged at 3000 \times g (4 °C) for 5 min to get the plasma. Liver and white muscle (sampled just under the dorsal fin) of juveniles were dissected and immediately frozen in liquid nitrogen, then stored in –80 °C until analyses.

2.3. Chemical composition and water stability of the diets

The chemical composition analysis of NC, HC and challenge diets were performed as previously described by Liu et al. (2017b). The stability of the diet was proven simply by immersing the pellets in a glass of water and deemed suitable as soon as a minute has elapsed without the pellet having begun to disintegrate. We also evaluated the buoyancy of the pellet, however tanks in which the fish are raised allow the use of floating and non-floating food.

2.4. Analysis of plasma metabolites

Glucose RTU (BioMerieux), Triglycerides-GPO PED (Sobioda), Lactate PAP (Biomerieux) and NEFA C (Wako Chemicals GmbH) kits were used for measuring the plasma glucose, triglycerides, lactate and NEFA (non-esterified fatty acid) respectively, according to the manufacturer's instructions.

2.5. qRT-PCR analysis

Total RNA of whole-body alevins (Fig. 1, samplings 1 and 2), liver and white muscle of juveniles (Fig. 1, sampling 3) were extracted with Trizol method following the manufacturer instructions (Invitrogen). Samples were homogenised as described by Marandel et al. (2016). In alevin samples, 5 pg of *luciferase* control RNA (Promega) per mg of tissues was added for data normalisation as previously described (Desvignes et al., 2011; Marandel et al., 2012). Quality test and the reverse transcription of RNA were performed as previously described by Liu et al. (2017a).

Primers used for quantitative real-time PCR were previously published (Liu et al., 2017a; Marandel et al., 2015; Marandel et al., 2016). qPCR assays were carried out as previously performed by Liu et al. (2017a). *Luciferase* and *ef1a* genes were chosen as reference genes for normalisation (*luciferase* for alevins, *ef1a* for liver and muscle of juveniles) to investigate the relative mRNA level of target gene by the E-method on Light Cycler software as previously described (Marandel et al., 2012).

2.6. Statistical analysis

Statistical analyses were performed using R software (v3.1.0)/R Commander. Normality of distributions was assessed using the Shapiro–Wilk test. Data were then analysed by a Kruskal–Wallis non-parametric test (Fig. 1, sampling 1) or two-way ANOVA analysis (Fig. 1, samplings 2 and 3) with oxygen and diet as independent variables. When oxygen \times diet interaction was significant, a Tukey's *post hoc* analysis was performed. Results are presented as mean \pm SD (standard deviation).

3. Results

3.1. Survival and growth

No significant difference in survival was noticed between treatments both at end of the hypoxic stimulus (Fig. 1, stimulus 1) (percent of survival: Normoxia group: 97.93% \pm 0.99; Hypoxia group: 98.57% \pm 0.32) and at the end of the HC dietary stimulus (Fig. 1, stimulus 2) (percent of survival: Normoxia history-NC diet: 99.04% \pm 0.87; Normoxia history-HC diet: 98.53% \pm 0.43; Hypoxia history-NC diet: 99.28% \pm 0.48; Hypoxia history-HC diet: 99.42% \pm 0.45).

Growth performance of fish during the 27-weeks growth trial was shown in Table 2. At the beginning of the growth trial, fish with the HC-dietary history displayed a higher body weight compared to fish with the NC-dietary history. From 0 to 9 weeks of the growth trail, fish with the HC-dietary history displayed a lower specific growth rate (SGR)

Table 2Effects of chronic hypoxia and high-carbohydrate diet stimuli on growth performance of trout during growth trial. Values are presented as mean \pm SD (n = 3).

Time	Index	NC dietary history		HC dietary history		p of Two-way ANOVA		
		Normoxia history	Hypoxia history	Normoxia history	Hypoxia history	Dietary history	Oxygen history	D*O
Weeks 0 to 9	Beginning body weight (g)	0.12 \pm 0.01	0.12 \pm 0.01	0.14 \pm 0.01	0.15 \pm 0.00	< .001*	0.456	0.909
	Final body weight(g)	0.90 \pm 0.01	0.90 \pm 0.01	0.98 \pm 0.03	0.98 \pm 0.04	< .001*	0.683	0.849
	SGR (%/d)	2.40 \pm 0.10	2.43 \pm 0.06	2.29 \pm 0.06	2.32 \pm 0.07	.012*	0.374	0.943
Weeks 9 to 21	Final body weight(g)	21.27 \pm 0.91	20.63 \pm 0.55	22.6 \pm 0.72	22.30 \pm 1.73	.043*	0.475	0.796
	SGR(%/d)	3.81 \pm 0.05	3.77 \pm 0.03	3.79 \pm 0.04	3.76 \pm 0.09	.681	0.339	0.842
	Feed intake (%/d)	1.75 \pm 0.02	1.78 \pm 0.02	1.75 \pm 0.00	1.72 \pm 0.03	.036*	0.882	0.061
Weeks 21 to 27	Feed efficiency	1.27 \pm 0.01	1.25 \pm 0.03	1.29 \pm 0.02	1.27 \pm 0.01	.160	0.860	0.154
	Final body weight(g)	53.67 \pm 2.83	51.24 \pm 6.91	58.38 \pm 8.21	52.58 \pm 3.79	.259	0.398	0.631
	SGR(%/d)	1.96 \pm 0.08	1.89 \pm 0.13	2.02 \pm 0.28	1.93 \pm 0.24	.650	0.506	0.926
	Feed intake (%/d)	1.81 \pm 0.08	1.89 \pm 0.08	1.89 \pm 0.05	1.88 \pm 0.10	.413	0.456	0.394
	Feed efficiency	1.01 \pm 0.03	0.98 \pm 0.03	1.01 \pm 0.04	1.02 \pm 0.07	.365	0.188	0.809

Stars and bold together indicate that the p value < 0.05.

than fish with the NC-dietary history but also a higher final body weight. From 9 to 21 weeks of growth trial, a higher final body weight and a lower feed intake were found in fish with the HC-dietary history compared with their control group. There was no significant difference in growth index among treatments during the last period (21 to 27 weeks) of the growth trail.

3.2. Molecular confirmation of the effectiveness of hypoxic and dietary high-carbohydrates stimuli

The mRNA levels of hypoxic-related genes were measured in alevins to ensure the effectiveness of hypoxic stimulus (Fig. 1, stimulus 1). As was shown in Table 3, significant differences were found in the mRNA levels of *egln3a* (egl-9 family hypoxia-inducible factor 3 a), *egln3b* and *glut1aa* (glucose transporter-1a a) between hypoxic and normoxic stimulus group at the end of the stimulus 1. Compared to the normoxic controls, alevins which were subjected to the 15-days hypoxic stimulus (50% of O₂, Fig. 1) displayed significantly higher mRNA levels of *egln3a* and *egln3b*. In contrast, *glut1aa* (glucose transporter-1a a) displayed a significant lower mRNA level in alevins exposed to the hypoxic stimulus compared to the control normoxic fish. Apart from this, there was no difference in the mRNA level of other hypoxia-related genes among treatments.

To ensure the effectiveness of HC dietary stimulus (Fig. 1, stimulus

Table 3Effects of chronic hypoxia stimuli applied after hatching (312°D) on mRNA levels of hypoxic and glucose metabolism-related genes in alevins at the end of stimulus 1. Values are presented as mean \pm SD (n = 6).

Target gene	Normoxia (10 mg·L ⁻¹)	Hypoxia (5 mg·L ⁻¹)	p
<i>hif1ab1</i>	1.45 \pm 0.92	1.19 \pm 0.59	0.631
<i>hif1ab2</i>	1.29 \pm 0.74	1.17 \pm 0.74	0.521
<i>egln3a</i>	0.55 \pm 0.18	2.02 \pm 1.25	0.003*
<i>egln3b</i>	0.90 \pm 0.66	1.73 \pm 1.01	0.037*
<i>pdcl</i>	1.15 \pm 0.50	1.40 \pm 0.77	0.521
<i>ldhaa</i>	1.33 \pm 0.63	1.22 \pm 0.80	0.800
<i>ldhab</i>	1.20 \pm 0.51	1.01 \pm 0.80	0.633
<i>slc16a3a</i>	1.30 \pm 0.73	1.06 \pm 0.80	0.615
<i>slc16a3b</i>	1.55 \pm 0.78	1.18 \pm 0.51	0.354
<i>glut1aa</i>	1.97 \pm 1.70	0.01 \pm 0.005	0.016*
<i>glut1ab</i>	1.41 \pm 1.14	1.15 \pm 0.57	0.872
<i>glut1ba</i>	1.23 \pm 0.64	1.32 \pm 0.74	0.728
<i>glut1bb</i>	1.27 \pm 0.66	1.31 \pm 0.63	0.631
<i>glut2a</i>	1.33 \pm 0.87	1.37 \pm 0.86	0.631
<i>glut2b</i>	1.30 \pm 0.67	1.31 \pm 0.65	1.000
<i>glut4a</i>	1.20 \pm 0.42	1.22 \pm 0.61	0.951
<i>glut4b</i>	1.22 \pm 0.53	1.09 \pm 0.58	0.631

Stars and bold together indicate that the p value < 0.05.

2), mRNA levels of several glucose metabolism-related genes were analysed (Table 4) in alevins at the end of the dietary stimulus (Fig. 1, stimulus 2). mRNA levels of the glycolytic genes *gcka* (glucokinase a), *gckb* (glucokinase b) and *pfkmaa* (phosphofructokinase (muscle)-aa) were significantly higher in fish fed the HC diet compared to fish fed NC diet. By contrast, compared to fish fed the NC diet, fish fed the HC diet showed lower mRNA levels of gluconeogenic genes such as *pck1* (phosphoenol pyruvate carboxykinases 1), *fbp1b1* (fructose 1, 6-bisphosphatase-1b 1) and *fbp1a* (fructose 1, 6-bisphosphatase-1a), but a higher mRNA level of *g6pcb2a* (glucose 6-phosphatase-b2 a).

3.3. Plasma metabolites at the end of the 5-days challenge test in trout juveniles

After the 5-days challenge test (Fig. 1), fish with the hypoxic history displayed significant lower plasma triglycerides (TG) level compared to fish with normoxic history (Table 5). Besides, there was no significant difference in plasma glucose, lactate and NEFA concentration whatever the oxygen or dietary histories of fish.

3.4. mRNA levels of glucose metabolism-related genes in liver and muscle of trout juveniles at the end of the 5-days challenge test

In liver (Table 6), fish which were subjected to a chronic hypoxia stimulus at hatching showed a lower mRNA level of *glut1bb* (glucose transporter-1b b) than fish with the normoxic history. Apart from this, no other significant difference was observed in the liver of juvenile fish.

In muscle (Table 7), the mRNA level of *pfkmaa* was significantly higher in fish with the HC-dietary history than in fish with the NC-dietary history. Regarding to the effect of oxygen history, the mRNA level of *glut4b* (glucose transporter-4b) in fish with the hypoxic history was significantly lower than in fish with the normoxic history. Moreover, an interaction effect between oxygen and dietary histories was found for *pfkmab* (phosphofructokinase (muscle)-ab) in the muscle of fish juveniles.

4. Discussion

In recent years, the concept of metabolic programming has been considered as a promising strategy to enhance the use of alternative ingredients for fish meal and fish oil in aquafeed formula. As first studies in this field mainly investigated programming effects of strict nutritional stimuli (Geurden et al., 2007; Geurden et al., 2014; Gong et al., 2015; Izquierdo et al., 2015; Lazzarotto et al., 2015), an increasing interest recently arose for non-nutritional stimuli (Liu et al., 2017a; Liu et al., 2017b; Rocha et al., 2015) including hypoxia when considering the programming of glucose metabolism. Indeed, an acute early

Table 4

Effects of the HC dietary stimulus applied at first-feeding (536°D) on mRNA levels of glucose metabolism-related genes in alevins at the end of stimulus 2. Values are presented as mean \pm SD (n = 6).

Target gene	NC dietary history		HC dietary history		p of Two-way ANOVA		
	Normoxia history	Hypoxia history	Normoxia history	Hypoxia history	Dietary history	Oxygen history	D*O
<i>gcka</i>	0.05 \pm 0.10	0.01 \pm 0.00	1.92 \pm 0.40	1.46 \pm 1.19	< .001*	0.340	0.422
<i>gckb</i>	0.08 \pm 0.07	0.02 \pm 0.00	1.34 \pm 0.45	1.34 \pm 1.13	< .001*	0.905	0.900
<i>pfkmaa</i>	0.56 \pm 0.09	0.56 \pm 0.13	0.86 \pm 0.21	0.75 \pm 0.11	.001*	0.349	0.364
<i>pck1</i>	1.92 \pm 0.93	2.07 \pm 0.57	0.20 \pm 0.07	0.47 \pm 0.61	< .001*	0.419	0.817
<i>fbp1a</i>	0.70 \pm 0.25	0.91 \pm 0.25	0.56 \pm 0.09	0.57 \pm 0.15	.008*	0.170	0.225
<i>fbp1b1</i>	0.80 \pm 0.17	0.92 \pm 0.17	0.52 \pm 0.14	0.50 \pm 0.18	< .001*	0.468	0.346
<i>g6pcb2a</i>	0.37 \pm 0.08	0.34 \pm 0.08	0.92 \pm 0.46	1.20 \pm 1.32	.023*	0.667	0.599

Stars and bold together indicate that the p value < 0.05.

hypoxic stimulus was shown to affect the glucose metabolism of trout in the long-term but with moderate effects (Liu et al., 2017b). As the difference in duration and severity of hypoxia may induce distinct outcomes in physiology and metabolism (Richards, 2009). Hence, understanding how chronic hypoxia affects the long-term glucose metabolism-related gene expression in trout juveniles will likely contribute to our knowledge of metabolic programming strategy in this species.

4.1. The chronic hypoxic (stimulus 1) and HC dietary stimuli (stimulus 2) were effective

With the purpose of testing the effectiveness of the chronic hypoxic stimulus, hypoxia-sensitive genes were analysed in whole-body alevins at the end of chronic hypoxic stimulus. *Egln3* is considered as a cellular oxygen sensor, which can be quickly induced under hypoxic condition (Aprelikova et al., 2004; Manchenkov et al., 2015; Marxsen et al., 2004; Matagreenwood et al., 2017). As expected, in the present study, we observed an increase in mRNA levels of *egln3a* and *egln3b* under hypoxic conditions. This result confirmed the physiological response of alevins to the hypoxic stimulus, and is in accordance with the acute hypoxic study conducted by Liu et al. (2017a). Also, as well as in the study of Liu et al. (2017a) conducted with an acute hypoxic stimulus, no significant difference among treatments was found in the mRNA levels of *Hif1* (Hypoxia inducible factor 1) encoding genes, the master regulators of the cellular hypoxia response. This result was not completely surprising because *hif1* is reported to be regulated predominantly at the post-translational level. In the contrary and surprisingly, a significantly lower mRNA level of one glucose transport gene, *glut1a*, in whole-body alevins exposed to chronic hypoxia was noticed in the present study when compared with their normoxic controls. Glucose transporters (*gluts*) are indeed known to play an important role in hypoxic responses of aerobic animals by facilitating glucose uptake (Bunn and Poyton, 1996; Iyer et al., 1998; Zhang et al., 2003). However, low *Glut* mRNA level was previously reported by Xia et al. (1997), in adult rats under chronic hypoxia. These authors proposed that GLUT regulation depends on the duration of O₂ deprivation and showed that GLUT are also regulated at the protein level. These findings could explain at least partially our results. All in all, the obtained results here

demonstrated that the chronic hypoxia stimulus (Fig. 1, stimulus 1) is effective.

On the other hand, the effectiveness of HC dietary stimulus was confirmed by analysing the mRNA levels of glucose metabolism-related genes in trout alevins at first-feeding (536°D). These effects include the increase in mRNA levels of glycolytic genes *gcka*, *gckb*, the inhibition of gluconeogenic genes *pck1*, *fbp1b1*, and an atypical up-regulation of *g6pcb2a*, these results were consistent with the previous studies (Liu et al., 2017a; Marandel et al., 2016). In addition, an increase or a decrease in mRNA levels of *pfkmaa* and *fbp1a* were found in trout alevins received the HC diet respectively, which were not significantly affected in previous studies (Liu et al., 2017a). The longer duration of the HC dietary stimulus performed in the present study (8 days versus 5 days in Liu et al., 2017a) can be an explanation of these differences. All these results thus confirmed that the HC dietary stimulus worked on fish. Together, our results confirmed that both hypoxic and dietary stimuli were being sensed by alevins and were effective in the present study.

4.2. An early chronic hypoxic stimulus alone or combined with a high carbohydrate stimulus at first feeding has moderate programming effect in the long term

Both dietary and oxygen histories have first independent long-term effects on zootechnical parameters but also at the molecular level.

Concerning the programming effect of dietary stimulus (Fig. 1, stimulus 2) at the first-feeding (536°D) on growth performance of fish, our results showed that the HC dietary stimulus received at first feeding (536°D) had a positive effect on growth performance during the first 21 weeks of the growth trial in juveniles. However, this effect was erased at the end of the growth trial period, indicating that the dietary history has no persistent programming effect on growth performance. In comparison, Liu and collaborators observed a negative programming effect of a 5-days HC-dietary stimulus at first-feeding on growth performance in fish juveniles at the end of 24-weeks growth trial (Liu et al., 2017b). The difference between the results obtained in these two studies may be attributed to the difference in the durations of the stimulus period.

Besides, the long-lasting changes in gene expression patterns are one of extremely crucial biological mechanisms for mediating the

Table 5

Effects of chronic hypoxia and high-carbohydrate diet stimuli on plasma metabolites of trout juveniles after the 5-days challenge test. Values are presented as mean \pm SD (n = 9).

Plasma metabolites (mmol/L)	NC dietary history		HC dietary history		p of Two-way ANOVA		
	Normoxia history	Hypoxia history	Normoxia history	Hypoxia history	Dietary history	Oxygen history	D*O
Glucose	6.28 \pm 1.93	6.79 \pm 1.84	8.69 \pm 3.71	7.39 \pm 2.64	0.102	0.679	0.314
Triglycerides	4.58 \pm 1.94	3.24 \pm 1.39	4.07 \pm 2.10	3.04 \pm 1.36	0.555	0.048*	0.080
Lactate	52.96 \pm 14.87	52.37 \pm 4.45	54.98 \pm 19.84	55.58 \pm 14.54	0.592	0.998	0.904
NEFA ¹	0.36 \pm 0.09	0.33 \pm 0.08	0.40 \pm 0.12	0.35 \pm 0.12	0.389	0.282	0.782

Stars and bold together indicate that the p value < 0.05.

¹ NEFA: non-esterified fatty acid.

Table 6

Effects of chronic hypoxia and high-carbohydrate diet stimuli on the mRNA levels of glucose metabolism-related genes in liver of trout juveniles after the 5-days challenge test. Values are presented as mean ± SD (n = 9).

Target gene	NC dietary history		HC dietary history		p of Two-way ANOVA		
	Normoxia history	Hypoxia history	Normoxia history	Hypoxia history	Dietary history	Oxygen history	D*O
<i>gcka</i>	0.86 ± 0.69	0.87 ± 0.75	1.12 ± 0.71	0.89 ± 0.82	0.569	0.659	0.625
<i>gckb</i>	0.95 ± 0.56	1.06 ± 0.96	1.08 ± 0.67	0.74 ± 0.66	0.709	0.631	0.351
<i>pfkla</i>	1.68 ± 0.37	1.80 ± 0.62	1.79 ± 0.62	1.89 ± 0.56	0.572	0.560	0.949
<i>pfklb</i>	1.06 ± 0.22	1.06 ± 0.28	1.05 ± 0.24	0.98 ± 0.26	0.559	0.691	0.707
<i>pklr</i>	0.82 ± 0.26	0.91 ± 0.32	0.92 ± 0.30	0.86 ± 0.31	0.765	0.890	0.435
<i>pck1</i>	0.72 ± 0.54	0.69 ± 0.44	1.26 ± 0.78	0.62 ± 0.41	0.223	0.083	0.110
<i>pck2</i>	1.26 ± 0.49	1.02 ± 0.41	1.19 ± 0.54	0.96 ± 0.32	0.664	0.137	0.977
<i>fbp1a</i>	0.68 ± 0.34	0.71 ± 0.23	0.78 ± 0.65	0.66 ± 0.27	0.832	0.729	0.606
<i>fbp1b1</i>	0.86 ± 0.37	0.80 ± 0.36	0.91 ± 0.37	0.95 ± 0.38	0.415	0.951	0.685
<i>fbp1b2</i>	0.90 ± 0.36	1.02 ± 0.50	0.84 ± 0.34	0.92 ± 0.34	0.537	0.455	0.907
<i>g6pca</i>	0.81 ± 0.38	0.83 ± 0.28	0.79 ± 0.32	0.86 ± 0.36	0.983	0.688	0.861
<i>g6pcb1a</i>	0.87 ± 0.80	1.05 ± 0.79	0.92 ± 0.81	0.80 ± 0.90	0.717	0.902	0.596
<i>g6pcb1b</i>	0.90 ± 0.75	0.75 ± 0.60	0.96 ± 0.77	1.08 ± 0.52	0.379	0.926	0.550
<i>g6pcb2a</i>	1.73 ± 2.52	0.40 ± 0.36	1.16 ± 1.07	0.63 ± 0.71	0.731	0.060	0.405
<i>g6pcb2b</i>	0.92 ± 0.32	0.88 ± 0.22	1.09 ± 0.31	0.83 ± 0.14	0.494	0.101	0.211
<i>glut1ba</i>	1.14 ± 0.46	0.85 ± 0.52	1.18 ± 0.42	1.07 ± 0.92	0.547	0.330	0.658
<i>glut1bb</i>	0.98 ± 0.44	0.85 ± 0.27	1.20 ± 0.35	0.79 ± 0.19	0.490	0.020*	0.196
<i>glut2a</i>	0.87 ± 0.15	0.88 ± 0.23	0.93 ± 0.18	0.95 ± 0.24	0.344	0.834	0.885
<i>glut2b</i>	0.91 ± 0.35	1.07 ± 0.24	0.99 ± 0.22	0.95 ± 0.27	0.813	0.532	0.274

Stars and bold together indicate that the p value < 0.05.

persistence of programming effects (Lucas, 1998). In the present study, muscular glycolytic *pfkmaa* was up-regulated in the long-term in trout juveniles with HC dietary history. This finding was inconsistent with previous studies using a HC stimulus at first-feeding, which demonstrated that mRNA levels of *pfkm* genes decreased in the muscle of fish juveniles with a HC dietary history (Geurden et al., 2014; Liu et al., 2017b). To be noticed that, the duration of the HC dietary stimulus was different between previous (Geurden et al., 2014; Liu et al., 2017b) and the present studies, so combined with foregoing results, it can hypothesis that the duration of the HC dietary stimulus played a role in their long-term programming effects. At this point, it should be also noted that, similar to previous studies (Liu et al., 2017a) the present study balanced the dietary carbohydrate proportion against protein content to keep the same energy level in diets. Therefore, cautions should be taken when considering the exact causal factor for the long-term effect of dietary history: high dietary carbohydrates, low dietary protein, or both.

With respect to the effect of oxygen history (Fig. 1, stimulus 1), from our results, we concluded that hypoxia had a direct impact on reducing the plasma TG content, whereas such results were not

described by Liu et al. (2017b). Although to our knowledge no data are available in trout, previous studies in mammals reported that chronic hypoxia can alter TG concentration, with both up- or down-regulating effects (Hashimoto et al., 2013; Siques et al., 2014). Thus, in the current state of knowledges it is difficult to interpret this data. Apart from that, in the present study, the oxygen history had also an existing long-term effect on mRNA levels of glucose metabolism-related genes both in liver and muscle of juvenile trout. The drastically decreased mRNA levels of *glut1bb* in liver and *glut4b* in muscle demonstrated a decrease in glucose uptake in response to long-term hypoxic stimulus history. These results suggested that a chronic hypoxic stimulus had a long-term programming effect on glucose transport in trout juveniles. Such results were in contrast with previous studies, which reported that an early acute hypoxic stimulus increased glucose transport in the long term in trout (Liu et al., 2017a; Liu et al., 2017b) and to our knowledge no comparable results have ever been reported in teleost fish. As a conclusion, our findings combined highlighted that the duration, and/or intensity of the hypoxic stimulus lead to differential long-term programming effect as well as the duration of a dietary HC stimulus at first-feeding.

Table 7

Effects of chronic hypoxia and high-carbohydrate diet stimuli on the mRNA levels of glucose metabolism-related genes in muscle of trout juveniles after the 5-days challenge test. Values are presented as mean ± SD (n = 9).

Target gene	NC dietary history		HC dietary history		p of Two-way ANOVA		
	Normoxia history	Hypoxia history	Normoxia history	Hypoxia history	Dietary history	Oxygen history	D*O
<i>pfkmaa</i>	0.87 ± 0.41	1.05 ± 0.25	1.34 ± 0.46	1.18 ± 0.39	0.024*	0.913	0.199
<i>pfkmab</i>	0.88 ± 0.42	1.28 ± 0.56	1.06 ± 0.34	0.85 ± 0.40	0.407	0.502	0.041*
<i>pfkmba</i>	0.91 ± 0.76	1.17 ± 0.46	1.27 ± 0.58	1.51 ± 0.58	0.095	0.223	0.960
<i>pfkmbb</i>	1.06 ± 0.62	1.04 ± 0.40	1.25 ± 0.80	1.02 ± 0.32	0.655	0.508	0.585
<i>pkmaa</i>	0.86 ± 0.46	0.83 ± 0.27	1.29 ± 0.88	0.86 ± 0.37	0.212	0.220	0.273
<i>pkmab</i>	1.06 ± 0.43	1.01 ± 0.29	1.17 ± 0.28	1.03 ± 0.24	0.407	0.545	0.677
<i>pkmba</i>	0.85 ± 0.55	1.08 ± 0.33	1.13 ± 0.46	1.21 ± 0.36	0.170	0.288	0.643
<i>pkmbb</i>	0.93 ± 0.53	1.20 ± 0.40	0.98 ± 0.32	1.14 ± 0.52	0.978	0.156	0.732
<i>glut1ba</i>	1.00 ± 0.46	1.09 ± 0.62	1.22 ± 0.26	0.92 ± 0.24	0.866	0.467	0.163
<i>glut1bb</i>	0.92 ± 0.32	0.88 ± 0.22	1.09 ± 0.31	0.83 ± 0.14	0.494	0.101	0.211
<i>glut4a</i>	0.86 ± 0.40	0.89 ± 0.44	0.96 ± 0.38	0.85 ± 0.31	0.054	0.114	0.286
<i>glut4b</i>	1.09 ± 0.97	0.78 ± 0.24	1.29 ± 0.57	0.66 ± 0.17	0.816	0.021*	0.401
<i>slc16a3a</i>	0.81 ± 0.68	0.98 ± 0.43	0.84 ± 0.59	0.79 ± 0.25	0.658	0.737	0.511
<i>slc16a3b</i>	0.95 ± 0.62	0.60 ± 0.39	1.25 ± 0.89	1.04 ± 0.68	0.104	0.214	0.772

Stars and bold together indicate that the p value < 0.05.

Finally and noteworthy, an interactive effect of the early chronic hypoxic stimulus at hatching and the diet received at first-feeding (536°D) were observed on the mRNA level of *pfkmab* in the muscle of juvenile fish. The significance of such an interaction on the glycolysis capacity of the muscle in these juvenile trout remained to be elucidated.

5. Conclusions

Taken together, our results demonstrated that a chronic hypoxic stimulus applied at hatching alone or combined with a HC dietary stimulus at first-feeding can lead to long-term programming effects on plasma metabolism parameters and on mRNA levels of glucose metabolism-related genes in trout juveniles.

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Competing interests

The authors declare that there are no conflicts of interests.

Authors' contributions

L.M. and S.P. designed the study. L.M. and J. L. managed the study. H.H. performed the experiment, sample analysis, statistical analysis and wrote the manuscript. E.P.J. provided technical assistance in molecular biology assays. A.H. performed plasma analysis. I.L. and L.G. developed the protocol to maintain long-term hypoxia and sampled alevins after hypoxia (sampling 1). I.G. sampled the hatched fish and designed the diet formulae. L.M., S.P. and J.L. contributed to the manuscript corrections.

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CHAPTER IV

DISCUSSION & PERSPECTIVES

CHAPTER 4 DISCUSSION AND PERSPECTIVES

4.1 Part I Further investigation about the roles of DNA methylation/demethylation related genes in trout

4.1.1 Conservation of the DNA methylation-related enzymes in trout (even for the paralogs) but neo-/sub-functionalisation

Finding all the paralogues for target genes is of great importance when working on animal models with a complex genome such as rainbow trout, since the retained duplicated genes may have divergent expression patterns and specialised regulatory functions (Conrad and Antonarakis, 2007; Kuang et al., 2016; Storz et al., 2013).

In the present thesis, we identified all the mammalian *DNMT1*, *DNMT3*, *TET1/2/3* and *TDG* orthologues in trout genome (2 *dnmt1*, 8 *dnmt3*, 2 *tet1*, 3 *tet2*, 2 *tet3* and 4 *tdg*) and clarified their respective evolutionary history in chordate phylum. Our results highlighted the fixation of multiple *dnmt3* genes in trout after successive WGD events. These results may support the contribution of WGDs to the expansion of gene families and also to the genetic and phenotypic diversity in teleost (Voldoire et al., 2017). However, it is still unclear why *dnmt3* family is more prone to be retained after WGD than other investigated genes like *dnmt1* and *tet*. Previous studies have demonstrated that the probability of gene retention was found to be positively correlated with the level of GC content and the level of gene expression (McGrath et al., 2014). It has been demonstrated that genes encoding ribosomal proteins, transcription factors and intracellular signalling proteins are overrepresented among maintained duplicates (McGrath et al., 2014). By contrast, genes linked to housekeeping and DNA repair and replication tended to quickly revert back to a singleton gene copy state following duplication (De Smet et al., 2013).

The sub- or neo-functionalisation represent common fates of retained duplicated genes after a WGD event. Through analysing the transcriptional expression patterns of all DNA methylation/ demethylation related genes during gametogenesis and embryogenesis in trout, our results found divergent expression profiles within certain paralogous genes. One of the most striking evidence is that, we observed opposite expression patterns between two *dnmt3ba* orthologues (*dnmt3ba1* and *dnmt3ba2*) during oogenesis in trout (**Fig. 4-1**). Besides, our data also showed a particular high mRNA level of *dnmt3bbb* during early ontogenesis as well as a unique high expression of *dnmt3aa* after hatching (**Fig. 4-2**). These results further

confirmed that the duplicates of DNA methylation/ demethylation related genes in trout may be sub- or neo-functionalised after WGD, although these divergences in transcriptional expression patterns seem less pronounced during ontogenesis than gametogenesis.

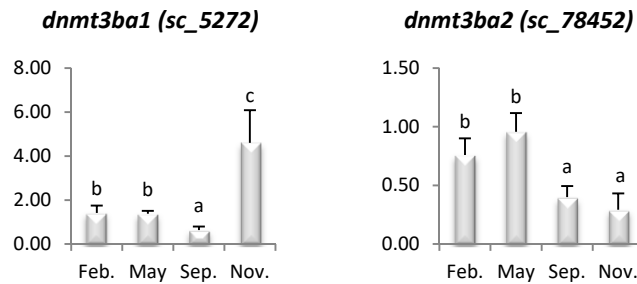


Figure 4-1 Expression patterns of *dnmt3ba* ohnologues during oogenesis in trout Ovaries were sampled (6 individuals per time point) in February, May, September and November, 2017, corresponding to pre-early vitellogenesis, vitellogenesis, late vitellogenesis, and spawn stage, respectively. Data are presented as the mean \pm SD (n=6 individuals), significant differences were evaluated by nonparametric Kruskal-Wallis test followed by a Tukey's test ($p < 0.05$).

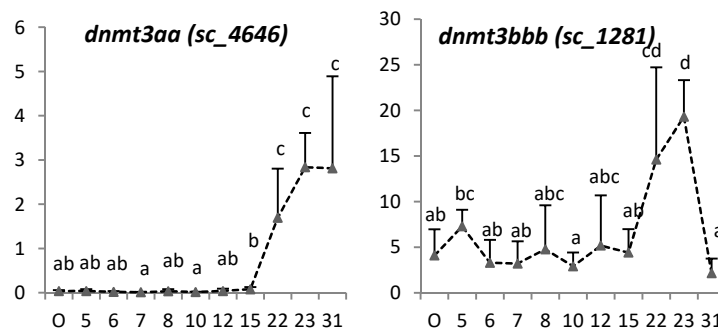


Figure 4-2 The mRNA level of *dnmt3aa* and *dnmt3bbb* during ontogenesis in trout Samplings were performed according to Vernier (1969) at stages O (oocyte), 5, 6, 7, 8, 10, 12, 15, 22, 23 and 31 (hatching alevin). The relative expression levels of target genes were calculated as the concentration ratio between target gene and exogenous luciferase. Data are presented as the mean \pm SD (n=3 pools of embryos or 6 individual alevins per spawn), significant differences were evaluated by nonparametric Kruskal-Wallis test followed by a Tukey's test ($p < 0.05$).

In order to explain what may contribute to the differential transcriptional patterns of duplicated genes involved in DNA methylation in trout, we further investigated if there was any difference in conserved motifs in their protein sequences. Our results showed that similar to the closed expression profiles during gametogenesis and ontogenesis, the duplicates of *dnmt1* on one hand and *tdga/b* on the other hand shared high identity in their respective

conserved protein sequences (Chapter III, Publication1, supplementary figure S2 and S9 for *dnmt1* and *tdg*, respectively). Besides, although there was low primary sequence similarity among Tet proteins in trout, zebrafish and mouse, the conserved motifs in catalytic domain of Tet families (*i.e.* iron and 2-OG binding sites) can be found (Fig. 4-3). In addition, our results further confirmed that Tet2b and Tet2c share only part of the sequence with Tet2a sequence in trout, with Tet2b lacking the cysteine-rich domain whereas Tet2c lacking the DBSH domain in the C-terminal catalytic domain. In this sense, it seems that only *tet2a* has an intact C-terminal catalytic domain thus representing as the valid *tet2* copy in trout.

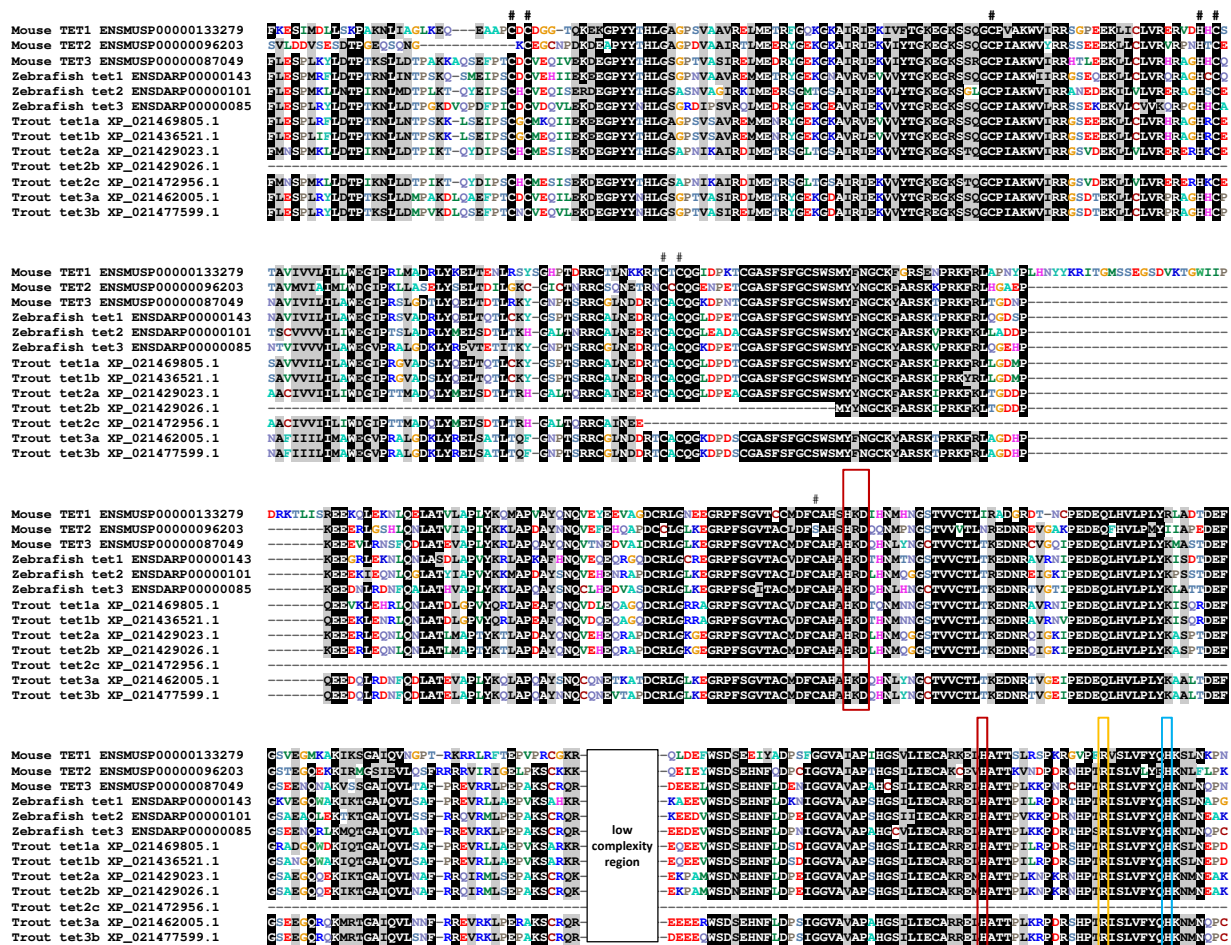


Figure 4-3 Catalytic domain alignment for tet enzymes in mouse, zebrafish and trout. # Zn binding sites; red box, Fe binding sites; yellow box, 2-OG binding sites; blue box, 5mC binding sites.

By contrast, in terms of Dnmt3 related sequences, our results highlighted particular variations in protein sequences of some paralogues in trout. As was previously mentioned in Chapter I of the present thesis, the Dnmt3 family of enzymes have a PWWP domain and an ADD domain in the N-terminal as well as 6 conserved DNMT motifs in C-terminal (I, IV, VI,

VIII, IX and X). One of the most interesting findings is that, conserved motif IX was not found in trout Dnmt3aa, although the other motifs are highly conserved even in different species (Fig. 4-4). It seems that no specific binding sites were identified around this motif. However, previous studies have demonstrated that the conserved motif IX in DNMT family could be responsible for the enhancement of catalytic activity (Kumar et al., 1994). In addition, the N-terminal ADD domain of Dnmt3aa in trout also exhibited divergent protein sequences compared to other DNMT3 family proteins (Fig. 4-5). It remains unclear how the loss of motif IX in catalytic domain and the lack of conservation for N-terminal ADD regulatory domain in Dnmt3aa would affect its function in *de novo* methylation in trout. It could be hypothesised that the alterations in these conserved domains in Dnmt3aa of trout may be a reason for its differential expression profile during ontogenesis.

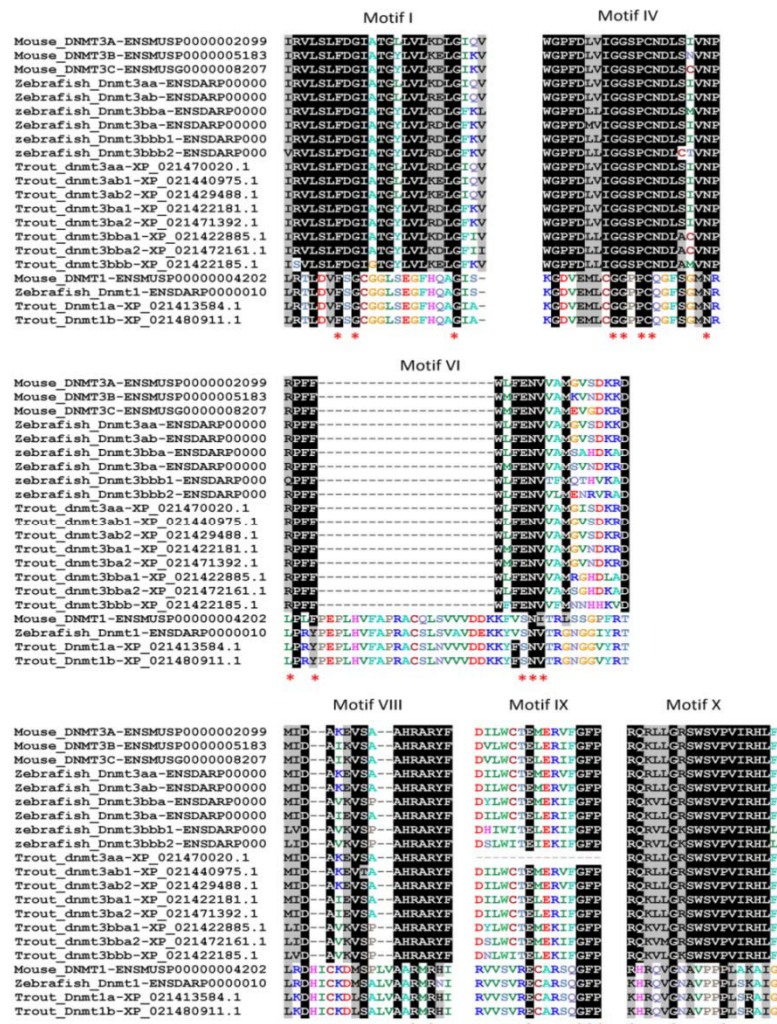


Figure 4-4 Conserved motif sites in catalytic domain of DNMTs of mouse, zebrafish and trout. Conserved motifs are marked with red asterisk.

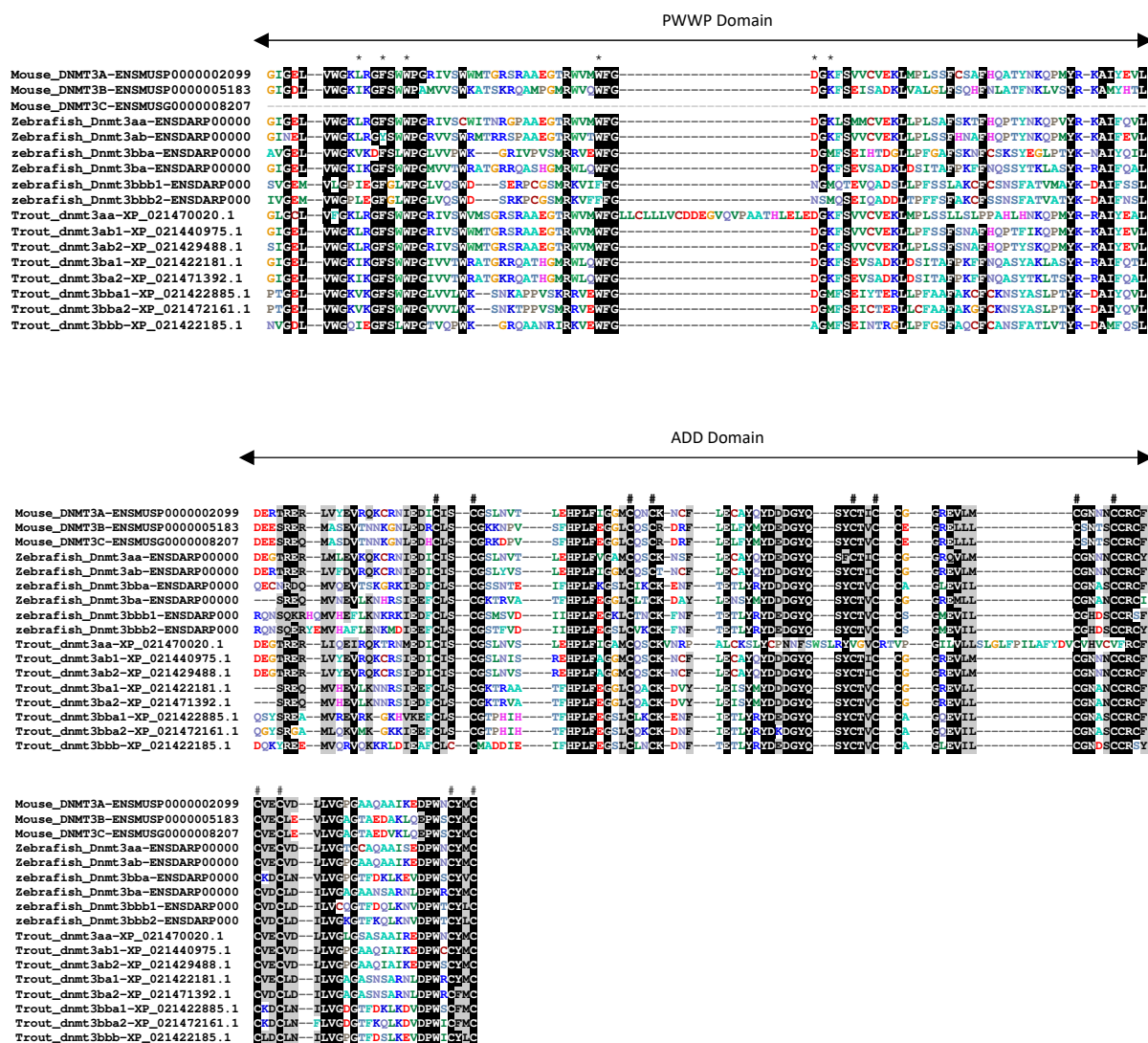


Figure 4-5 N-terminal conserved domains in DNMT3s of mouse, zebrafish and trout. Putative chromatin binding sites are marked with asterisk; Zn binding sites are marked with #.

Apart from the difference in protein sequences, other reasons which may lead to the different transcriptional patterns of duplicated *dnmt*, *tet* and *tdg* genes especially during gametogenesis in trout could be due to the **potential distinct transcriptional regulatory that dependent on sex**. Indeed, results obtained from the present thesis showed that the transcriptional expression patterns of DNA methylation/demethylation related genes were differently regulated during **oogenesis** and **spermatogenesis**. Sexually dimorphic expression profile of *de novo* DNA methyltransferases during gonadal development has also been previously reported in Nile tilapia (Wang et al., 2018). These diverse expression profiles of DNA methylation/demethylation related genes in gonads during gametogenesis could be

involved in the establishment of a sex specific methylation pattern (La Salle and Trasler, 2006).

4.1.2 Evolution of the expression territories of *dnmt*, *tet* and *tdg* genes

Previous studies showed that the sub- or neo- functionalisation of duplicated genes after WGD is mostly occurred at the gene regulatory level instead of protein level, and the fixation of duplicated genes enable more-specialised regulatory control of development through acquisition of new spatiotemporal expression territories (Kassahn et al., 2009). Previous studies in mammals have widely documented that DNA methylation related genes have been found to be highly expressed in reproduction related tissues (*i.e.* testis, ovary, oocyte) and embryos (Hales et al., 2011; Labbé et al., 2017). However, relevant information remains scarce in teleost, especially regarding the possible divergent roles of duplicated genes after WGD. Comparative spatial expression pattern studies may provide unique insights into the functional evolution of duplicated genes. We thus investigated the tissue expression pattern of genes involved in DNA methylation/demethylation pathways through *in silico* analysis of RNA-seq data in several teleost species including rainbow trout using the Phylofish database (Pasquier et al., 2016) (**Fig. 4-6**).

Data obtained in the present study specifically showed that almost all *dnmt*, *tet* and *tdg* genes were highly expressed in testes and/or ovaries and/or embryos and/or unfertilised eggs in salmonid species and other teleost species depending on the tissues available for analysis in Phylofish. These findings confirmed the importance of these genes within the reproduction process and embryonic development and as such, seemed to have been conserved between teleost and mammals. In addition, our data showed a higher abundance of *tet* genes in gills of all teleost species, indicating that these genes might gain new function in this aquatic species-specific tissue. One hypothesis to explain the relatively high transcriptional expression of *tet* genes in gills of teleost could be due to the fact that this tissue interacts with the external water environment frequently and thus a sensitive response in active demethylation could be important for the quick acclimation to environmental changes. Future studies concerning the different functional roles of these duplicated genes in epigenome remodelling under different nutritional/environmental conditions could be of great interest.

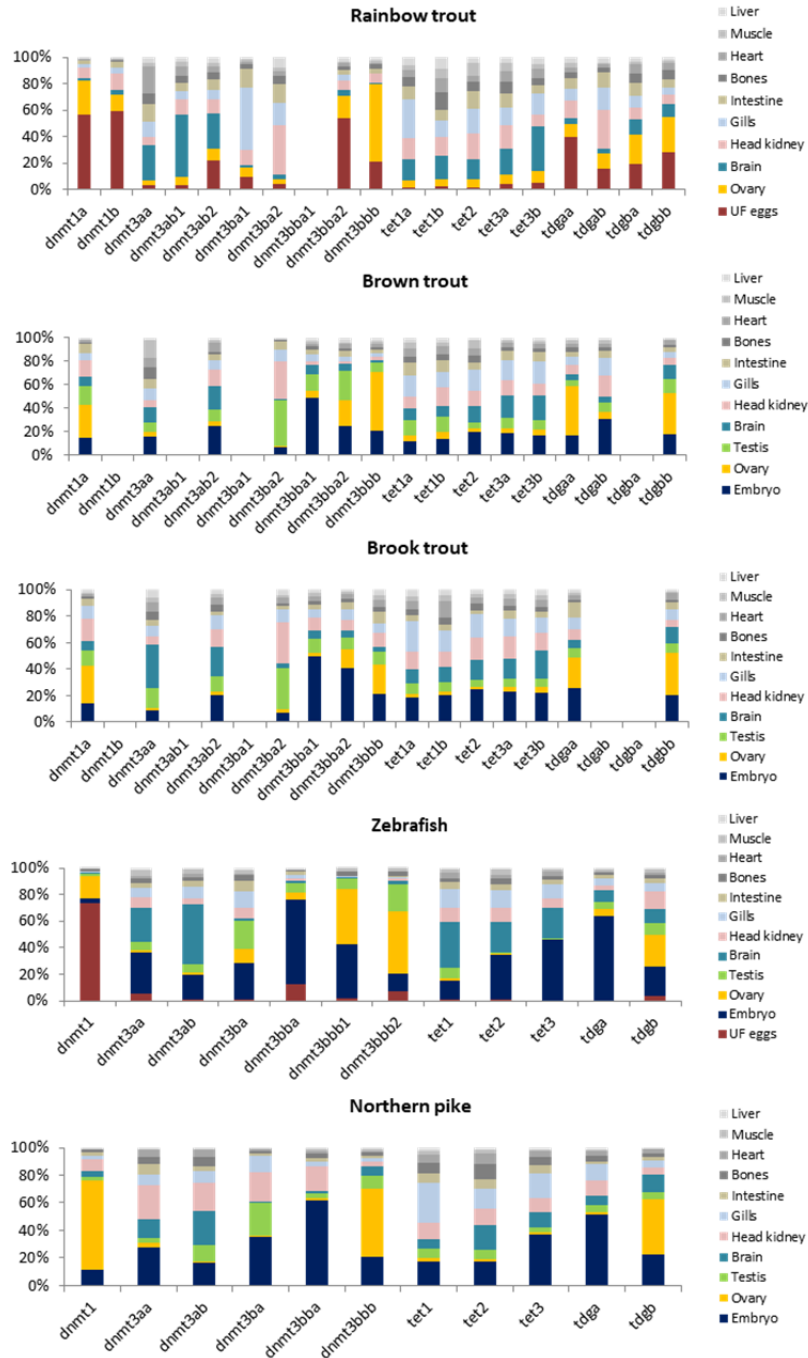


Figure 4-6 Tissue expression pattern of *dnmt*, *tet* and *tdg* genes in selected teleost species. Data collected from the Phylofish database (Pasquier et al., 2016).

4.2 Part II Hepatic global DNA hypomethylation after feeding a high carbohydrate/low protein diet in trout: causes and beyond

4.2.1 New insights on dietary factors that induced global non-CpG hypomethylation in the liver of trout

An inspiring finding of the present thesis is that our results revealed the potential of remodelling the hepatic global DNA methylation landscape through a short-term (4 days) stimulus by dietary changes in carbohydrates and/or dietary protein level in trout. Remarkably, data obtained from our work highlighted that this dietary factors induced alteration in hepatic global DNA methylome, namely the global DNA hypomethylation, took place primarily at non-CpG sites and CpG sites out of C^mCGG sequences in the genome. Moreover, this dietary factors induced loss of global 5-mdC content was believed to proceed through an active demethylation pathway and thus leading to a concomitant accumulation of 5-cadC level. Indeed, our results found that the decrease in dietary protein level induced stronger effects in the loss of hepatic global methylation over the increase in dietary carbohydrate level. Nonetheless, 4-day feeding with both low protein and high carbohydrate diets are considered to be efficient in provoking a hepatic global hypomethylation phenotype in this fish. Although the high carbohydrate diet accompanied hyperglycaemia seems not to affect the global methylation patterns (both C^mCGG and 5-mdC levels) in our feeding trial, a significant global C^mCGG hypomethylation was observed in the liver of fish 24 h after receiving a glucose injection (**Fig. 4-7**). Since the hyperglycaemia level in glucose-injected fish was around one fold higher compared to those fed with a high carbohydrate diet, it could be hypothesised that the hyperglycaemia induced hepatic global CpG hypomethylation could be observed in a dose-dependent manner. **To the best of our knowledge, it is indeed the first animal study which uncovers the relationship between a short-term manipulation in dietary macronutrients composition *in vivo* and the hepatic global methylation status focusing both C^mCGG and 5-mdC oxidative intermediates levels.**

Our data showed that estimation of CpG methylation given by LUMA assay did not seem to reflect a good overview of what happen at global level, *i.e* at all cytosine sites. Nonetheless, the proportion of C^mCGG methylation in all CpG methylation in trout genome remain unclear. In our case DNA hypomethylation can occur at CpG sites located out of C^mCGG sites but also at non-CpG sites. Up to date, there was limited information about the functional significance concerning **non-CpG methylation/demethylation**. However, increasing

evidence has demonstrated that non-CpG methylation is particularly prevalent in low proliferating tissues including liver and muscle (Patil et al., 2014) and in metabolic context (Barres et al., 2009). In addition, it seems that non-CpG methylation is more responsive to environmental factors than CpG methylation, and thus representing as a primary mediator of environmental stimuli, *i.e.* diet, induced alterations epigenetic landscapes and metabolism (Fuso and Lucarelli, 2019). It will be thus interesting to evaluate in the future if the hypomethylation that we highlighted here occurred mostly at CpG sites out of C^mCGG sequences or at non-CpG sites. These points of view combining with the findings obtained from the present study thus providing new insights on the importance of modification in rearing protocols in affecting the epigenome and metabolism of economic fish species during aquaculture practice. For instance, it is well known that during the trout culture process, they were fed with commercial diets of different composition according to their weights. Regarding our data, it is uncertain what epigenetic consequences for this transition of diet may induce, and whether the potential alterations in epigenetic landscape will finally contribute to a specific metabolic phenotype.

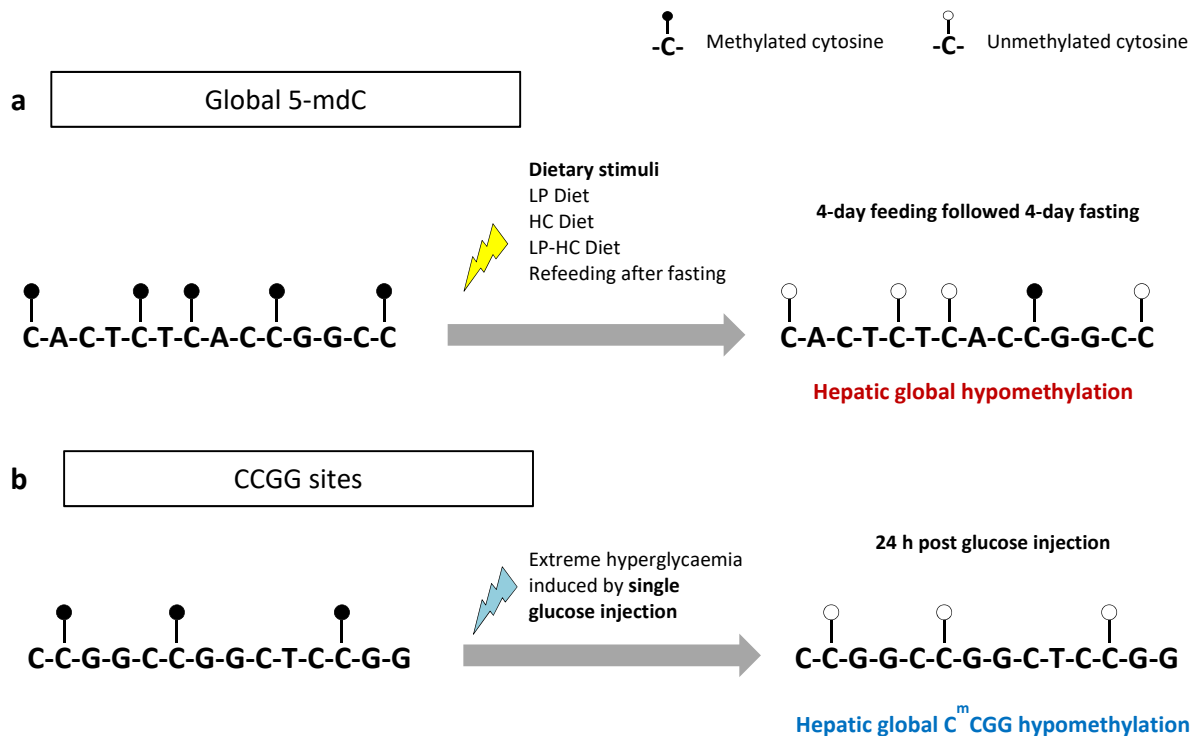


Figure 4-7 Main findings concerning the effects of dietary carbohydrates, dietary protein and hyperglycaemia on global DNA methylation in liver of trout. HC, high carbohydrate; LP, low protein.

Results obtained from the present thesis also found that **the transition between fasting and feeding status** has powerful influence in regulating the **global DNA methylome as well as the transcription of genes involved in DNA methylation/demethylation pathways**. For instance, in both feeding trial, the global 5-mdC level was significantly higher in the liver of fasted fish than fed fish regardless of the dietary composition. Also, there were more significant differences between fasted and fed fish in the mRNA level of *dnmt*, *tet* and *tdg* genes in the liver. Similar finding has never been reported in trout and other teleost species before. The detail mechanisms underlying this nutritional status induced alterations in hepatic global DNA methylation level remain elusive. However, it could be hypothesised that the nutritional status-related hormones such as insulin may play important roles DNA methylation/demethylation. Previous studies in human hepatocyte showed that methionine deficiency in the culture medium resulted in a 1.36 fold decrease in 5-mdC content of hepatocyte, whereas this decrease was more obvious when stimulated with insulin (1.46 fold) (Chiang et al., 2009). In addition, previous studies have demonstrated that the energy homeostasis is closely related to the regulation of DNA methylation/demethylation (Gongol et al., 2018).

To be noted that, teleost species generally evolved to endure long-lasting food-deprivation conditions. For many species, a fasting period consists part of the natural life cycle, *i.e.* winter months, spawning migration, and pre-spawning stage (Navarro and Gutiérrez, 1995). Besides, fasting is also frequently applied before some routine handling processes in aquaculture such as transportation, vaccination. It remains unclear to what extent the increased hepatic global DNA methylation level observed during fasting condition in the present thesis may contribute to the adaptive evolution of teleost under durable starvation. Besides, whether or not the nutritional status/dietary factors induced modifications in epigenome are reversible later in life after removing the original stimuli still need to be investigated in the future.

4.2.2 Limitations and obstacles in the experimental design of the present study

As pair-feeding is hard to implement in fish, **increasing one ingredient in a diet formula induced the decrease of another ingredient and vice versa**. Thus in the aim of trying to keep the energy and dietary lipid content equivalent from one diet to the other to free from the effect of these parameters, as well as covered the fish nutritional requirement, compromises have to be done with the diet formula. Indeed, in the feeding trial 2, aiming at

discriminating the effect of high carbohydrate or low protein content on the methylome, low digestible cellulose and crude starch were included with different proportion in the CTR and LP diets as bulk stuffs and thus, allowing CTR and the HC diets have similar crude protein level (40%) whereas the CTR and the LP diets have similar carbohydrates level. Twenty percent of crude starch was included in these two diets to avoid extreme high inclusion of cellulose, since high level of cellulose can change the hardness of the diet (Hansen and Storebakken, 2007) and particularly can disturb fish digestion in the long term. Theoretically, we considered the cellulose and crude starch cannot be used as dietary carbohydrate source thus the CTR and LP diets should have very low digestible carbohydrates. However, although we tried to decrease the machine temperature for extruding process during diet preparation, the crude starch seemed to be partly ‘cooked’, as was revealed by the slightly increased glycaemia level in trout fed these two diets (**Fig. 4-8a**). Nonetheless, our experiment could be considered efficient because the glycaemia level in HC group was much higher compared to those in trout fed the LP and CTR diets. The lack of statistically significant level could be due to the high individual variance within HC group. In addition, we also analysed the glycogen content in liver (**Fig. 4-8b**), which confirmed that the HC group received more glucose compared to the other two feeding groups.

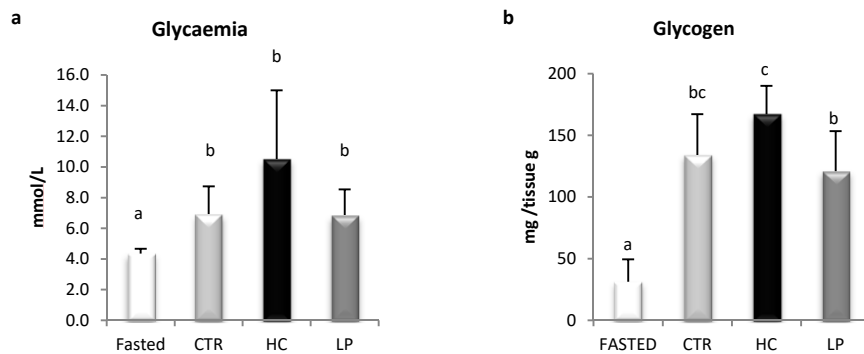


Figure 4-8 Glycaemia (a) and glycogen content (b) in trout subjected to 4-day fasting followed by 4-day feeding (feeding trial 2). Glycaemia and glycogen were measured after fasting (n=9) or at 6 h after last meal (n=12). CTR, control; HC, high carbohydrate; LP, low protein.

Furthermore, although the three diets are isolipidic, they have different digestible energy due to the unequal digestibility of gelatinised starch, crude starch and cellulose. The energy level could be a potential factor that can affect global DNA methylation. However, we were unable to improve this formula because it was the inherent constraint of all the *in vivo* experiment when studying macronutrients.

In terms of investigating the effects of hyperglycaemia on global DNA methylation level in liver, our **glucose/saline injection may be improved to overcome encountered bottlenecks**. First, the first sampling time at 6 h post injection could be already too late to observe potential changes in 5-mdC and other oxidised intermediates at this time point. Indeed, a previous study did in mice reported that the genome-wide 5-hmdC levels were rapidly increased in various mouse tissues within 30 min of glucose injection, and rapidly declined/recovered at 60 min post glucose injection (Yang et al., 2014a). Second, the glycaemia of trout received saline injection was also increased at 6 h post injection when compared to the fasted fish, although it was significantly lower when compared to their glucose injection counterparts. These data could implicate that the injection handling gave rise to stressful condition for the fish, and thereby affecting the global DNA methylation level (Blaze et al., 2018). Third, due to the differences in individual responses to handling stress, the experimental sample size (n=4 or 5) for this trial might be not enough. This could be revealed by the large standard deviation for most of the markers analysed.

Future work could centre on **how to improve the above limitations and refine the experimental design**. For instance, we may perform a kinetic analyse of the global DNA methylation dynamics after glucose injection, and find out a best window for monitoring the effects. Subsequently, the injection trial could be redo within the time range of the ‘best window’, as well as increasing the sample size to minimise the effects of individual differences. Furthermore, *in vitro* experiment could be considered as an alternative path to answer the question of which factor triggers the hepatic global DNA hypomethylation in trout fed a high carbohydrate diet. Through *in vitro* experiment, we can manipulate the concentration of glucose, amino acids as well as insulin and investigate the consequences on global DNA methylation status of hepatocytes. Nonetheless, cautions should be taken when comparing results obtained form *in vitro* and *in vivo* studies, as *in vitro* investigation has limitations due to the disconnection from a number of hormonal and neuronal inputs that *in vivo* essentially contribute to metabolic regulation. Finally, since our data concerning the qPCR analyses of DNA methylation/demethylation related genes, hepatic SAM-SAH content and aminothiols content did not seem to be explicative for the high carbohydrate/low protein diet induced hepatic global DNA hypomethylation phenotype in trout, further analyses about the contribution of TCA metabolites dynamics, oxidative stress, or AMPK signalling in regulating global DNA methylation could be of great interest.

4.3 Part III Programming of glucose metabolism in trout: achievement and challenges

4.3.1 How to ‘program’ fish: selection of the type of stimulus and the “windows of metabolic plasticity”

In metabolic programming studies, the most important questions should be considered are i) what type of stimulus could be appropriate, ii) what intensity/duration of the stimulus could be effective, iii) at which developmental stage could the stimulus be applied, namely choosing the ‘windows of metabolic plasticity’. Indeed, these three issues are interrelated with the last one serves as the base and should be considered prior to the other two questions.

In teleost species, the embryonic development exhibits high level of developmental plasticity as mammals since several important ontogenetic events begin to occur focusing on the development of organs buds and the onset of key regulatory pathways in metabolism (Anderson and Podrabsky, 2014; Schnurr et al., 2014; Soanes et al., 2011). Besides, the period of mouth opening during early larval stage, namely the first-feeding stage, is frequently used in most of the metabolic programming studies in teleost (Fang et al., 2014; Geurden et al., 2007; Geurden et al., 2014; Rocha et al., 2016a; Rocha et al., 2016b; Vagner et al., 2009; Vagner et al., 2007). The first-feeding stage is the earliest stage for receiving a nutritional stimulus in teleost species, since the majorities of them are oviparous and the embryos/newly hatched larvae rely exclusively on their endogenous yolk reserves before mouth opening (Fig. 4-9).

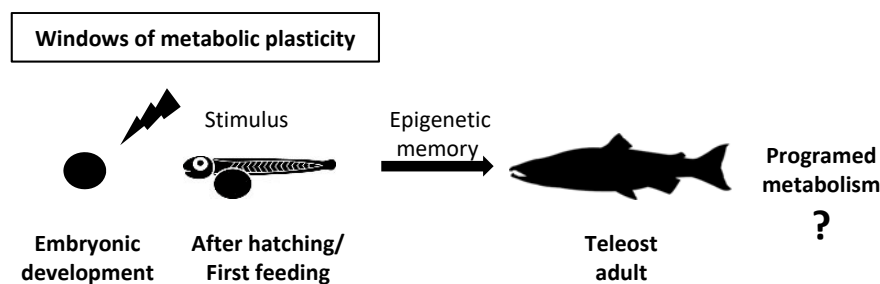


Figure 4-9 Windows of metabolic plasticity in teleost

In the present thesis, we hypothesised that first-feeding stage may be too late to program the glucose metabolism in juvenile trout (Marandel et al., 2016b). It could be assumed that other ‘windows of metabolic plasticity’ earlier than the first-feeding stage may be more interesting to modulate the long-term glucose metabolism in trout. Consequently, **two new**

‘windows of metabolic plasticity’ during early development were tested in the present thesis, namely i) embryonic stage before or around the period when the onset of glucose metabolism-related genes and the development of several vital organs including primitive liver occur, and ii) after hatching.

Concerning the types of stimulus, to deliver a nutritional stimulus to teleost before mouth opening seems not possible, since at this stage the alevins were fed only with their endogenous yolk reserve. Metabolic programming studies through manipulations on parental nutrition thus modulating the nutrient transfer to the yolk reserved of embryos could be one solution. Nonetheless, if choosing to conduct the metabolic programming study before the first-feeding stage, **a non-nutritional environmental factor such as oxygen**, temperature, and light, *etc.* could be considered **as promising candidates for stimulus**. Previous studies proved that a glucose stimulus through microinjection during embryogenesis can program the glucose metabolism in juvenile zebrafish (Rocha et al., 2015). However, it is actually hard to manipulate and also difficult to be applied in other species (*i.e.* sea bream) due to the fragility of embryos (Rocha, 2015). In the present thesis, hypoxia was selected as the stimulus because it is demonstrated to be a powerful environmental factor that can modulate both glucose metabolism (Dunn and Hochachka, 1987; Rissanen et al., 2006; Wright et al., 1989) and the epigenetic landscapes (Melvin and Rocha, 2012; Watson et al., 2010). Besides, previous studies in mammals have proved that hypoxia during early life can induce long-lasting programming effects on glucose metabolism in later life (Iqbal and Ciriello, 2013; Osumek et al., 2014).

In terms of the **intensity and the duration of the hypoxic stimulus**, the present thesis tested the long-term programming effects of a severe acute hypoxia (25% dissolved oxygen, 24h) at embryonic stage (152 °D) or a moderate chronic hypoxia (50% dissolved oxygen, 15 days) after hatching (312 °D) on glucose metabolism in trout juveniles. Indeed, a pre-experiment were done previous to the thesis to test the tolerance of embryos and alevins to different hypoxia level (25%, 50% and 75% dissolved oxygen during 30 min, 4 h, 6 h and 24 h). Results from this pre-experiment showed no significant difference for survival regardless of the hypoxia intensity or duration when applied at embryos as well as hatching alevins (data not shown). However, when applied an acute hypoxic stimulus (both 25% and 50% dissolved oxygen level) in hatching alevins (552 °D), a significant increase in mortality was observed as the decrease in oxygen level in the present thesis (Chapter III, publication 3). We thus stopped this treatment after first feeding stage to avoid the potential selection effects. But in

chronic hypoxia experiment of the present thesis, a 15-day chronic hypoxia at 50% dissolved oxygen level applied to 312 °D alevins did not affect their survival. Interestingly, results obtained from the present thesis proved that even inside the chorion, trout embryo can sense an environmental stimulus as was showed by the up-regulated mRNA level of hypoxia responsive genes. However, this physical barrier seems to soften the environmental impact since a higher mortality was observed when applied the same level of hypoxic stimulus (20% dissolved oxygen, 24h) in hatched alevins.

4.3.2 Metabolic programming of trout with hypoxia: early hypoxic history induced alteration in glucose metabolism in juvenile trout- highlights of the effects on glucose transporter related genes (the gluts)

In the present thesis, one of the most interesting results concerning the programming effects of early hypoxic stimulus during early life on glucose metabolism in juvenile trout is that, we found persistent up- or down-regulating effects for glucose transporter genes in trout juveniles several months after hypoxic exposure (**Fig. 4-10**). For instance, after a 24 h acute hypoxic stimulus ($2.5 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$, 25% dissolved oxygen), an increase mRNA level of *glut1ba* was observed in hypoxic whole embryos compared to their normoxic counterparts, and this elevation was persistently observed in the liver of juvenile trout with hypoxic history when challenged with a high-carbohydrate (HC) diet (30%). It might be postulated that the exposure to an acute hypoxic stimulus during embryonic development could modify the epigenetic signatures of *glut1ba* gene (*i.e.* promoter hypomethylation), thus leading to a permanent activation of its transcription. However, no similar findings have been reported in previous study, thus it is really a preliminary speculation. Besides, when challenged with a HC diet, increased mRNA levels of other glucose transporters (hepatic *glut2b* and muscular *glut4* ohnologues) were also observed in juvenile trout previously exposed to acute hypoxia, although no direct effect of hypoxic stimulus was found in any of these genes in trout whole embryos at the last hour of stimulus. Similarly but also contrarily, we observed a down-regulated mRNA level of *glut1aa* in the whole-body of trout alevins at the end of a 15-day chronic hypoxic stimulus ($5.0 \text{ mg}\cdot\text{L}^{-1} \text{ O}_2$, 50% dissolved oxygen), as well as the decreased mRNA level of hepatic *glut1bb* and muscular *glut4b* after a 5-day HC dietary challenge test in juvenile trout with a chronic hypoxic history. These results indicated that an early hypoxic stimulus may not alter the transcription of these genes directly during the stimulus but can induce changes in their transcription in later life when fed with a HC diet. The detail mechanisms underlying the early hypoxia induced long-term programming effects on glucose

transporters in trout juveniles remain elusive. Nonetheless, our results demonstrated that hypoxic history during early life could alter the glucose transport in juvenile trout several months after exposure. In addition, the acute and chronic hypoxic stimuli led to completely opposite effects on the transcription of glucose transporter genes. There could also be sub-or neo-functionalisation of different *glut* paralogues in terms of the sensitivity to acute or chronic hypoxia, since they exhibited different expression patterns in response to hypoxic stimulus.

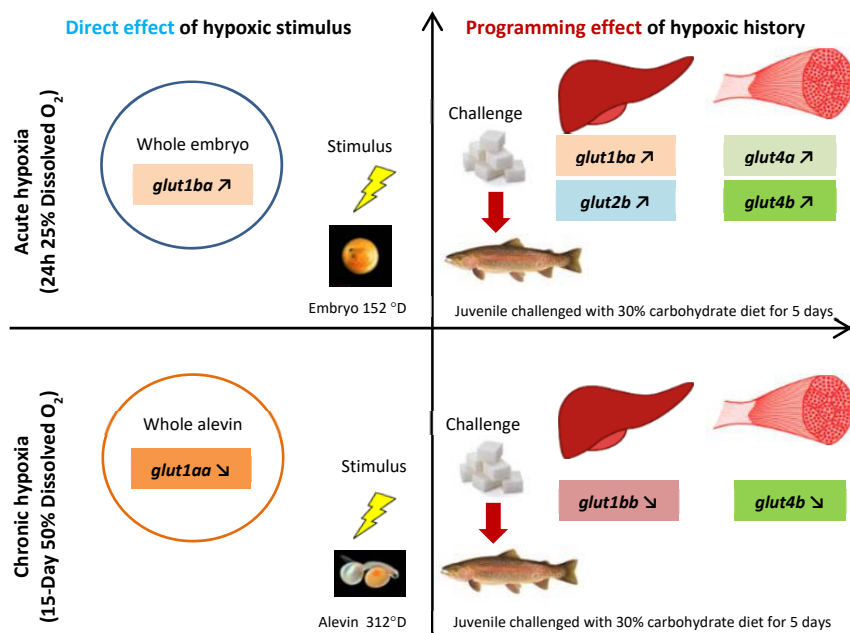


Figure 4-10 Direct and programming effect of acute and chronic hypoxia on the mRNA level of glucose transporter genes in rainbow trout

In terms of the **direct effects of hypoxia on the transcription of glucose transporter genes**, previous studies in mammals have widely documented that reductions in oxygen tension can lead to dose-dependent increases in the expression of glucose transporter at both mRNA level (Chen et al., 2001; Ouiddir et al., 1999; Park et al., 2016; Terova et al., 2009) and protein level (Bashan et al., 1992; Ouiddir et al., 1999; Park et al., 2016). The enhancement of glucose transport has been deemed to be important for the adaptive response to hypoxia, because the shift from oxidative phosphorylation to anaerobic glycolysis for ATP production necessitates the acceleration of glucose transport (Ismail-Beigi, 1993; Zhang et al., 1999). This hypoxia inducing glucose transporter expression is regulated at multiple levels, including transcriptional (through HIF signalling pathway) (Baumann et al., 2007; Kilic et al., 2006), mRNA stabilisation (Stein et al., 1995), translational (Wood et al., 2007), and

posttranslational mechanisms (*i.e.* translocation of transporters to the plasma membrane) (Guan et al., 2008; Siques et al., 2018; Zhang et al., 1999).

In addition, previous studies also showed that the transcriptional expression of *Glut1* can be stimulated by both hypoxia and the inhibition of oxidative phosphorylation, whereas the other members of *Glut* genes cannot. This unique feature makes *Glut1* a special member of *Glut* gene family whose transcription is dually controlled by hypoxia (Zhang et al., 1999). In contrast to acute hypoxia, the down-regulated transcriptional expression of one mammalian *GLUT1* orthologue *glut1aa* in trout alevins subjected to 15-day chronic hypoxia in the present thesis was unexpected. There was scarce evidence which reported an inhibiting effect of hypoxic condition on *glut* transcription both in mammals and teleost. One example in human study reported that high altitude hypoxia during pregnancy inducing low birth weight is linked to the reduced GLUT1 protein expression in basal membrane fractions of placentas (Zamudio et al., 2006). Because *in vivo* GLUT1 expression has been shown to be regulated by extracellular glucose concentrations, insulin, insulin-like growth factors and other effectors apart from hypoxia (Baumann et al., 2002), thus the decreased GLUT1 protein expression could be the net result of multiple factors, and that these signals can override the hypoxia-induced upregulation of the GLUT1 expression (Zamudio et al., 2006). Another example obtained in Atlantic cod, which showed that hypoxia induced an increase of the mRNA level of *glut1* in gill but also a decrease of *glut1* and *glut3* in spleen (Hall et al., 2014). The authors pointed out that this decrease in *glut* expression in spleen potentially reflected a general decrease in rates of transcription (Hall et al., 2014).

Nonetheless, there could be some other interpretations of the down-regulated mRNA level of *glut1bb* in trout alevins which received chronic hypoxia in the present thesis. First, the contrasting effects of acute and chronic hypoxia on the transcriptional expression of glucose transporter genes reveal that the cellular responses to hypoxia may depend on the intensity/duration of the hypoxic stimulus. The transcriptional upregulation of *glut* genes under hypoxia is mainly mediated through the activation of Hif-1 α signalling pathway. However, previous studies in mice have shown that the chronic hypoxia is unable to upregulate hypoxia-responsive processes (including glucose transport) due to the destabilisation of HIF-1 α protein (Moine et al., 2011). In this sense, the distinct regulating effects of acute and chronic hypoxia on glucose transporter genes in the present thesis maybe in line of these previous findings. Second, the decreased mRNA level of *glut1bb* in trout alevins subjected to chronic hypoxia could be potentially due to a lower demand for oxygen,

since prolonged hypoxia could induce the adverse effects on organ size and cell number through a reduction in proliferation rate or an increase in apoptosis (Bae et al., 2003; Botting et al., 2014; Cao et al., 2012; Louey et al., 2007).

Regarding the **long-term programming effects of hypoxic history on the mRNA level of glucose transporter genes**, our results highlighted that a moderate chronic hypoxia experienced after hatching seems to lead to an inferior transcriptional expression of *glut1bb* and *glut4b* compared to those without hypoxia exposure. However, a severe acute hypoxia exposed during embryonic development can activate the transcription of several *glut* genes (*glut1ba*, *glut2b*, *glut4a* and *glut4b*) in juvenile trout. Previous studies in mammals have reported that prenatal hypoxia can result in developmental programming of insulin resistance in offspring, which could be associated with the reduced protein expression of muscular GLUT4 (Camm et al., 2011). A study in rats demonstrated that hypoxia during pregnancy can induce an increase of GLUT2 protein expression in the liver of foetus, but a decrease in its expression in the liver of offspring (Cao et al., 2012). They also found that prenatal hypoxia may lead to ultra-structure damage in hepatic parenchyma cells, the alterations in hepatic insulin signalling, and finally the changes in hepatic function in insulin sensitivity and glucose metabolism in postnatal life (Cao et al., 2012). Similar results have been found in European sea bass, which showed that the *glut2* mRNA was increased after a moderate hypoxic episode during the larval stage but decreased in the liver of juvenile fish (Vanderplancke et al., 2015).

Taken together, results from the previous studies as well as the present thesis confirmed that **early hypoxia exposure can program glucose transport in later life**. However, these programming effects may vary in dependent of the hypoxic exposure intensity/duration, as well as the developmental stages when exposed to hypoxia. Besides, none of the acute and chronic hypoxia exposure during early life impaired the growth performance of juvenile trout in the present thesis, indicating that hypoxia could be a powerful stimulus for metabolic programming studies, although further improvement of the strategy should be considered. Moreover, the increased mRNA levels of glucose transporter genes in both liver and muscle of juvenile trout with an acute hypoxic history in the present thesis may imply that a better glucose utilisation in these fish, even though evidence was achieved only at the transcriptional level.

4.3.3 Mechanisms of programming: potential epigenetic mechanisms linked to the long-term programming effects

Mechanisms involved in metabolic programming effects have not yet been fully understood. Nonetheless, epigenetic regulation has attracted more and more attention for explaining the long-lasting alterations in metabolism caused by stimuli exposure during early life (Vickers, 2014). It has been widely reported that alterations in DNA methylation/demethylation and histone modifications could be associated with these long-term programming effects in metabolism. Epigenetic researches in teleost species are less extensive, especially concerning the field of metabolic programming studies. However, existing evidence has proved that both nutritional and non-nutritional factors can modify the epigenetic landscapes and thereby altering gene expression profile in teleost (Burgerhout et al., 2017; Cavalieri and Spinelli, 2017; Craig and Moon, 2013; Marandel et al., 2016a; Perera and Yúfera, 2017).

Most data concerning the epigenetic regulations of gene expression in metabolic programming researches focus on specific target genes involved in critical metabolic pathways. However, increasing studies have proved that modifications of the epigenome at global genomic level (*i.e.* global DNA methylation and global histone modifications) can also play important roles in various biological processes and programmed changes in metabolism (Laird and Jaenisch, 1994; Panserat et al., 2017; Perera and Yúfera, 2017; Seligson et al., 2005). Other than being targeted to promoters, methylation of DNA molecules or modifications of histones, can occur over large regions of chromatin including coding regions and non-promoter sequences, which are referred to as global DNA methylation or histone modifications (Nelson et al., 2011; Vogelauer et al., 2000). Aberrant epigenomic status is associated with genome instability. Changes in global levels of DNA methylation or individual histone modifications are believed to be predictive of clinical outcome for diseases (Fouad et al., 2018; Seligson et al., 2005).

In the present thesis (Chapter III, publication4), analyses of global DNA methylation and certain global histone modifications were performed in juvenile trout in order to explore long-term programming effects of an acute hypoxic stimulus during embryogenesis and/or a hyperglucidic dietary stimulus at the first-feeding on glucose metabolism and the global epigenetic landscapes (**Fig. 4-11**). Our results highlighted a significant interacting effect for oxygen history and dietary history on hepatic H3K9me3 level in juvenile trout. Although

further analyses showed no statistically difference between each individual group, the programming effects of hypoxic history tended to be more obvious when combined with a high dietary carbohydrate history. Indeed, in trout experiencing a high-carbohydrate dietary stimulus at the first-feeding stage, those which were previously exposed to hypoxic stimulus during embryonic development showed slightly higher hepatic H3K9me3 level in juvenile fish compared to those with a normoxic history when challenged a high carbohydrate diet.

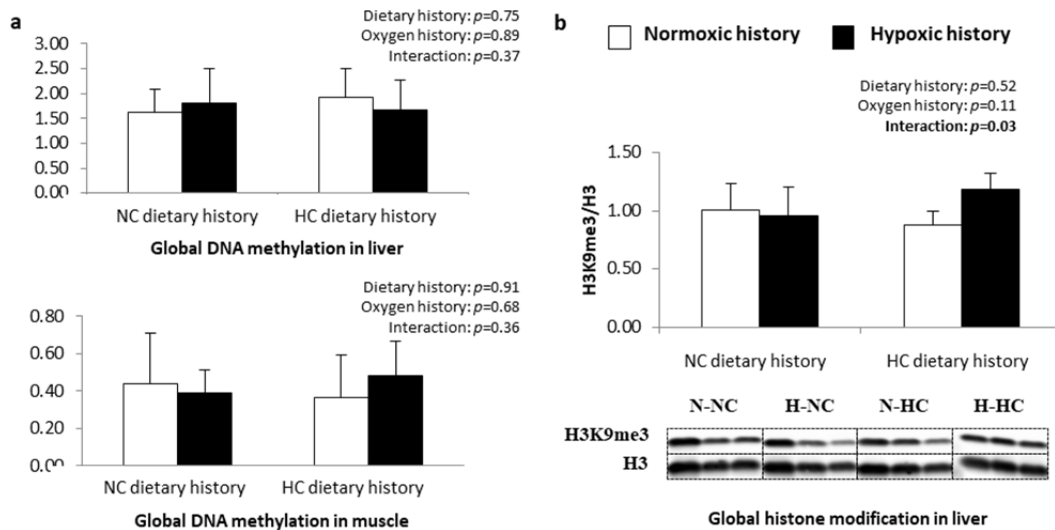


Figure 4-11 Effects of embryonic hypoxia and HC diet at first-feeding on global DNA methylation (a) and trimethylation of histone 3 lysine 9 levels (b) in juvenile trout. HC, high carbohydrate; NC no carbohydrate; N-NC, normoxia-no carbohydrate diet; H-NC, hypoxia

On one hand, previous studies in mammals demonstrated that **histone modifications** can be induced by high glucose (Honma et al., 2007; Ishikawa-Kobayashi et al., 2012; Lin et al., 2016; Yu et al., 2012b), although the detail mechanisms through which the modifications proceed are not clear. On the other hand, hypoxia is also a powerful regulator for histone modifications. Oxygen serves as a cofactor for the function of both two classes of histone demethylases: the histone demethylases KDM1 (lysine demethylase 1) family and the Jumonji C (JmjC) domain containing demethylases (JHDMs) (Klose et al., 2006; Kooistra and Helin, 2012). H3K9me3 is known to be a key repressive and relatively stable epigenetic chromatin mark in mammals (Kimura, 2013). Decreased of H3K9me3 at promoter region of key inflammatory genes is suggested to be involved in metabolic memory of high glucose induced proinflammatory phenotype of diabetic cells (Villeneuve et al., 2008; Yu et al., 2012b). An increase in di- and tri-methylation of H3K9, two typically repressive marks, are among the best characterised global modifications induced under hypoxia (Chen et al., 2006;

Tausendschön et al., 2011). Taken together, concerning the interaction effects of dietary history and oxygen history on hepatic global H3K9me3 level, it might be hypothesised that an early acute hypoxic stimulus induced increase in global H3K9me3 level may attenuate over time, but when combined subsequently a HC-diet stimulus at first-feeding, this alteration was strengthened and maintained durably. Although we don't know what would be the consequences for the increasing of global H3K9me3 level on gene expression, the overall higher hepatic global H3K9me3 level in trout with both hypoxic and HC dietary histories may indicate a more compacted genome status in these fish. In this sense, it seems that a hyperglucidic stimulus at first-feeding is probably not recommended after an acute hypoxic stimulus during embryonic development.

In terms of the analyses of **global DNA methylation**, no significant difference was observed in both liver and muscle of juvenile trout when challenged with a high carbohydrate diet, regardless of the dietary and oxygen histories. Notably, the Part3 of my thesis was actually conducted prior to the other two parts, and the quantification of global DNA methylation level was performed with ELISA method. Moreover, the significant contributions of global non-CpG hypomethylation to the poor dietary carbohydrates utilisation phenotype in trout, as well as the development of HPLC method in measuring global 5-mdC and its oxidative intermediates in our lab were only achieved during the Part 2 of my thesis at the end of my PhD study. Therefore, we did not analyse the global 5-mdC methylation level in these samples. We cannot exclude the possibility that the acute hypoxic history and dietary high carbohydrate history during early life can program the global methylation at non-CpG or CpG sites out of C^mCGG sequences in juvenile trout. Further studies need to be done in order to answer clearly the question.

In addition, we did not monitor the epigenetic changes in chronic hypoxia experiment (Chapter III, publication 5), as there were actually few alterations in gene expression at the transcriptional level of genes analysed in the long-term. The effects of the initial programming event may be transient and reversible after a long-term exposure to a range of other external factors in later-life. However, further studies concerning the long-term programming effects on gene-specific DNA methylation alterations (*i.e.* glucose transporter genes) could be an outlook. Because there are large number of genes involved in early development, but each of them is likely to exhibit unique changes in methylation patterns in response to early environmental modifications. Depending on the direction of those changes, effects could be washed out in a global assessment (Doherty et al., 2016).



CHAPTER V

GENERAL CONCLUSIONS

CHAPTER 5 GENERAL CONCLUSIONS: NEW MAJOR KNOWLEDGE LEARNED FROM THE PRESENT THESIS

5.1 What new knowledge did we obtain to explain the poor carbohydrate utilisation in trout?

Results obtained from the present thesis found that 4-day feeding of a high carbohydrate diet can trigger a hepatic global DNA hypomethylation phenotype in trout. This global erase of DNA methylation mark was occurred most likely at non-CpG or CpG sites out of C^mCGG sequences through an active demethylation pathway, since a concomitant accumulation of global 5-cadC level was also observed. Additionally, we found that hyperglycaemia provoked by glucose injection alone can also induce hepatic global DNA hypomethylation in trout, but this demethylation process mostly took place at C^mCGG sequences. Our data for the first time discovered that a short-term dietary carbohydrates feeding can change the global non-CpG methylation pattern in trout, which may be potentially linked to the poor dietary carbohydrate utilisation in this species by inducing potential genome instability. The detail mechanisms underlying this dietary high carbohydrates and/or low proteins induced demethylation process remain unclear.

5.2 Is it possible to improve the dietary carbohydrate utilisation of trout with metabolic programming strategy?

The present thesis showed that it is possible to use a non-nutritional stimulus, hypoxia, during early developmental stage to program the glucose metabolism in liver and muscle of juvenile trout without reducing growth performance. Results highlighted that an acute or chronic hypoxia experienced during early developmental stage can profoundly regulate the mRNA levels of glucose transporter genes in both liver and muscle of juvenile trout. However, these latter genes were modulated in different way depending on the intensity/duration of the hypoxic stimulus. Indeed, the increased mRNA levels of glucose transporter genes in both liver and muscle of juvenile trout with an acute hypoxic history may imply a better glucose utilisation in these fish, even though evidence was achieved only at the transcriptional level. Nonetheless, concerning the programming effects of an acute hypoxic stimulus on global DNA methylome, our results showed that when challenged with a high carbohydrate diet at juvenile stage, there was no significant difference in global DNA methylation level in both liver and muscle regardless of their oxygen history. One of the

limitations for the present thesis is that we should analyse more deeply the epigenetic mechanisms in our programming experiments.

5.3 What are the potential impacts of the present thesis on aquaculture?

To sum up, data obtained from the present thesis may deepen our understanding of how dietary manipulations induced epigenetic changes could help to elucidate the origin of phenotypes in teleost, enlighten the exploration of novel breeding strategies for tailoring their adaptation to new feed stuffs, and finally promote the prosperity of aquaculture industry.

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ANNEXES

LIST OF PUBLICATIONS & COMMUNICATIONS

Publications during PhD

- (1) **Liu, J.**, Plagnes-Juan, E., Geurden, I., Panserat, S. & Marandel, L. (2017). Exposure to an acute hypoxic stimulus during early life affects the expression of glucose metabolism-related genes at first-feeding in trout. *Scientific reports*, 7(1), 363.
- (2) **Liu, J.**, Dias, K., Plagnes-Juan, E., Veron, V., Panserat, S. & Marandel, L. (2017). Long-term programming effect of embryonic hypoxia exposure and high-carbohydrate diet at first feeding on glucose metabolism in juvenile rainbow trout. *Journal of Experimental Biology*, 220(20), 3686-3694.
- (3) Hu, H.*, **Liu, J.***, Plagnes-Juan, E., Herman, A., Leguen, I., Goardon, L., Geurden, I., Panserat, S. & Marandel, L. (2018). Programming of the glucose metabolism in rainbow trout juveniles after chronic hypoxia at hatching stage combined with a high dietary carbohydrate: Protein ratios intake at first-feeding. *Aquaculture*, 488, 1-8. (*Co-first author)
- (4) Véron, V.*, Marandel, L.*, **Liu, J.**, Vélez E.J., Lepais O., Panserat S., Skiba, S. & Sciliez, I. (2018). Epigenetic regulation of *bnip3* and *bnip3l* genes induced by metabolic programming. *BMC Genomics*, 19(1), 677 (*Co-first author)
- (5) Marandel, L.*, Kostyniuk, D. J. *, Best, C., Forbes, J. L. I., **Liu, J.**, Panserat, S., & Mennigen, J. A. (2019). Pck-ing up steam: Widening the salmonid gluconeogenic gene duplication trail. *Gene*, 698, 129-140. (*Co-first author)
- (6) **Liu, J.**, Hu, H., Panserat, S. & Marandel, L. (2019). Evolutionary history of DNA methylation related genes in chordates: new insights from multiple whole genome duplications. (**Under review**)
- (7) Marandel, L., Hu, H., Larroquet, L., Surget, A., **Liu, J.**, Plagnes-Juan, E., Maunas, P., Turonnet, N., Mennigen, J.A., Bobe, J., Burel, C., Corraze, G. & Panserat, S. (2019). A high carbohydrate broodstock nutrition during a whole reproductive cycle differentially affects the physiology and metabolism of males and females rainbow trout but does not impact reproductive performances (**Under review**)
- (8) **Liu, J.**, Burel C., Herault C., Véron V., Panserat, S. & Marandel, L. Factors triggering hepatic global DNA hypomethylation phenotype in trout fed a high-carbohydrate diet (**In preparation**)

Communications (* presenter)

- **Liu, J.***, Hu H., Panserat S. & Marandel L., Expression pattern of DNA methyltransferase genes during gametogenesis and early development in rainbow trout *Oncorhynchus mykiss*. World Aquaculture Society AQUA 2018, August 25-29, Montpellier, France (**Oral presentation**)
- **Liu, J.***, Plagnes-Juan, E., Geurden, I., Panserat, S. & Marandel, L., An acute hypoxic exposure during early life affects the mRNA level of glucose metabolism-related genes at the first-feeding in rainbow trout (*oncorhynchus mykiss*). Aquaculture Europe 2017, October 17 - 20, 2017, Dubrovnik, Croatia (**Oral presentation**)
- Marandel, L.*, Hu, H., **Liu, J.**, Mennigen, J.A., Bobe, J., Corraze, G., Burel, C. & Panserat, S., Consequences on Gametogenesis and Reproduction Performances of a High Carbohydrate Nutrition During the Whole Reproductive Cycle of Males and Females Trout., Experimental Biology 2019, April 6-9, 2019, Orlando, USA (**Poster presentation**)
- Heraud, C.*, **Liu, J.**, Véron, V., Panserat, S. & Marandel, L.*, Approches complémentaires de l'étude de la méthylation des cytosines de l'ADN - Application au génome de la truite : LuMA vs LC-UV vs ELISA, Journées EpiPHASE 2019, June 26–27, 2019, Toulouse, France (**Oral presentation**)

RESEARCH ARTICLE

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DNA methylation of the promoter region of *bnip3* and *bnip3l* genes induced by metabolic programming

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Abstract

Background: Environmental changes of biotic or abiotic nature during critical periods of early development may exert a profound influence on physiological functions later in life. This process, named developmental programming can also be driven through parental nutrition. At molecular level, epigenetic modifications are the most likely candidate for persistent modulation of genes expression in later life.

Results: In order to investigate epigenetic modifications induced by programming in rainbow trout, we focused on *bnip3* and *bnip3l* paralogous genes known to be sensitive to environmental changes but also regulated by epigenetic modifications. Two specific stimuli were used: (i) early acute hypoxia applied at embryo stage and (ii) broodstock and fry methionine deficient diet, considering methionine as one of the main methyl-group donor needed for DNA methylation. We observed a programming effect of hypoxia with an increase of *bnip3a* and the four paralogs of *bnip3l* expression level in fry. In addition, parental methionine nutrition was correlated to *bnip3a* and *bnip3lb1* expression showing evidence for early fry programming. We highlighted that both stimuli modified DNA methylation levels at some specific loci of *bnip3a* and *bnip3lb1*.

Conclusion: Overall, these data demonstrate that methionine level and hypoxia stimulus can be of critical importance in metabolic programming. Both stimuli affected DNA methylation of specific loci, among them, an interesting CpG site have been identified, namely – 884 bp site of *bnip3a*, and may be positively related with mRNA levels.

Keywords: Rainbow trout, Metabolic programming, *bnip3*, *bnip3l*, Hypoxia, Methionine, DNA methylation

Background

Biotic and abiotic environmental changes encountered during critical early windows of developmental plasticity may exert a profound influence on physiological functions later in life [1]. This process named “developmental programming” has been proposed to prepare adult phenotypes to better cope with specific environment [2–4]. Over the past decades, developmental programming has been widely studied in mammals [5]. In contrast, the first studies on this topic in fish were initiated only recently and were mainly focused on nutritional and metabolic programming. In 2007, Vagner et al. showed higher and

persistent delta 6 desaturase mRNA levels when European sea bass larvae were fed a low levels of n-3 HUEFA diet [6]. In addition, an hyperglucidic diet applied at first feeding was found to lead to persistent changes in levels of transcripts related to glucose digestion and utilisation in zebrafish, gilthead seabream and rainbow trout juvenile [2, 3, 7, 8]. More recently long term effects of first feeding with plant-based diet have been tested successfully in trout, salmon and seabass, confirming the concept of nutritional programming in fish [9–12].

Most of these studies applied a nutritional stimulus at first exogenous feeding. However it is known that embryogenesis also represents a sensitive window of metabolic plasticity during which a stimulus may have a more efficient long-term effect [4, 13]. For instance, an acute hypoxic stimulus applied in rainbow trout at

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embryo stage modified mRNA levels of several glucose-metabolism related genes at first feeding [14]. Moreover, after the embryonic hypoxic stimulus, the glucose metabolism in liver and muscle of rainbow trout juvenile was impacted, confirming a long term programming effect of a very early stimulus [15]. Programming can also be driven through parental nutrition. For instance, feeding rainbow trout broodstock for 6 months with a methionine deficient diet affected the expression of genes involved into methionine metabolism in fry fed for 21 days [16]. Moreover, genes involved in gluconeogenesis and autophagy were also affected by parental methionine deficiency [17]. These data confirm that developmental programming with nutritional and non-nutritional stimuli applied directly at embryo stage or through broodstock nutrition can induce long-term metabolic programming in fish.

Different biological mechanisms have been shown to be involved in developmental programming. Clonal selection of adapted cells during differential proliferation of tissue cell type could explain physiological adaptations in later life [18]. At molecular level, epigenetic modifications are the likeliest candidates to consider in the context of nutrition and more particularly when working on programming. Indeed, these mechanisms are influenced by metabolic state as well as environmental changes [19] and can be maintained at long-term during cellular divisions at least through mitosis [20], even through meiosis. In mammals, many studies have focused on mechanisms involving epigenetic regulation of genes expression and their role in nutritional or metabolic programming [21–23]. By contrast, in fish, few data are available on epigenetic mechanisms which potentially underlie programming phenotypes and are mainly limited to modifications occurring at the whole epigenome level [15, 24, 25].

In order to deeper investigate epigenetic modifications induced by environmental programming and their involvement on resulting phenotypes in teleost at target gene level, it is essential to focus on a model gene known to be sensitive to environmental changes but also regulated by epigenetic modifications in a strong and repeatable way. In the present study, we focused on *bnip3* (bcl-2/E1B-19 K interacting protein 3), and *bnip3like* (also known as *nix*), two genes involved in mitochondrial mediated apoptosis and/or mitochondrial autophagy upon diverse cellular stress including hypoxia [26–28]. Changes in the expression of these two genes were previously demonstrated to be regulated by DNA methylation under hypoxic environment [29–32] but also to be associated to the activity of the one-carbon metabolism [33], the major metabolic supply route of methyl groups that are required for DNA and histone methylation.

Here, we aimed at studying epigenetic regulation of *bnip3* and *bnip3l* genes in rainbow trout subjected to two specific stimuli known to strongly affect these genes,

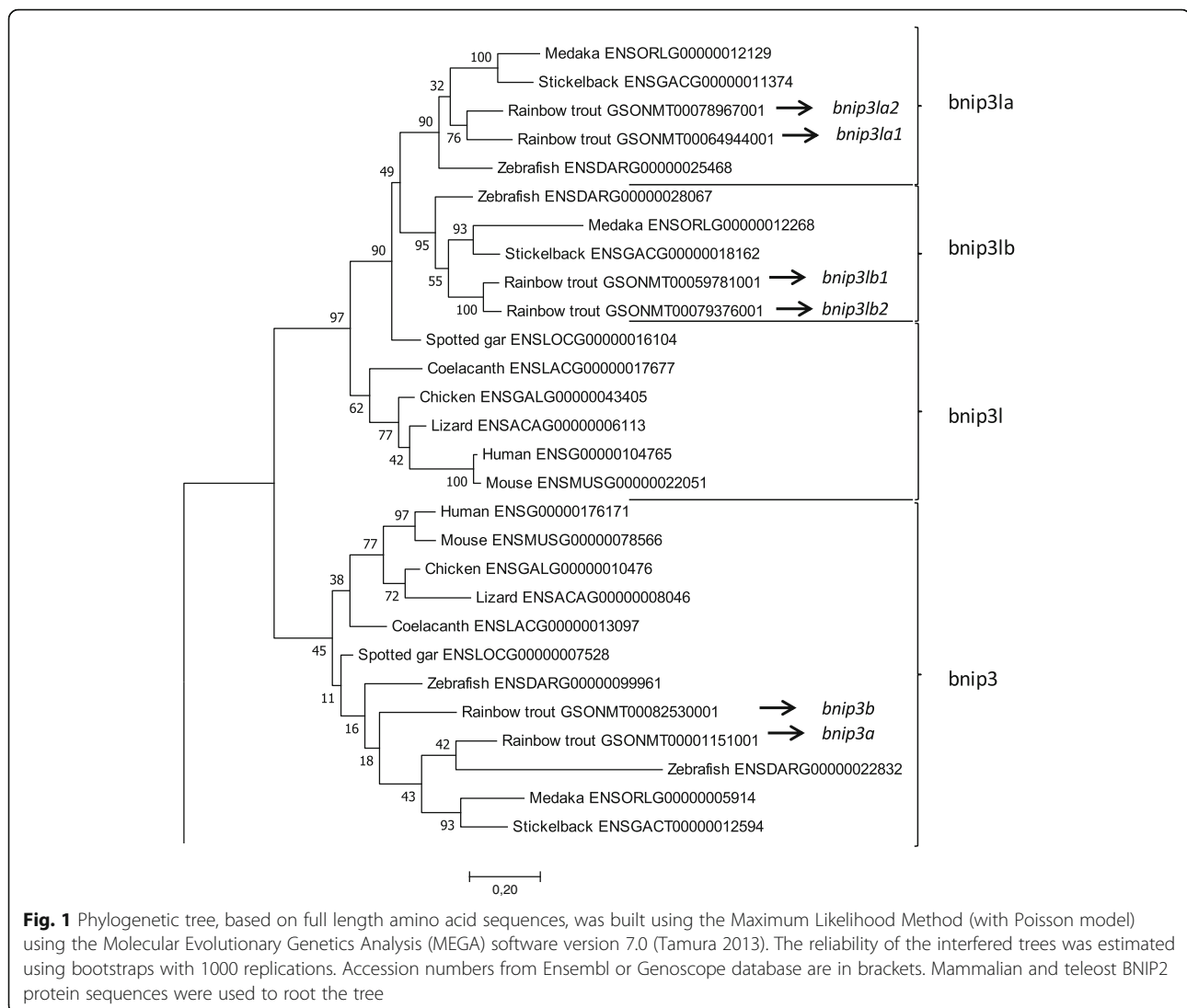
hypoxia and methionine deprivation, in a context of metabolic programming. First, as *bnip3* and *bnip3l* genes can be induced by hypoxia [34–37], we studied the programming effect of an early acute hypoxic stimulus applied at embryo stage on the regulation of *bnip3* and *bnip3l* genes at fry stage. Secondly, regarding the role of methionine as methyl donor for epigenetic modifications [38, 39], we investigated the programming consequences on the regulation of *bnip3* and *bnip3l* genes on progeny of parents fed a methionine deficient diet during gametogenesis. This last step allowed investigating for the first time intergenerational programming at the epigenetic level in rainbow trout.

Results

Identification of *bnip3* and *bnip3l* genes in rainbow trout

Using the recent availability of the rainbow trout genome assembly [40], we identified two genes (Genoscope accession number: GSONMT00001151001 and GSONMT00082530001) sharing a high sequence homology (E-value $>2e-09$, Sigenae tblasn <http://www.sigenae.org/>) with the zebrafish *bnip3* available in Ensembl (ENST00000368636.8). Similarly, we found 4 genes (Genoscope accession number: GSONMT00078967001, GSONMT00064944001, GSONMT00079376001 and GSONMT00059781001) sharing a high sequence homology (E-value $>2e-16$) with the zebrafish *bnip3la* available in Ensembl (ENSDART00000035676.4).

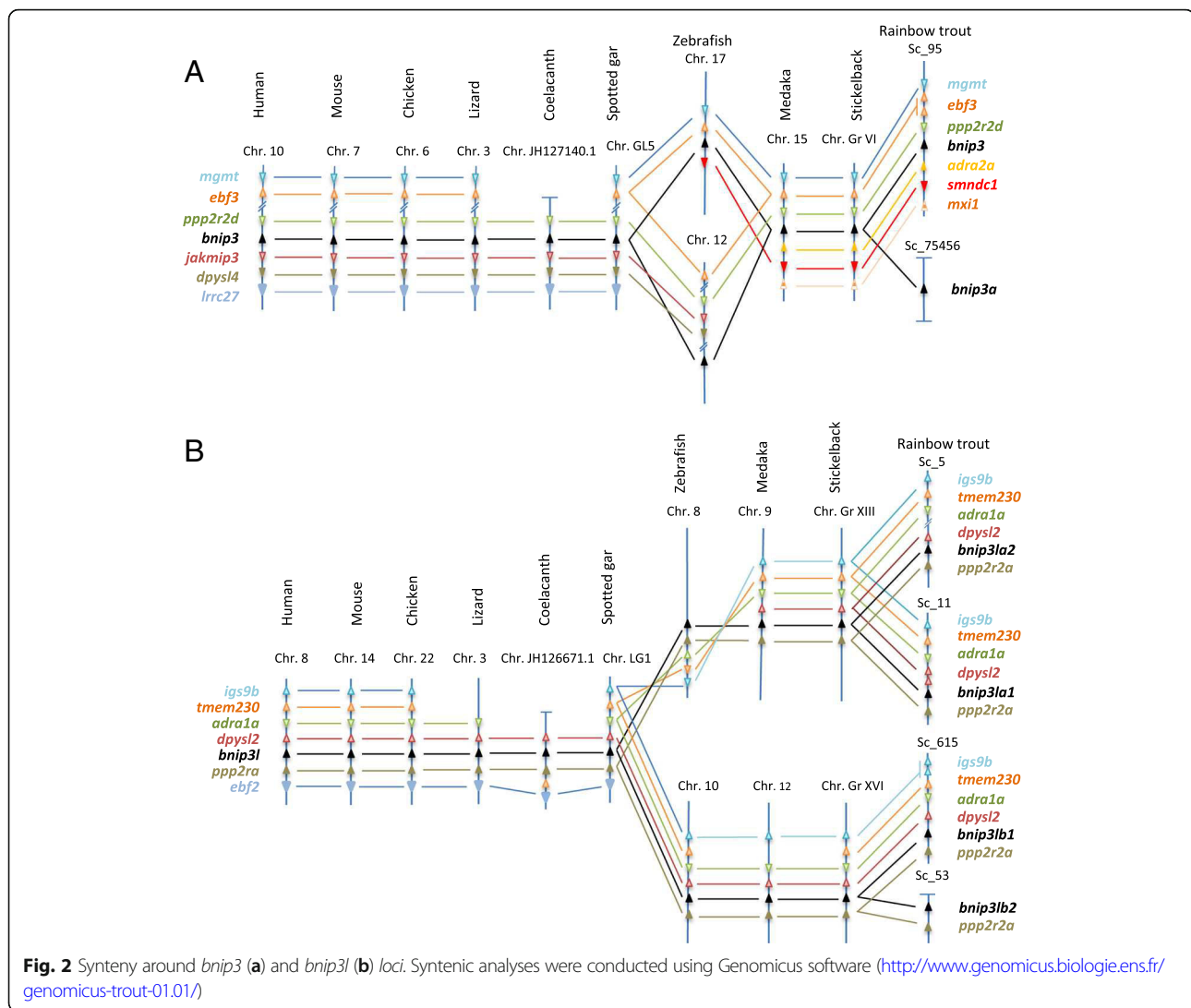
In order to confirm the identity of the identified genes, a percentage identity matrix was established after alignment of the deduced amino acids (aa) sequences of these genes with those of *BNIP3* and *BNIP3L* from other vertebrate species including human, mouse, chicken, lizard, coelacanth spotted gar, zebrafish, medaka and stickleback (Additional file 1: Figure S1). The identity matrix showed that the deduced aa sequences of GSONMT00001151001 and GSONMT00082530001 shared a higher percent of homology with *BNIP3* (mean of 57.6% of homology) than *BNIP3L* (mean of 49.1% of homology). Inversely, sequences GSONMT00078967001, GSONMT00064944001, GSONMT00079376001 and GSONMT00059781001 presented a higher homology with *BNIP3L* (mean of 59.8% of homology) than *BNIP3* (mean of 50.6% of homology) when we compared trout sequences with other studied species. Accordingly, the phylogenetic analysis performed by the Maximum Likelihood method (Poisson model, 1000 bootstraps) showed that the two trout sequences (GSONMT00001151001 and GSONMT00082530001), sharing the highest percent of homology with *BNIP3*, clustered with vertebrates *BNIP3*, while the four last sequences (GSONMT00078967001, GSONMT00064944001, GSONMT00079376001 and GSONMT00059781001) grouped together with vertebrates *BNIP3L* (Fig. 1). These results suggested that the two former



trout genes (GSONMT00001151001 and GSONMT0082530001) are paralogous genes and co-orthologous to vertebrates *BNIP3*, while the four last genes (GSONMT00078967001, GSONMT00064944001, GSONMT00079376001 and GSONMT00059781001) are co-orthologous to vertebrates *BNIP3L*. Moreover, this phylogenetic tree also revealed that two trout *bnip3l* proteins (GSONMT00064944001 and GSONMT00078967001) rooted with teleosts *bnip3la* (defining them as *bnip3la1* and *bnip3la2*, respectively), while the two others (GSONMT00059781001 and GSONMT00079376001) were co-orthologous to *bnip3lb*, also identifying them as *bnip3lb1* and *bnip3lb2*, respectively.

We then performed a syntenic analysis to clarify the evolutionary history of *BNIP3* and *BNIP3L* in vertebrates. In all non-teleost species analysed here, *bnip3* was included in the *ppp2r2d-jakmip3-dpysl4-lrrc27* syntenic group highly conserved across species (Fig. 2a).

Interestingly, a syntenic conservation of this region was found in two distinct chromosomes (17 and 12) of the zebrafish genome whereas only one syntenic region containing *bnip3* in medaka and stickleback was identified. Considering the newly sequenced rainbow trout genome, our syntenic analysis showed that GSONMT00001151001 (*bnip3a*) and GSONMT0082530001 (*bnip3b*) genes were localized on two distinct scaffolds (scaffold_75,456 and scaffold_95, respectively). The synteny around *bnip3b* locus was well conserved but the scaffold_75,456 bearing *bnip3a* was too short to provide relevant syntenic information. As regard *bnip3l*, our analysis showed a conservation of synteny in the vicinity of this locus. In all the studied vertebrates, *bnip3l* is included in *dpysl2-ppp2r2a* syntenic group (Fig. 2b). It should be noted that this conserved region bearing *bnip3l* is present in two distinct chromosomes in zebrafish, medaka and stickleback and four different scaffolds in rainbow trout.



bnip3 and *bnip3l* mRNA levels

In mammals, hypoxia was shown to increase the expression of *bnip3* and *bnip3l* [27, 34–36, 41, 42]. Here, we aimed to determine whether the different *bnip3* and *bnip3l* paralogs identified in trout were affected by hypoxia as well as the possible existence of a “middle term” (until fry stage) programming effect of this stimulus on the expression of these genes. As shown in Fig. 3a, *bnip3lb1* and *bnip3lb2* were the only analysed genes exhibiting a significantly higher expression in embryos directly subjected to a 24 h hypoxic stress compared to control embryos kept at normoxic condition. However, surprisingly, at longer term (502 °D after hypoxia stimulus) fry from the hypoxic-embryos displayed significantly higher mRNA levels of not only *bnip3lb1* and *bnip3lb2* but also of *bnip3a*, *bnip3la1* and *bnip3la2* compared to the control group (Fig. 3b).

Met emerged as a key factor in modulating the cellular availability of the main biological methyl donor

S-adenosylmethionine (SAM) needed for all biological methylation reactions including DNA and histone methylation. As such, it represents a potential critical factor in nutritional programming. We therefore monitored the effect of feeding broodstock with a diet deficient in Met on the expression of identified *bnip3* and *bnip3l* genes in offspring subjected to different dietary Met levels. We found that *bnip3a* and *bnip3lb1* mRNA levels were induced by the Met deficiency in the diet only in offspring from Met deficient broodstock (BD-FD group significantly higher to the 3 other groups) (Fig. 4). We also found that Met deficiency applied in fry enhanced the expression of *bnip3lb2* whatever the broodstock diet. All the other studied genes did not display significant changes of their expression.

Together, these results support the possibility of programming the expression of some *bnip3* and *bnip3l* genes in rainbow trout by means of both early hypoxia stimulus and broodstock nutritional stimulus, thereby

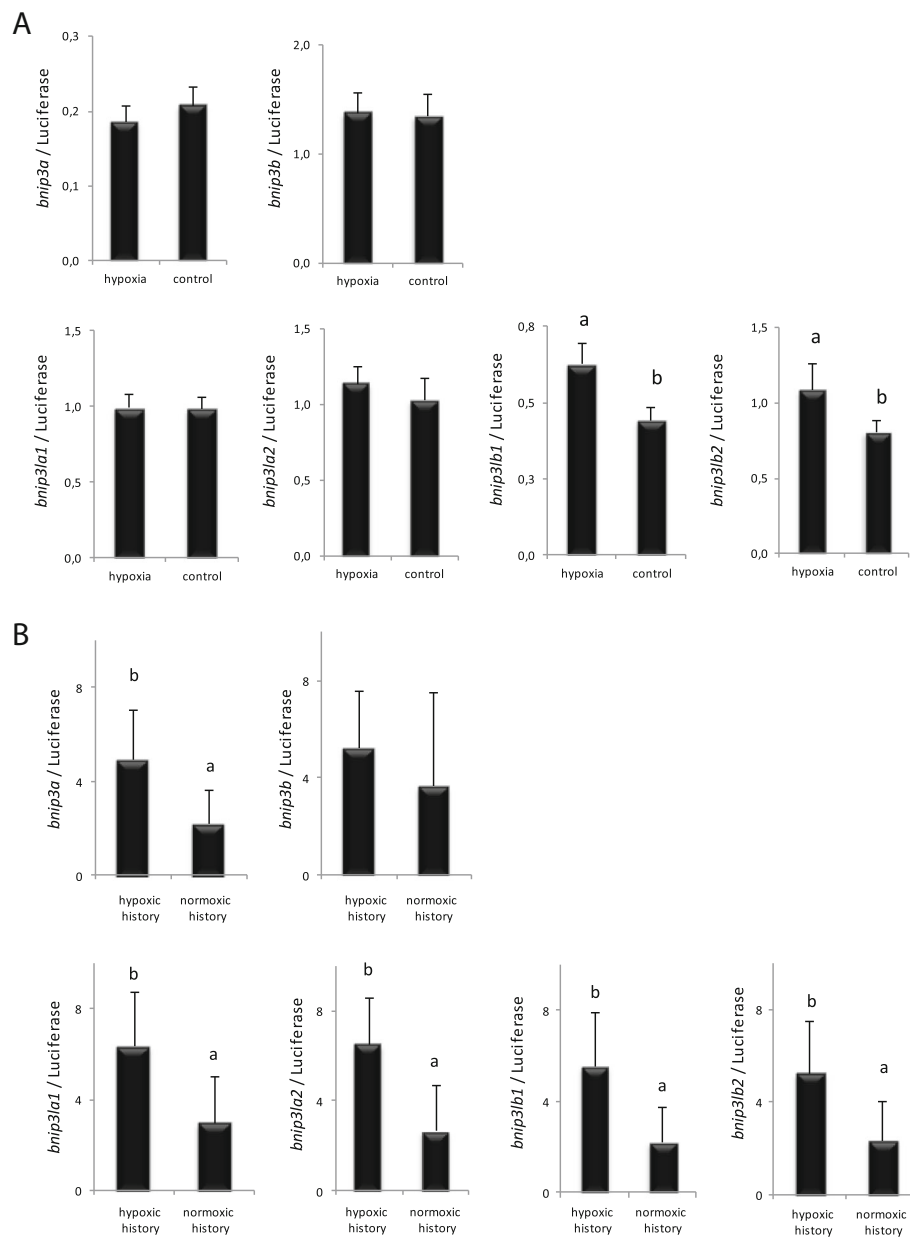


Fig. 3 Effect of hypoxic stimulus at embryo stage on mRNA levels of *bnip3* and *bnip3l* genes in rainbow trout embryos (a) and fry (b). Data are expressed as mean \pm SE ($n = 6$). Letters indicate significant differences between conditions ($p < 0.05$)

offering a good model to determine the underlying epigenetic mechanisms.

bnip3 and *bnip3l* promoters DNA methylation levels

We studied the DNA methylation of *bnip3* and *bnip3l* genes upstream region in fry of both experiments (hypoxia and Met deficiency). We focused on genes which expressions were affected by both the hypoxic stimulus and the Met deficient diet; namely *bnip3a* and *bnip3lb1*.

Using target gene Next Generation Bisulfite Sequencing, we analysed DNA methylation at specific CpG sites along the 5' upstream region of these two genes.

Effect of hypoxic history on DNA methylation level of *bnip3a* and *bnip3lb1*

For both genes, we observed that the methylation level was low (from 0 to 3%) around the ATG predicted codon and until -600 bp whatever the condition (data not shown). In the region upstream to -600 bp, DNA

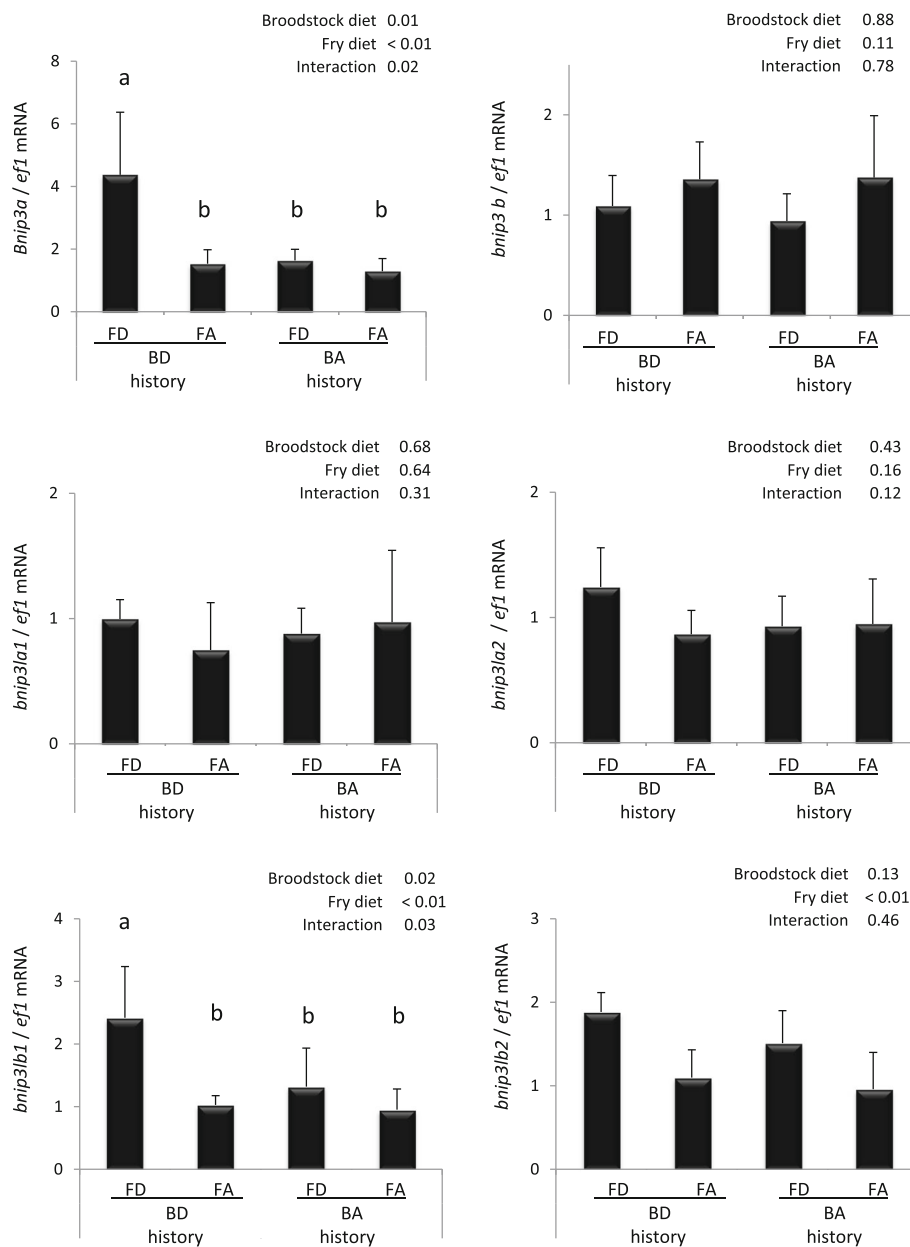
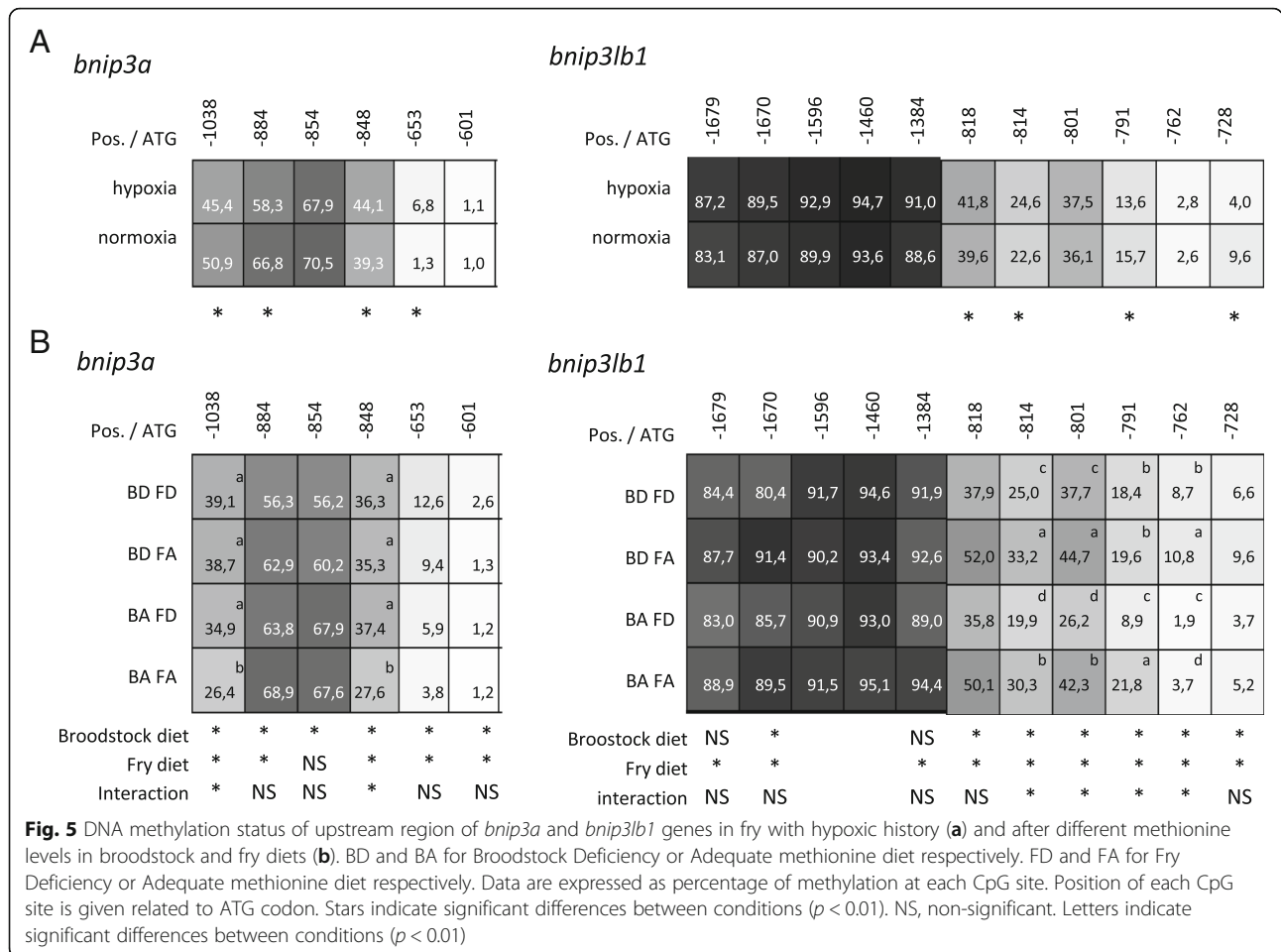


Fig. 4 Effect of a 3 weeks Met deficiency in rainbow trout fry from BD and BA broodstock groups on mRNA levels of *bnip3* and *bnip3l* genes. BD and BA for Broodstock Deficiency and Adequate methionine diet, respectively. FD and FA for Fry Deficiency and Adequate methionine diet, respectively. Data are expressed as mean ± SE (n=6). Letters indicate significant differences between conditions (p < 0.05)

methylation levels strongly increased to reach a maximum of 70.5% and 94.5% of methylated CpG for *bnip3* and *bnip3l*, respectively (Fig. 5a). Within this region, two CpG sites of *bnip3a* (at -1038 and -884 bp) and of *bnip3l* (at -791 and -728 bp) exhibited significantly lower methylation levels in fry with the hypoxic-history compared to their control counterparts. By contrast, the CpG sites at -848 and -653 bp of *bnip3a* and -818 and -814 bp of *bnip3lb1* presented higher methylation rates in fry with the hypoxia history.

Effect of broodstock and/or fry fed met deficient nutrition on the level of DNA methylation of *bnip3a* and *bnip3lb1*

As for hypoxic stimulation, we observed for both genes that the DNA methylation level was very low around the ATG codon (from 0 to 2.5%) and until -600 bp (data not shown) whatever the condition. We also detected a strong increase of methylation levels in the regions upstream to -600 bp. As regard *bnip3a*, we detected two trends. The first concerns the CpG sites at -884 and -854 bp, which showed an inhibitory effect of both the parental and fry



methionine deficiency on DNA methylation levels (Fig. 5b). In contrast, the CpG sites at -1038, -848, -653 and -601 bp showed higher DNA methylation levels upon both parental and fry methionine deficiency. As regard *bnip3lb1*, the CpG sites downstream of -1384 bp showed higher DNA methylation in fry from BD broodstock compared to those from BA broodstock (Fig. 5b). In contrast, the upstream CpG sites showed no effect of broodstock nutrition or even an opposite effect at -1670 bp. In all cases (all analysed CpG sites), regardless of the broodstock diet, we observed a significantly lower DNA methylation levels in fry fed the methionine deficient diet.

Discussion

In this work, we aimed at studying in rainbow trout epigenetic mechanisms underlying persistent changes of *bnip3* and *bnip3l* expression in a context of metabolic programming.

We first proceeded to the identification and characterization of the *bnip3* and *bnip3l* genes in trout. A previous phylogenetic analysis suggested that *bnip3* and *bnip3l* was restricted to metazoan lineage and both

genes would have emerged from whole genome duplication (WGD) in the vertebrate ancestor [43]. The authors highlighted a low divergent selection pressure between both genes which was in accordance with their similar functions in apoptosis and mitophagy [26–28]. In the present study, we identified two *bnip3* paralogs in rainbow trout genome assembly [40]. *bnip3a* and *bnip3b*, clustered with *bnip3* teleost sequences on phylogenetic analysis. Moreover, we observed a high conserved synteny in the vicinity of *bnip3* genes between the studied species. This suggests that the two rainbow trout paralogs are co-orthologous to vertebrates *BNIP3* genes. It was not possible to study *bnip3a* synteny in rainbow trout due to a short scaffold length. However, both phylogenetic analysis and synteny suggest that the presence of two *bnip3* paralogs in rainbow trout genome may be due to the recent 4th salmonid specific WGD (named Ss4R) which occurred around 80–100 million years ago [40, 44, 45] suggesting that *bnip3a* and *bnip3b* were ohnologous genes.

We also identified four *bnip3l* paralogs in rainbow trout genome. Two sequences clustered with *bnip3la* and the remaining two with *bnip3lb* teleost sequences

on the established phylogenetic tree. Furthermore, in all studied vertebrates we observed conservation of genes synteny in the vicinity of *bnip3l* locus. This suggests that rainbow trout *bnip3l* paralogs are co-orthologous to vertebrates *bnip3l* genes. This syntenic group is present in two distinct chromosomes in teleosts (zebrafish, medaka and stickleback) and in a unique copy in holostei (represented here by the spotted gar). Phylogenetic and syntenic data supported that the teleost-specific WGD (Ts3R) gave rise to *bnip3la* and *bnip3lb* paralogs [46–50]. Our results also showed that *bnip3la1* and *bnip3la2* identified in rainbow trout are co-orthologs to zebrafish, medaka and stickleback *bnip3la*. Similarly, *bnip3lb1* and *bnip3lb2* are co-orthologous with zebrafish, medaka and stickleback *bnip3lb*. Regarding the phylogenetic and syntenic analysis, the duplication of rainbow trout *bnip3la* and *bnip3lb* genes into *bnip3la1* and *bnip3la2*, and *bnip3lb1* and *bnip3lb2*, respectively, arose at the Ss4R. Therefore, existence of four *bnip3l* paralogs in rainbow trout could be explained by two rounds of WGD, Ts3R and Ss4R. Overall, these gene duplication events of *bnip3l* and *bnip3* offered an interesting model to study potential divergences in both the function and the expression of the related paralogs.

Numerous studies in mammals reported that early developmental stages can be critical windows of metabolic plasticity [1]. In this regard, early life events such as nutritional or environmental changes can affect growth, health and metabolic status later in life [3, 4]. Here, we show in rainbow trout that hypoxia (applied to embryo for 24 h) can induce, at least until fry stage, the expression of *bnip3a* and the four *bnip3l* paralogs. Previously, genes involved in gluconeogenesis pathway and glucose transport were reported to be affected at fry stage in same experimental conditions [15]. Therefore we confirm that hypoxia act as a developmental programming stimulus.

Our results also showed that hypoxia lead to significant changes in DNA methylation levels of upstream regions of both *bnip3a* and *bnip3lb1*. DNA methylation is often associated with gene silencing [51, 52]. It has been observed in mammalian cancer cells, especially in hypoxic micro-environment, that *BNIP3* expression is repressed by promoter aberrant hypermethylation [29, 31, 53–56]. This specific epigenetic alteration allows cancer cells to escape to *BNIP3* and *BNIP3L* proapoptotic activity. Silencing of *BNIP3* expression was associated with methylation of the hypoxia-responsive element (HRE) site that in turn inhibited the binding of hypoxia-inducible factor 1 (HIF1 α) to the *BNIP3* promoter [56]. In our study, hypoxia induced lower methylation level at CpG sites – 1038 and – 884 of *bnip3a* and – 791 and – 728 of *bnip3lb1*. Interestingly, *bnip3a* displays two possible HRE at sites – 1038 and – 884 (data not shown), fitting consensus sequence of HRE1 and HRE2 (R-CGTC and R-CGTG, respectively), described previously [34, 57–59], and making possible a

direct link between the hypomethylation at these specific *loci* and the upregulation of *bnip3a* expression observed in fry from the hypoxic-embryos. However, the higher methylation levels observed in neighbouring CpG sites (– 848 and – 653 for *bnip3a* and – 818 and – 814 for *bnip3lb1*) highlights the complexity of epigenetic mechanisms at play in the control of gene expression [60].

Similarly to early developmental events, parental nutrition can have an impact on growth potential, health and metabolism of the offspring [1, 61]. Here, we observed higher expression of both *bnip3a* and *bnip3lb1* genes in the BD-FD group compared to other fry, supporting the existence of an early fry programming by the mean of parental nutrition. Previously, in the same experimental conditions, Seiliez et al. also reported that it was possible to drive nutritional programming in fish through parental nutrition. Met deficiency in broodstock diet affected gene expression of fatty acid, cholesterol synthesis and autophagy in fry [17]. Overall, our results confirmed that Met level can be of critical importance in metabolic programming and prompted us to investigate epigenetic mechanisms at play in the effect observed.

In mammals, early Met nutrition was shown to affect DNA methylation later on life by controlling the one-carbon metabolism [62, 63]. More recently, in zebrafish, parental deficiency of one-carbon metabolism-related metabolites was observed to affect DNA methylation levels at some specific genes *loci* of the offspring [64]. In the present study, we clearly demonstrated that both broodstock and early fry Met nutrition affected the methylation of several CpG sites of *bnip3a* and *bnip3lb1* genes. However, as for hypoxia, the effect of Met deficiency (applied to both broodstock and fry) differs between the different CpG, making difficult to depict a real picture of mechanism at play in this event. It should be noted, however, that the methylation levels at the CpG site – 884 of *bnip3a* (which exhibit an HRE-like sequence) may be positively related with the mRNA levels of *bnip3a*.

In the future, additional investigations by ChIP and promoter fusion analysis approaches should be done to better understand function of key CpG identified here. Gene regulation is complex and most of the time results from the combination of different mechanisms. For instance, histone modifications have a major impact on expression regulation by opening or condensing chromatin which enable or not transcription factor binding. Another interesting subject will be to determine whether the effects observed at fry stage persist until adult stage for both stimuli applied.

Conclusion

Collectively, we highlighted in this study modifications of DNA methylation levels of *bnip3a* and *bnip3lb1* genes in a context of metabolic programming. An interesting

zone has been identified, namely – 884 bp site of *bnip3a*, which would deserve additional functional analyses in the future.

Methods

Ethical issues and approval

The INRA facilities are authorized for animal experimentation under French regulations (B 29–277-02). The experiments were carried out in accordance with the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree N_2001–464, May 29, 2001). The project was approved by the French National Consultative Ethics Committee (reference number 2015112018112159 and 201511201756973).

In silico analysis

The genoscope database (<http://www.genoscope.cns.fr/trout>) was used to identify *bnip3* and *bnip3l* related genes in the rainbow trout genome using BLAST analysis. Sequences are available under the accession numbers GSONMT00001151001 and GSONMT00082530001 for *bnip3* and GSONMT00064944001, GSONMT00078967001, GSONMT00059781001 and GSONMT00079376001 for *bnip3l*, respectively.

Ensembl database (<http://www.ensembl.org/index.html>) was also used to collect amino acids deduced sequences of *bnip3* and *bnip3l* for human, mouse, chicken, lizard, spotted gar, medaka, stickleback, zebrafish and coelacanth.

Protein alignment and the percentage Identity Matrix established with amino acids deduced sequences were performed using MUSCLE software (<http://www.ebi.ac.uk/Tools/msa/muscle/>).

Phylogenetic tree, based on full length amino acid sequences, was built using the Maximum Likelihood method (with Poisson model) and confirmed by Neighbor-Joining method (data not shown) using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 [65]. The reliability of the interfered trees was estimated using the bootstrap method with 1000 replications. Mammalian and teleost *bnip2* protein sequences were used to root the tree.

Syntenic analyses were conducted using Genomicus software (<http://www.genomicus.biologie.ens.fr/genomicus-trout-01.01/>) to confirm the identity of rainbow trout genes.

Experimental designs

All fish used in this study were acquired from our INRA experimental fish farm facilities (Lées-Athas, France).

Hypoxia stimulus

The experimental design was previously described by Liu and collaborators [14] where the efficiency of the hypoxic stimulus was validated. Rainbow trout oocytes

were fertilised and reared at 8 °C in 12 separate tanks in the INRA facilities (Lées-Athas, France). A 24 h hypoxia stimulus (2.5 mg.l⁻¹ dissolved oxygen, ~22%) was applied in 6 tanks to embryos at 152 degree days (°D) (Fig. 6a). The remaining 6 tanks of embryos were kept under normoxic conditions (11 mg.l⁻¹ dissolved oxygen) as a control group. After the 24 h hypoxic stimulation, embryos were kept under normoxic conditions. After hatching, alevins were reared with natural spring water in tanks at 18 °C under natural photoperiod. At 654 °D fry fed their first meal (Additional file 2: Table S1). After 85 °D (5 days) of feeding, fry were sampled 3 h after last meal by terminal anesthetization by bathing in benzocaine (30 mg/l then a bath at 60 mg/l) and were then frozen in liquid nitrogen and stored at – 80 °C until analysis. Fry which received the hypoxia at embryonic stage will be called later “fry with hypoxic-history”.

Methionine deficiency stimulus

The experimental design was detailed in Fontagné-Dicharry et al. [16] and summarized in Fig. 6b. Briefly, female and male rainbow trout broodstock were reared in our experimental fish farm facilities (INRA, Lées-Athas, France), in a flow-through rearing system supplied with natural spring water (8 °C) under natural photoperiod (April to October). Fish were randomly divided into two dietary groups (35 fish/group) that were fed for 1440 °D (6 months) before spawning one of the two plant-based diet providing two dietary methionine (Met) levels, set at 0.5 or 1% of the diet in the deficient (BD) or adequate (BA) diets, respectively (Additional file 2: Tables S2 and S3). Broodstock growth, relative fecundity, egg size and egg amino acid composition were analyzed and reported in Fontagné-Dicharry et al. [16]. Approximately 3000–5000 eggs collected for each female of each nutritional group were fertilized with a pool of sperm (collected from 5 to 8 males fed the corresponding diet). Embryos were reared at INRA experimental facilities at Lées-Athas in 8 °C stream water until the swim-up fry stage at the age of 528 °D; hatching occurred at the age of 352 °D. Following this, the swim-up fry were transferred to the experimental facilities at INRA Donzacq (France) and randomly distributed into 12 circular tanks (50 l; 400 fish/tank) supplied with natural spring water (17 °C) under natural photoperiod. The first-feeding fry were fed during 357 °D (3 weeks) a fry diet containing Met in adequate (FA) or deficient (FD) amounts ($n = 3$ tank/diet/broodstock group) (Additional file 2: Tables S2 and S3). After 3 weeks, $n = 9$ fish/tank were killed 8 h after the last meal by terminal anesthetization by bathing in benzocaine (30 mg/l then a bath at 60 mg/l) and were then frozen in liquid nitrogen and stored at – 80 °C before further mRNA and DNA analyses.

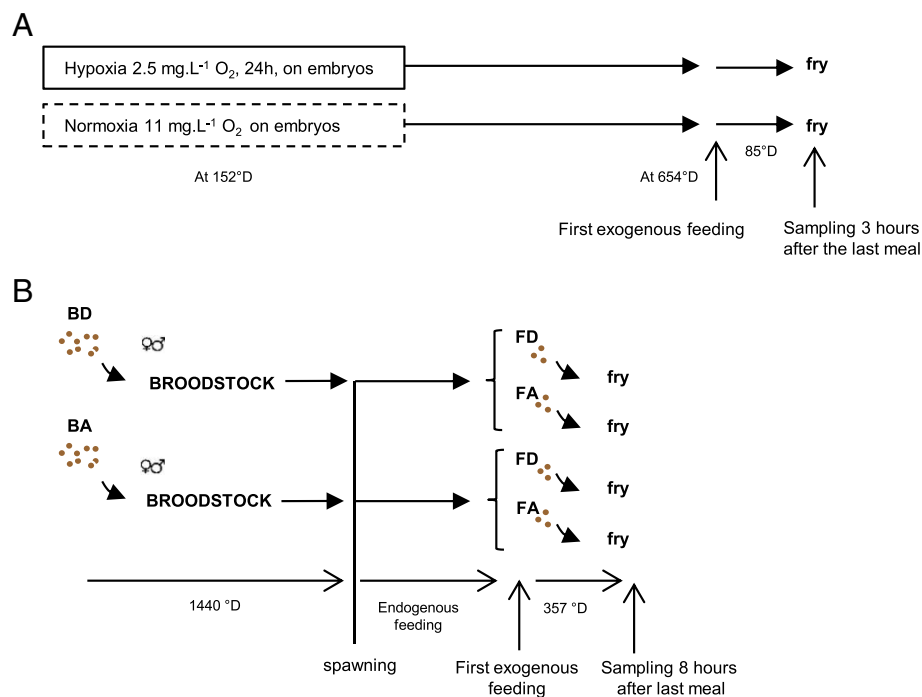


Fig. 6 Experimental design. **a** 24 h hypoxia stimulus (2.5 mg.L⁻¹ O₂) was applied to embryos (152 °D). Fish were fed their first meal at 654 °D. After 85 °D of feeding, fry were sampled 3 h after last meal. A normoxia group was used as control. **b** Broodstock fish were fed for 1440 °D with either a methionine deficient diet (BD) or a control (Adequate, BA) diet. Males and females of each feeding group were then crossed and the obtained fry were fed with either the FD (Deficient) or the FA (Adequate) diet for 357 °D from the first exogenous feeding

RNA extraction and rtPCR

Total RNA was extracted from the whole body of fry (six fry were extracted per condition) using the TRIzol reagent method (Invitrogen, Carlsbad, CA) with Precellys 24 (Bertin technologies, Montigny le Bretonneux, France) following Trizol manufacturer's instructions. 1 µg of total RNA was reverse transcribed in duplicate with SuperScript III RNase H Reverse Transcriptase kit (Invitrogen) and random primer (Promega) according to the manufacturer's instructions. mRNA levels were assayed using LightCycler 480 SYBR Green I Master (Roche Diagnostics, Neuilly sur Seine, France), 0.4 mM final of each primer, 2 µl of cDNA in a total volume of 6 µl. A LightCycler 480 II thermocycler (Roche) was used. The qPCR protocol was initiated by an initial denaturation step at 95 °C for 10 min followed by 45 cycles of a two steps amplification programme (15 s at 95 °C and 10 s at 60 °C). For each sample RT and qPCR was run in duplicate, negative controls for RT and qPCR were included.

For hypoxia stimulus, luciferase control RNA (Promega) was used, 10 pg per 1.9 mg of fry added to each sample to allow data normalisation as previously described [66]. For Met deficient diet stimulus, transcripts were normalized using Elongation Factor 1α (*ef1a*) as reference gene transcript following the Pfaffl method [67]. Primer sequences

used to amplify *bnip3* and *bnip3l* paralogs are presented in Table 1.

Target gene DNA methylation study

DNA extraction

Whole body fry were digested in 5 ml of ice cold extraction buffer (125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea, 10 mM tris-HCl, pH = 8) with 80 µg.ml⁻¹ final of proteinase K (P6556, Sigma-Aldrich) overnight at 37 °C under agitation (250 rpm). Six replicates per conditions were performed.

After overnight digestion, 5 ml of phenol chloroform isoamyl alcohol (25:24:1) was added to each sample. After mixing by inverting tubes, samples were centrifuged 15 min at 10000 g at room temperature. Aqueous phases were kept and 675 µl 5 M NaCl and 5 ml 100% ice-cold ethanol were added. After a 15 min centrifugation at 10000 g and 4 °C, pellets were washed with 1 mL 75% ice-cold ethanol and then centrifuge again 15 min, 10,000 g at 4 °C. Pellets were dried and re-suspend in H₂O DNase free and treated containing 2 µg of RNase (R4642, Sigma) for 1 h at 37 °C. Quality of DNA was checked on 1% agarose gel and quantification was made using Nanodrop (ThermoFisher, USA).

Table 1 Primers used for mRNA levels measurement

Gene	Primer (5' to 3')	T _m in °C
<i>bnip3a</i>	F: CCTGTGACAGTCTCCGAGA	60
	R: CCACTTCACGTCTCCGTTCT	
<i>bnip3b</i>	F: GAGAACAACCCACCAAGGA	60
	R: GTATATCCCCAGGCCAACTG	
<i>bnip3la1</i>	F: CAACTCCACCACACCCTCT	60
	R: CTGATCTGGACTGGGAGTGC	
<i>bnip3la2</i>	F: GGAGAGTCAGGCCCTCAG	61
	R: TCCTGATCTGGACTGGAAGG	
<i>bnip3lb1</i>	F: GAACAACGGAGACGCTGGA	61
	R: GGTGGAGGTAGACTGGGACA	
<i>bnip3lb2</i>	F: GCTGTATCAGAGAACAACGGACTA	60
	R: CATGCTGAGCGTCCAGTAGA	

Analysis of target gene methylation by targeted next-generation bisulfite sequencing

The upstream sequence of *bnip3* located on the scaffold 75,456 (GSONMT00001151001) and *bnip3l* located on the scaffold 615 (GSONMT00059781001) were assessed.

The sequence GSONMT00001151001 of *bnip3* started 513 base pairs before the ATG codon so we sequenced the 5'UTR until - 1148 bp from ATG using the Universal GenomeWalker 2.0 (Clontech Laboratories, USA) and following manufacturer's instructions. Sanger sequencing

was done by Eurofins Genomics (Paris) to identify 5'UTR region. Then, MethPrimer software (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) [68] was used to design primers (Table 2) targeting *bnip3* and *bnip3l* upstream regions. Each extracted DNA was bisulfite converted using EZ DNA Methylation-Gold Kit (D5005, Zymo Research, USA) following manufacturer's instructions. In order to prevent any PCR artefact, three PCR replicates were run for each bisulfite converted DNA. Advantage 2 polymerase Mix (639,206, Clontech laboratories, USA) was used for amplification. PCR conditions were 94 °C for 2 min, and 40 cycles at 94 °C for 25 s, melting temperature (T_m mentioned for each primers set in Table 2) for 1 min and 72 °C for 2 min followed by a final step of 7 min at 72 °C. For each condition, all 10 amplicons (5 for each studied gene) run in triplicates were pooled. Libraries were generated using KAPA library preparation Kit (KAPA Biosystems, USA) at EpigenDx (Hopkington, USA). Sequencing was performed at EpigenDx on Ion Torrent PGM using 314 Chip kit v2. The NGS QC Toolkit v2.3.3 [69] was used to trim data removing part of the sequences with a quality score lower than 18 followed by a removal of reads smaller than 35 nucleotides using Bowtie 2 [70] using gene sequences in silico bisulfite converted as a reference. Alignment BAM files were then sorted by target and condition using BAM tools [71] split

Table 2 Primers used for target gene DNA methylation study

Gene	Location from ATG	Primers (5' to 3')	T _m in °C
<i>bnip3a</i>	- 1085 / -762	F: TGATGGAATATTTAGTTTTAGTAGGATAA R: TCCAAACCATCCAAAATATTTAA	57
	- 717 / -498	F: TTTTATGGATGGAGGAAATATTTGT R: TAAACAACCTCTCTAAACTATTAAC	57
	-513 / -283	F: TAGAGAGTTGTTTTATATAGGAAAA R: ATCACTACTAATATATTCATTAATC	57
	- 383 / -120	F: TTTGAATTTGTTAATAGAAATTTT R: ATATTATTCTAATACCTCTAAATTA	54
	-202 / + 43	F: TGTTTTGGTAGTTTAGTGTT R: CCTGCAAATTTTCCT	58
	<i>bnip3lb1</i>	- 1735 / -1358	F: AAAGAGATAGATATTTTGGAGATTTGTTATA R: TAATAAATAAATTCACCTTCACTCC
- 1435 / -1038		F: TGAAGAATTGTTATGAAAGAGGTAATGT R: TCTCCAAAACCTATATTTACCATAAAC	57
- 867 / -590		F: GGGTATTTTAAAATTTTATTAATTTTTTATT R: ACACCTATTTAACAATTTAACACTTATTTA	57
- 594 / -222		F: GTGTGAGGTGAATTTAAGTTGT R: AATAATCCAATTCCTTAATAACAAAAACA	59
-252 / + 26		F: TGTTTTTGTATTAAAGAATTGGATTATT R: TCAACTACAACAACACTTCAAAC	58

function. Sorted reads were analyzed in BiQ Analyzer HT [72] setting parameters at 100% of the read length, and bisulfite conversion efficiency $\geq 98\%$ and lower cut-off at 20 reads per CpG site analyzed. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. Data are expressed as percentage of methylation at each CpG site. Positions of CpG sites were determined from ATG site.

Statistical analysis

Statistical analysis of mRNA measure. For hypoxia stimulus, normality of distributions was assessed using the Shapiro-Wilk test and data were then analysed by Kruskal-Wallis non-parametric test followed by Tukey's test as *post hoc* analysis. For Met deficient diet stimulus, effects of broodstock diet, effects of fry diet and interaction of both factors were tested by two-way ANOVA. *post hoc* tests were performed using Tukey's multiple mean comparisons.

Statistical analysis of gene target methylation measure. For hypoxia stimulus, data were analyzed by a binomial generalized linear model followed by a Tukey test as a *post-hoc* analysis in order to test statistical significance of difference of DNA methylation observed between conditions at each CpG. For Met deficient diet stimulus, effects of broodstock diet, effects of fry diet and interaction of both factors were analysed by a binomial generalized linear model followed by Chi-square test. Tukey test as a *post-hoc* analysis were run to test difference between each conditions.

All statistical calculations were made using R software (v3.1.0)/R Commander Package [73].

Additional files

Additional file 1: Figure S1. Protein alignment and the percentage Identity Matrix established with amino acids deduced sequences were performed using MUSCLE software (<http://www.ebi.ac.uk/Tools/msa/muscle/>). RT for rainbow trout. In brackets is given gene identity, 3 for BNIP3 and 3 I for BNIP3L. (PDF 167 kb)

Additional file 2: Table S1. Formulation and proximate composition of diet used in hypoxia stimulus [14]. **Table S2.** Formulation and proximate composition of diet used in methionine deficiency stimulus [16]. **Table S3.** Analyzed amino acid composition of the diets as g/100 g dry feed used in methionine deficiency stimulus [16]. (DOCX 24 kb)

Abbreviations

BNIP3: bcl-2/E1B-19 K interacting protein3; Chip: Chromatin Immunoprecipitation; HUFA: Highly unsaturated fatty acids; Met: methionine

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Availability of data and materials

Bioinformatic data and mRNA measures generated or analysed during this study are included in this published article and its supplementary information files.

The datasets used and analysed related to targeted next-generation bisulfite sequencing during the current study are available from the corresponding author on reasonable request.

Authors' contributions

I.S, S.P and S.S designed the study. I.S and L.M managed the study. J.L and E.J.V performed the gene expression measures and statistical analysis. V.V performed methylation measures and epigenetic statistical analysis, O.L contributed to bioinformatics and statistical analysis of epigenetic data. V.V and L.M wrote the manuscript. I.S, S.P, J.L, E.J.V and S.S contributed to critically revising the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The INRA facilities are authorized for animal experimentation under French regulations (B 29–277-02). The experiments were carried out in accordance with the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree N_2001–464, May 29, 2001). The project was approved by the French National Consultative Ethics Committee (reference number 2015112018112159 and 201511201756973).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Around the poor use of dietary carbohydrate phenotype in trout (*Oncorhynchus mykiss*): its epigenetic consequences and metabolic modulation through a programming strategy

Abstract: The carnivorous rainbow trout (*Oncorhynchus mykiss*) is considered as a poor user of dietary carbohydrates. Recent studies showed that a high-carbohydrate/low protein diet inducing hepatic global DNA hypomethylation could be involved in the establishment/maintenance of the poor dietary carbohydrate utilisation phenotype in trout, but the detail mechanisms remain unclear. The present thesis aimed at investigating the epigenetic mechanisms underlying this poor dietary carbohydrate utilisation phenotype in trout, and exploring if the glucose metabolism and the epigenome in juveniles can be programmed through a hypoxic stimulus during early life. We first identified all the paralogous genes related to DNA methylation/demethylation pathways (*dnmt*, *tet* and *tdg*) in trout genome, clarified their molecular evolution histories and monitored their transcriptional expression patterns during gametogenesis and embryogenesis in trout. Besides, we investigated further the causes, processes and potential mechanisms about the hepatic global DNA hypomethylation in trout after feeding a high carbohydrate/low protein diet. Results for the first time demonstrated that a decrease in protein content and an increase in carbohydrate content in the diet can independently as well as interactively induce hepatic global hypomethylation in trout. This global loss of methylation is probably established through an active demethylation pathway. We also found that a strong hyperglycaemia induced by glucose injection induces global C^mCGG hypomethylation in the liver of trout. The detailed mechanisms of these demethylation processes remain to be elucidated. Finally, through metabolic programming strategy, we confirmed for the first time that using a non-nutritional stimulus, hypoxia, during early life stage persistently modulates the transcription of glucose metabolism-related genes in juvenile trout without negative effects on growth performance. Moreover, acute and chronic hypoxia tended to induce opposite programming effects on glucose-transporter encoding genes in both liver and muscle of juvenile trout. Together, the present thesis brings forward our understandings about the roles of epigenetics in contributing to the low ability to use dietary carbohydrates in trout, and sheds light on the potential of using hypoxia as the stimulus in metabolic programming strategy to tailor the glucose metabolism, the epigenome and dietary carbohydrate utilisation in rainbow trout.

Phénotype de faible utilisation des glucides alimentaires chez la truite arc-en-ciel (*Oncorhynchus mykiss*): ses conséquences épigénétiques et sa modulation métabolique *via* une stratégie de programmation

Résumé: La truite arc-en-ciel carnivore (*Oncorhynchus mykiss*) est considérée comme une espèce pauvre utilisatrice de glucides alimentaires. Des études récentes ont montré qu'une hypométhylation globale de l'ADN hépatique induite par un régime alimentaire riche en glucides et pauvre en protéines pourrait être impliquée dans l'établissement / le maintien de ce phénotype chez la truite, mais le détail des mécanismes sous-jacents reste inconnu. La thèse vise à étudier les mécanismes épigénétiques sous-jacents à ce phénotype de faible utilisation des glucides alimentaire chez la truite et à examiner si le métabolisme du glucose et l'épigénome chez les juvéniles peuvent être programmés par un stimulus hypoxique précoce. Nous avons d'abord identifié tous les gènes paralogues liés aux voies de méthylation / déméthylation de l'ADN (*dnmt*, *tet* et *tdg*) dans le génome de la truite, clarifié leurs histoires évolutives et analysé leurs profils d'expression au cours de la gamétogenèse et de l'embryogenèse chez la truite. Nous avons ensuite étudiés plus en détail les processus et les mécanismes potentiellement à l'origine de l'hypométhylation de l'ADN hépatique globale constatée chez la truite après un régime riche en glucides et pauvre en protéines. Les résultats ont montré pour la première fois qu'une diminution du taux de protéines et une augmentation du taux de glucides dans l'aliment induisent de manière indépendante et en interaction une hypométhylation hépatique globale chez la truite, qui semble établie par le biais d'une voie de déméthylation active. Nous avons également constaté qu'une forte hyperglycémie induite par une injection de glucose induit une hypométhylation globale de l'ADN au niveau des sites C^mCGG dans le foie de la truite. Les mécanismes détaillés de ces processus de déméthylation restent à élucider. Enfin, grâce à la stratégie de programmation métabolique, nous avons pour la première fois confirmé que l'utilisation d'un stimulus non nutritionnel au début de la vie, l'hypoxie, pouvait moduler de façon persistante la transcription des gènes liés au métabolisme du glucose chez la truite juvénile sans nuire aux performances de croissance. De plus, selon sa nature chronique ou aiguë, l'hypoxie, a tendance à induire des effets de programmation opposés sur les gènes codants pour les transporteurs au glucose notamment dans le foie et le muscle de la truite juvénile. Dans son ensemble, la thèse met en avant notre compréhension du rôle du méthylome dans la contribution à la faible capacité d'utilisation des glucides alimentaires chez la truite. La thèse met aussi en lumière le potentiel d'utilisation de l'hypoxie comme stimulus pour programmer le métabolisme du glucose, l'épigénome et l'utilisation des glucides alimentaires chez la truite arc-en-ciel.

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