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D6.5 Effect of early life nutrition on experimental outcomes at later life in rainbow trout

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Executive Summary

Objectives:
Identification of long-lasting effects of early feeding with carbohydrates or with low dietary energy intake on performances (growth, metabolism, resistance to confinement stress) of juvenile rainbow trout

Rationale:
Previous studies in mammals and avian but also recently in fish have acknowledged that early life is a critical period for developmental plasticity. Thus, exposure to stressful events during early life stages may affect metabolic phenotype and stimulus received at early life can modulate metabolism in later life, a process which is called 'metabolic programming'. It can also disrupt brain development and consequently modifying brain-related processes such as behavior or stress response. It is well known that growth performance, metabolism and stress resistance in animals can also be regulated by early feeding. However, it is still largely unknown in aquaculture fish species to which extent the effects of sub-optimal nutrition during early life stages can adversely affect performances done later at the juvenile or adult stages. Information on such long-lasting effects is important to ensure proper management of experimental groups before undertaking experiments.

In order to assess the above objectives, a cohort of rainbow trout alevins was fed during 4 weeks with a high carbohydrate diet (first feeding). Another cohort was fed during 4 weeks every other day with a control diet (dietary restriction at first feeding). Later, all these fish were reared up to the juvenile stage during 26 weeks at INRA/NuMeA experimental farm (Donzacq) in the same conditions to test the programming of nutrition/metabolism. An acute challenge (acute confinement stress) was performed at the end of the experiment to test the programming of the stress responses.

Long-term effects on fish nutrition/metabolism and stress functions were studied in INRA/NuMeA and INRA/LPGP respectively. For fish nutrition and metabolism studies, liver and muscle were studied for molecular markers involved in glucose, lipid and amino acid metabolisms as well as those involved in growth functions. On the other hand, several parameters related to corticotrope axis, gill functions (homeostasis, stress and metabolism, immunity) or behaviour were studied in juvenile fish kept in normal conditions or in fish exposed to an acute challenge (acute confinement stress).

Main Results:
Better capacity of muscle Glucose metabolism in juveniles linked to high carbohydrate intake at first feeding: after the first feeding with high level of carbohydrates, in muscle of juvenile

fish, higher capacities for glucose metabolism (glycolysis, glycogen) have been observed very clearly. Moreover, there were also expressions of genes involved in myogenesis and growth which were modified due to the high carbohydrate diet intake at first feeding. This observation was associated with a lower level of DNA methylation in muscle suggesting the existence of epigenetics mechanisms linked to the intake of a new diet at first feeding.

Modified autophagy (mitophagy) and growth actors in muscle of juveniles linked to the feed restriction at first feeding: when the alevins have been fed 50% less than the control, higher capacity for autophagy (mainly mitophagy) had been detected at the molecular level suggesting that the feed restriction could impact at long term the capacity of the fish to modify the level of autophagy (a main function involved in metabolism, nutrition, growth, health etc…).

*Resistance to confinement stress***:** our data clearly indicated that nutrient or protein restrictions applied at first feeding have long term effects on coping ability assessed at the level of HPI axis and gill functions. These effects are moderate but significant: a similar situation have been observed in experiments on long term effects of early hypoxia stress, thus confirming that different kinds of stressors (environmental, nutritional) applied on early stages at first feeding have long term consequences on coping ability of the fish.

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1. INTRODUCTION AND RATIONALE

Excellent management of experimental fish stocks are required of European aquaculture research infrastructures, thus enabling ethical, rapid and flexible use of experimental fish as well as low experimental variance and reduced project costs. To improve these aquaculture services, we have to consider several issues in order to develop relevant tools and/or management rules. Among these issues, the impact of experimental fish management on the outcome of experiments may start already at the embryonic and larval stage where the construction of the phenotypes of the fish starts. Developmental events such as cell proliferation, migration or cell death during embryonic and larval stages determine adult tissue structure and function. These events are under the influence of genetic, epigenetic and environmental influence and possibly underlie individual differences.

Previous studies in mammals and avian but also recently in fish have acknowledged that early life is a critical period for developmental plasticity. Thus, exposure to stressful events during early life stages may also modify metabolic phenotype in later life, a process which is called 'metabolic programming' (Lucas et al., 1998). They can also disrupt brain development thus altering brain maturation endpoints and consequently modifying brain-related processes such as stress response (Auperin et al., 2008; Fokos et al., 2017). However, it is still largely unknown in aquaculture fish species to which extent the effects of sub-optimal environmental conditions during early life stages can adversely affect experiments done later at the juvenile or adult stage. I this deliverable we will test if early feeding of specific diet or the level of feeding (energy restruction) can affect the juvenile growth, metabolism and stress response: it is the concept called the nutritional programming (Lucas, 1998). In this context, filling knowledge gap on the later life consequences of dietary carbohydrate or dietary restriction exposure during first feeding would improve our understanding regarding the quality of the experimental fish for use later in life in aquaculture research infrastructure.

The aims of the present study was to assess in rainbow trout to which extend first feeding with either high carbohydrate diet or dietary energy restriction at first feeding early-life can affect permanent impact on growth performance, nutrient utilization and stress resistance in juveniles fish later in life. These issues have been approached through two complementary questions: 1) Does early feeding modify the nutrient use in later life? 2) Does early feeding modify the resistance to confinement stress in later life?

These studies were developed on two research infrastructures (INRA/NuMeA and INRA/LPGP but all fish used were issued from the same cohort exposed during alevin stage to first feeding with carbohydrate or energy restriction in INRA/DONZACQ.

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2. EXPOSURE TO HIGH CARBOHYDRATE STIMULI AT FIRST FEEDING AFFECTS GLUCOSE METABOLISM LATER IN LIFE

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2.1 Introduction

Environmental factors (nutritional or non-nutritional) experienced during the early development can lead to long-term influence on the physiology and metabolism functions of the organism, which is termed metabolic programming or developmental programming [1-5]. In aquaculture, the concept of metabolic programming has attracted broad attention in recent years [6-10]. However, more studies are necessary to better characterize the concept of metabolic programming in fish.

Rainbow trout, a "glucose-intolerant" carnivorous species, showed reduced growth performance and persistent hyperglycemia after intake of high carbohydrate diets [11, 12]. This is the main reason why a new strategy based on metabolic programming have been recently performed in rainbow trout using high dietary carbohydrate at first feeding as an early stimulus to improve their glucose-intolerant phenotype and its use of dietary carbohydrates in juveniles [13, 14]. Indeed, previous studies in rainbow trout demonstrated that a huge hyperglucidic stimulus (60% of dietary carbohydrates) during 3 up to 5 days at the first feeding was able to permanently improve carbohydrate digestive capacity but also, unexpectedly, decrease the muscle glucose transport and glycolysis at the juvenile stage [13, 14]. By contrast, using other fish species *i.e.* the gilthead seabream, early high-glucose stimulus during a longer period of rearing could improve ^{14}C starch utilization in seabream juveniles [15]. These data illustrate that differences in metabolic programming could appear that can be due to differences either in species-related responses (feeding habit, early development and rearing temperature) or to the early feeding protocol (composition and duration). In the present paper, we decided to use, for the first time, the following starter diet as a stimulus (20% of carbohydrates – 42% of protein (LP diet) *versus* 0% carbohydrates – 69% of proteins (HP diet)) during a longer period (4 weeks) to test the hypothesis of improving carbohydrate utilization in trout juveniles through nutritional programming.

Dietary energy restriction applied during early developmental stage could also induce long-term metabolic changes of individuals later in life [4]. Several studies in mammals showed that prenatal and neonatal dietary energy restriction can affect metabolism and physiology in the offspring. In rat, maternal energy restriction to fifty percent of *ad libitum* intake during the last week of pregnancy impaired β-cell development of offspring; continued maternal energy restriction during lactation caused a long-term reduction in β-cell mass and number, and resulted in glucose intolerance in the offspring [16, 17]. In goat weaned progenies, dietary energy restriction during early life have long-term detrimental effects on morphological

development of rumen and small intestine [18]. In their natural environment, trout alevins (fries) at the first feeding stage may face a poor-nutrient environment due to the decrease of natural preys (insects) and they can resist up to 9 days of fasting [19]. Up to date, no study has been performed to test the existence of a metabolic programming in juvenile fish induced by early dietary energy restriction (feed restriction), which is worth investigation especially in the context of fish nutrition in aquaculture. This is the second question of the present study.

Thus, the main objectives of the present study were to investigate the existence of a glucose metabolic programming linked to two early stimuli at the first feeding either with an early dietary carbohydrates intake or with a dietary energy restriction. The analysis of the metabolic programming will be assessed by measuring the growth performance, whole body composition, plasmatic metabolites and glucose metabolism in liver and muscle, two key tissues involved in glucose use. Indeed, as the liver is the center of the intermediary metabolism [11], muscle is the largest part of the fish weight and considered as a stronger user of glucose as a source of energy than other tissues [20, 21]. Moreover, remodeling of epigenetic landscapes is an important mechanism mediating persistent metabolic programming strategy [22]. Nutritional status can affect the way of gene transcription therefore biological processes through epigenetic alterations, such as global DNA methylation [23], which has been also found recently in rainbow trout [8, 24]. This is the main reason why we have measured also the global C^{m}CGG DNA methylation in trout juveniles linked to the two early stimuli.

2.2 Material and methods

Ethical issues and approval

The experiments were conducted according to French and European legislation for the use and care of laboratory animals (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 and the “Ministère de la Recherche et de l’Innovation”, number APAFIS#10803-2017071017221313v4.

Diet, fish and experimental design

Two experimental extruded diets for trout alevins, namely HP diet for no carbohydrate / high protein diet (the control diet) and LP diet for high carbohydrate / low protein diet, were prepared at INRA, Donzacq, France (**Table 1**). Fish meal was included as the unique protein source, gelatinized corn starch was used as the carbohydrate source, whereas dietary lipids were provided by fish oil and fish meal.

The experimental design is detailed in **Figure 1**. Rainbow trout eggs were obtained from Lees Athas, INRA fish farm facilities, France. After hatching, trout alevins (initial average weight is 0.11g) were randomly distributed into experimental tanks with the density of 140 fish per tank in the Donzacq experimental farm. For the early nutritional stimulus (at first feeding), trout alevins were fed with HP or LP diets during 4 weeks. Trout alevins called HPR (dietary energy restriction group) were fed with the HP diet every other day during 4 weeks also (1 day fed, 1 day unfed, 1 day fed, etc…). Each treatment was performed in triplicate (n

= 9 tanks in total). Fish were fed eight times daily for four weeks and the uneaten feed was collected after feeding. During the dietary stimulus at first feeding, the mortality of alevins was recorded every day in order to calculate the survival rate. After the early nutritional stimulus, all fish groups were fed with a commercial diet (Skretting; 62-58% proteins, 16-18% lipids, 9-12% of carbohydrates) during a period of 15 weeks (called in the present study the "growth trial"). Fish were fed twice a day. The residual feed pellets were collected for correcting the feed intake. At the end of the growth trial, juvenile fish were subjected to a dietary challenge for 11-weeks with LP diet in order to test the existence of a metabolic programming due to the early feeding ("challenge trial"). As for the growth trial, fish were fed twice a day. The residual feed pellets were also collected for correcting the feed intake. During the periods of nutritional stimulus, growth trial and challenge test, fish were reared in a flow-through rearing system supplied with natural spring water (18°C) under a natural photoperiod, and water quality was checked every two weeks.

Samplings and nutritional parameters

At the end of the nutritional stimulus, three fish per tank were sampled 3 h after the last meal (**Figure 1**, Sampling 1). Fish (whole body) were anaesthetized in a benzocaine bath at 30 $mg \cdot L^{-1}$ and then killed in a benzocaine bath at 90 $mg \cdot L^{-1}$ 3h after the last meal (postprandial peak of nutrition absorption for alevins). Then the samples were stored at -80 °C until analyses. At the end of the challenge trial, a second sampling was performed 6 h after the last meal (**Figure 1**, sampling 2). Three fish per tank were euthanized 6 h after the last meal (postprandial peak of nutrition absorption for juveniles) using the same method. Blood was collected from the caudal vein using heparinized syringes and then centrifuged (3000g, 4 °C, 5 min) to get the plasma. The obtained plasma was immediately frozen and stored at -20 °C until using. Liver and white muscle (sampled under the dorsal fin) were dissected and immediately frozen in liquid nitrogen, then stored at -80 °C pending analyses. Subsequently, three fish per tank were randomly sampled 48 h after the last meal, and then immediately frozen at -20 °C for whole body composition measurement.

The parameters for growth performance, such as survival, specific growth rate (SGR), feed intake (FI) and feed efficiency (FE), were measured every 3 weeks and calculated as follow: (1) Survival (%) = 100 × final fish number/initial fish number; (2) Specific growth rate (SGR, $\%d^{-1}$) = 100× [Ln (final average wet body mass)–Ln (initial average wet body mass)]/d; (3) Feed intake (%/d) = 100 × {dry feed intake/[(initial wet body mass+ final wet body mass)/2]}/d; (4) Feed efficiency (FE) = (the mass for dead fish + final wet body mass -initial wet body mass)/dry feed intake, Where d is the experimental period in days.

Analysis methods: metabolites and enzymatic activities

The chemical composition of diets and whole body composition were measured as previously described by Song *et al*. [12]. Plasma glucose, triglycerides, lactate and free fatty acids were analysed with Glucose RTU (BioMerieux), PAP 150 (Biomerieux), Lactate PAP (Biomerieux) and NEFA C (Wako Chemicals GmbH) kits, respectively, according to the recommendations of each manufacturer (n=9 samples per experimental treatment). Total plasma free amino acid concentrations were determined by the ninhydrin reaction according to the method of Moore with glycine as standard [25]. Muscle hexokinase (HK) and phosphofructokinase (PFK) activities were measured according to Borges *et al.* [26] (n=9

samples per experimental treatment). Muscle pyruvate kinase (PK) activity was determined as previously described by Panserat *et al.* [27] (n=9 samples per experimental treatment). Liver and muscle glycogen levels were measured by a hydrolysis technique as reported by Good et al. [28] (n=9 samples per experimental treatment). Liver and muscle glycogen was determined by a hydrolysis technique previously described by Good et al. [28] and Song et al. [12] (n=6 samples per experimental treatment).

qRT-PCR analysis

The analysis of mRNA levels was performed in trout alevins as well as liver and muscle of trout juveniles (n=9 samples per experimental treatment). Samples were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) with Precellys®24 (Bertin Technologies, Montigny-le-Bretonneux, France), and then total RNA was extracted following the manufacturer instructions. Total RNA (1 µg) was synthesized to cDNA in duplicate using the SuperScript III RNase H-Reverse Transcriptase kit (Invitrogen) with random primers (Promega, Charbonniéres, France). The primer sequences applied in quantitative real-time PCR (qPCR) assays for selected genes are shown in **Table 2**. The real-time RT-PCR assays were conducted according to Liu *et al*. [29]. *Luciferase* and ***ef1α*** genes were used as reference genes for normalization (luciferase for alevins, ef1α for liver and muscle) of mRNA levels of target genes in alevins and juvenile (liver and muscle) respectively through the E-method on Light Cycler software according to Panserat *et al*. [8] and Borges *et al*. [26].

DNA extraction and global DNA CpG methylation analysis

Genomic DNA extraction was performed on the muscle (juvenile fish) of nine fish per experimental treatment as previously described by Liu *et al*. [24]. DNA quality and quantity were assessed using 1% agarose gel and Qubit dsDNA HS assay kit, respectively. Global DNA C^{m}CGG methylation pattern was determined using the method of luminometric methylation assay (LUMA) according to Karimi *et al.* [30]. Each analysis was carried out in duplicate with 2.5 µg muscle DNA samples according to the manufacturer's instructions.

Statistical analysis

The effects of nutritional stimulus (HP, LP and HPR diets) at the first feeding on the different parameters were tested using one way ANOVA test (statistical R software/R Commander package). For statistical analyses, differences were considered significant at $p < 0.05$. Normality of distributions was assessed using the Shapiro-Wilk test whereas the homeostatiscity of variance was evaluated using the Brown-Forsyth test. The results are presented as mean ± SD (standard deviation).

2.3 Results

Survival and growth performance of rainbow trout from early stimulus up to the end of the dietary challenge

Survival and growth performance of fish during the 30-week growth trial are shown in **Table 3**. No significant difference in survival was observed between the three treatments at the end of stimulus, after the 5-19 weeks of growth trial with commercial diet and after the last dietary

challenge test (LP diet). Regarding the growth performance, from 0 to 4 weeks (stimulus period), fish fed LP diet had significantly higher final body mass, specific growth rate and feed efficiency than those fed HP diet (p<0.001, Anova test); fish fed HPR diet had significantly lower final body mass, specific growth rate and feed efficiency than those fed HP diet (p<0.001, Anova test). From 5 to 19 weeks of growth trial (with commercial diet), specific growth rate of fish subjected to LP diet was significantly lower than those fed HP diet whereas specific growth rate of fish in HPR group was significantly higher than those in HP group (p<0.001, Anova test). This was mainly due to the significant increase of specific growth rate just after the stimulus period for the HPR fish (**Figure 2**), a well-known process in fish called the compensatory growth [31]. By contrast, after the LP stimulus, fish decreased significantly their specific growth rate when fed with a commercial diet (**Figure 2)**. After the 20 days, all the fish groups presented the same specific growth rate. Moreover, at the end of the growth trial (commercial diet), *i.e.* just before the LP challenge, there was no more differences in fish weight due to higher SGR in fish with HP and HPR history. Finally, from 20 to 30 weeks of the growth trial with the LP challenge diet, there was no significant differences in all of the zootechnical parameters measured among the 3 different groups (p>0.05, Anova test).

Whole body composition and plasma metabolites at the end of the 11-week LP challenge test in rainbow trout juveniles

Whole body composition (crude protein content, crude lipid content and gross energy) and major plasma metabolites (glucose, triglycerides, free amino acids, free fatty acids and lactate levels) were measured in juveniles at the end of the 11-week challenge test (6 h after the last meal). As expected, the glycemia (around 7-8 mM) was higher than usual value (around 5 mM) due to the intake of the carbohydrate-rich LP diet during the challenge period. As shown in **Table 4** and **Table 5**, there was no significant difference among the three groups (HP, LP and HPR dietary histories) in whole body composition parameters and in plasma metabolites levels at the end of the LP challenge test in rainbow trout juveniles (p>0.05, Anova test).

Metabolic gene expressions in rainbow trout alevins at the end of the early stimulus

As shown in **Table 6**, the mRNA levels of glucose transport-related gene *glut2a*, glycolysis-related genes *gcka*, *gckb*, *pfkla* and *pfkmba* (p<0.01, Anova test) and pyruvate conversion-related genes *ldhaa* and *ldhab* (p<0.001, Anova test) as well as gluconeogenesis-related gene *fbp1a* (p<0.01, Anova test) were significantly higher in alevins which fed the LP diet at first feeding compared with those fed HP diet. Moreover, alevins fed HPR diet at the first feeding had significantly higher mRNA level of gluconeogenesis-related gene *pck1* than those fed HP diet (p<0.02, Anova test). All these observations demonstrated that the stimulus was effectively perceived by trout alevins and translated into molecular reponses.

Metabolic gene expressions in liver and muscle of rainbow trout juveniles at the end of the 11-weeks LP challenge test

Long-term adaptive changes in gene expression patterns are one of the extremely important biological mechanisms that can be at the origin of a programming effect [1]. We thus

analysed mRNA levels for glucose metabolic genes in liver and muscle of juveniles fish challenged with LP diet after early LP and HPR stimuli. As shown in **Table 7**, there was no significant effect among the three groups on mRNA levels of the metabolic genes involved in glucose metabolism in trout liver. By contrast, significant higher mRNA levels of glucose transport-related gene *glut4a* and glycolysis-related genes *hk2* and *pkmab* were observed in muscle of juvenile fish with LP dietary history compared to those with the HP dietary history (**Table 8)**.

Glucose enzymes and glycogen in muscle of rainbow trout juveniles at the end of the 11-weeks LP challenge test

In order to confirm the programming of the glucose metabolism in muscle observed at the molecular level, we analyzed the enzymatic activities of the 3 key glycolytic enzymes. As shown in **Figure 3A, 3B, 3C**, higher hexokinase, phosphofructokinase and pyruvate kinase activities were noticed in the muscle of fish with LP dietary history compared to those with HP dietary history (p<0.02, Anova test). Moreover, whereas there was no significant effect in the liver (**Figure 4A**), there was a significant lower level of glycogen in the muscle of fish with LP dietary history compared to those with HP dietary history (p<0.01, Anova test) (**Figure 4B**) .

Global DNA C^{m}CGG methylation level in the muscle of juvenile trout at the end of the 11-week LP challenge test

The modification of glucose metabolism (gene expression and activities of the glycolytic enzymes in muscle) associated to the early LP diet intake could be due to an epigenetic mechanism, especially to the level of the DNA C^{m}CGG methylation status. We thus estimated the level of such methylation by analysis C^{m}CGG content by LUMA assay. As shown in **Table 9**, global DNA C^{m}CGG methylation level in the muscle of trout juveniles with LP dietary history was significantly lower than those with HP dietary history (p<0.01, Anova test).

2.4 Discussion

Studies about nutritional programming in fish revealed that nutritional stimulus applied at critical developmental stages early in life had persistent effects on physiological/metabolic functions of the organism later in life [10]. Based on the concept of nutritional programming [1-3], the purpose of the present study was to investigate the effects on glucose metabolism of early nutritional stimuli in rainbow trout juveniles using HP, LP and HPR feeding conditions in alevins. Their putative programming effects were tested in juvenile rainbow trout based on growth performance, plasma metabolites, mRNA levels for genes encoding proteins involved in glucose metabolism as well as global epigenetic modification (global DNA C^{m}CGG methylation).

Early stimuli using LP diet and HP Restriction feeding have been effective in rainbow trout alevins without decrease of survival.

In the present study, higher growth performances (final body mass, specific growth rate and feed efficiency) were noticed in alevins fed LP diet compared with those fed HP diet at the first feeding, suggesting that LP diet at the first feeding could lead to a positive stimulus effect on growth performance in trout alevins. The positive effect of early LP dietary stimulus on growth performance is probably related to the higher lipid content in LP diet compared to the HP diet (16% *versus* 9.5% respectively) because it is well known that trout alevins easily use dietary lipids as an energy source [32]. On the other hand, lower growth performances (final body mass, specific growth rate and feed efficiency) were observed in alevins fed HPR diet at the first feeding compared with those fed the HP diet, indicating - as expected - that early dietary restriction (they are fed two times less) caused strong negative impacts on growth performance in trout alevins in the short term.

At the molecular level, results of this study showed that LP dietary stimulus significantly influenced the mRNA levels of some glucose metabolic genes in trout alevins at the end of the first feeding trial. Indeed, the increase in mRNA levels of glucose transporter gene (*glut2a*), glycolysis genes (*gcka*, *gckb*, *pfkla* and *pfkmba*) and pyruvate conversion genes (*ldhaa* and *ldhab*) were found in trout alevins subjected to LP diet compared to those fed HP diet. These data indicated that intake of LP diet, rich in carbohydrates (almost 30%), at the first feeding had expected effect on glycolysis in trout alevins as previously shown in juveniles [33]. However, the increase of one gluconeogenic gene (*fpb1a*) was also observed in LP fish, which was not expected. On the other hand, in trout alevins following the dietary restriction protocol (HPR), there was only one gene differentially expressed (the gluconeogenic *pck1*) compared to those fed HP diet. Although this result suggested that HPR diet at the first feeding results in an expected up-regulation of the first step of the gluconeogenesis in trout alevins, as previously observed during the fasting stage in alevins[19], the number of glucose metabolic genes modified by the dietary restriction protocol was quite low.

All together, these observations (growth performance and molecular data) indicated that both LP and HPR dietary stimuli were well received by trout alevins, especially at the glucose metabolism level. As no differences in survival were observed at the end of the stimuli, these alevins can thus be used to test the existence of a glucose metabolic programming in juveniles.

Growth performance and glucose metabolism of rainbow trout juveniles fed LP diet (challenge) were not affected by the early dietary HPR history

In this study, the mRNA levels of glucose metabolism-related genes and glycogen content in the liver and muscle were not influenced by the HPR dietary history, suggesting that using the concept of nutritional programming with early energy restriction to improve dietary carbohydrate utilization in rainbow trout is ineffective to program glucose metabolism. The absence of effects on growth performance, plasma metabolites and whole body composition confirmed the molecular data. We can compare with caution the HPR experimental groups with global caloric restriction models in mammals [3-5]. Moderate maternal caloric restriction

programs obesity and even fatty liver in mammals; this was not the case in our fish model, suggesting that the caloric restriction effects at long term is highly dependent of the animal species and maybe linked to the general level of fasting resistance of the species.

Glucose metabolism in muscle of rainbow trout juveniles fed LP diet (challenge) was largely modified by the early dietary LP history

Regarding the effects of early LP diet, no significant effect of early dietary LP stimulus at first feeding was found for the whole body biochemical composition (proteins, lipids, energy) in juvenile trout. In the same way, no significant variations of the plasmatic parameters (glycemia, lactate, triglycerides) were found between the experimental groups. Finally, during the challenge trial, we did not observed any significant effect of the diet used at first feeding on the growth performance of juvenile trout. In comparison, Liu *et al*. [24] reported a negative effect of a 5-days dietary 60% carbohydrates stimulus at first feeding on growth performance of juveniles trout at the end of 24-weeks growth trial whereas Geurden et al.[13, 14] did not observe any difference in growth performances in trout fed a 60% carbohydrates diet for 3 and 5 days at first feeding. It seems that the long-term effect of dietary carbohydrates stimulus on growth performance is highly dependent either of the duration or of the levels of dietary carbohydrates-proteins-lipids ratios of the stimulus.

Besides, the absence of effects on liver has been detected is in accordance with the studies performed by Geurden *et al.* [14] and Hu *et al*. [9] using rainbow trout fed high carbohydrate diet (60%) at the first feeding. By contrast, dietary LP stimulus at the first feeding had permanent effect on glucose metabolism in the muscle of juvenile trout. Indeed, higher mRNA levels of muscular glucose transport-related gene *glut4a* was detected in juvenile fish with the LP dietary history compared with those with HP dietary history, suggesting that trout may adapt glucose transport in muscle. Moreover, higher mRNA levels of muscular glycolysis-related genes *hk2* and *pkmab* were observed in juvenile fish with the LP dietary history compared with those with HP dietary history. Meanwhile, the activities of hexokinase and pyruvate kinase in the muscle of fish subjected to LP diet at the first feeding were also significantly higher than those fed HP diet. These above data indicated that dietary LP stimulus at first feeding can strongly regulate hexokinase and pyruvate kinase at mRNA transcript and enzymatic levels in the muscle of trout juveniles. In addition, even though the mRNA levels of phosphofructokinase genes (*pfkmaa*, *pfkmab*, *pfkmba* and *pfkmbb*) in the muscle were differentially expressed among the 3 dietary histories, fish with LP dietary history displayed significantly higher phosphofructokinase enzyme activity in the muscle compared to HP diet control. This indicated that LP dietary history group can only strongly regulate phosphofructokinase at the enzymatic level in the muscle of trout juveniles. Moreover, enzymatic activities for the glycolytic enzymes in fish with the LP history were also higher compared to fish with the HPR history. In our study, muscular glycogen content in the muscle of fish with LP history was also different *i.e*. lower level than those with the HP dietary history. Thus, we can associate this lower level of glycogen (storage of excess glucose) to the higher activities of glycolytic enzymes in LP-history fish. Together, all these data indicated that early LP dietary history could, unambiguously, induce higher activities and

expression of actors involved in glucose utilization in the muscle of trout juveniles. In fish fed with 60% of carbohydrates (and very low level of proteins 20%) as early stimulus in our previous study [14], we found reverse data *i.e.* a down regulation of *glut4*, *hk* and *pk* gene expression, suggesting that the programming of glucose metabolism in muscle is strongly dependent of the type of the stimulus (dietary carbohydrate/protein ratio for example). Indeed, in mammals [3-5], a low protein diet in early life is also associated with low levels of *glut4* mRNA and problem of glucose tolerance. As a whole, our findings strongly suggest that, when the levels of proteins is above the requirement (>35% proteins), the dietary carbohydrates can have a positive effect on glucose metabolism. However, we cannot also eliminate the idea that the level of dietary lipids (different between HP and LP diets) played a role in the muscle programming.

Level of global C^{m}CGG DNA methylation in muscle of rainbow trout juveniles fed LP diet (challenge) was lower in fish with the early dietary LP history

Epigenetic modification can be an important mechanism involved in the long-term metabolic adaptations by early-life environmental stimuli [34]. Global DNA methylation is one of the main markers used for reconstructing the epigenetic state of the genome [35]. In the present study, global DNA C^{m}CGG methylation level in the muscle of juvenile trout with LP dietary history was lower than in those with HP and HPR dietary histories, suggesting that this stimulus induced a modification of the genomic stability and may be involved in the modifications of glucose metabolism-related gene expressions observed in this fish. A global DNA hypomethylation (at 5-methylcytosine level) associated with an intake of carbohydrate-rich diet was noted in the previous study performed by Marandel *et al.* [36] with juvenile rainbow trout. Moreover, DNA hypomethylation was also been documented in metabolic diseases, such as diabetes [37, 38]. Our data clearly showed that a variation of at least one epigenetic mark (C^{m}CGG DNA methylation) occurred in muscle, which reinforces the fact that nutritional programming can be mediated through epigenetic mechanisms in rainbow trout as shown in mammals [3- 5], especially for the skeletal muscle memory [39].

Conclusion

In summary, our data demonstrate that LP dietary stimulus at first feeding led to the programming of glucose metabolism in the muscle of trout at the juvenile stage. Moreover, early LP stimulus induced global DNA C^{m}CGG hypomethylation in the muscle of juvenile trout. This altered global DNA C^{m}CGG methylation level may manifest a way through which dietary LP history permanently modified gene expressions in the muscle of trout juveniles. Nevertheless, our result showed also that dietary HPR stimulus at first feeding exerts no programming influence on glucose metabolism in both liver and muscle of trout juveniles (while being not deleterious for long term growth). Finally, these findings are promising for improving nutritional strategies based on early metabolic programming with different compositions of diets. Further researches are needed to focus on the optimization of the programming conditions in order to change the energy metabolism regulation in this carnivorous fish species.

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2.5 Legends to the figures

Figure 1. Experimental design. LP and HPR dietary stimulus were applied to rainbow trout alevins for 4 weeks at the first feeding, then the fish were fed the commercial diet. After a growth trial of 15 weeks, fish were subjected to a 11 weeks challenge test with LP diet. HP diet: without carbohydrates. LP diet: with carbohydrates. HPR: fish fed HP diet every other day.

Figure 2. Specific growth rates of the fish from HP, HPR and LP history. HP diet: without carbohydrates. LP diet: with carbohydrates. HPR: fish fed HP diet every other day.

Figure 3. Enzymatic activities of muscle glycolytic enzymes measured in juvenile trout at the end of the LP challenge. **A**. Hexokinase (HK) activity in the muscle of rainbow trout juveniles fed with HP, LP and HPR diets at the first feeding (p<0.02, Anova test). **B.** Phosphofructokinase (PFK) activity in the muscle of rainbow trout juveniles fed with HP, LP and HPR diets at the first feeding (p=3 10^{-10}, Anova test). **C.** Pyruvate kinase (PK) activity in the muscle of rainbow trout juveniles fed with HP, LP and HPR diets at the first feeding (p=0.001, Anova test). For all the activities, data were presented as mean ± SD (n=6), Values with different letters are significantly different (p < 0.05). HP diet: without carbohydrates. LP diet: with carbohydrates. HPR: fish fed HP diet every other day.

Figure 4. Glycogen contents measured at the end of the LP challenge in the liver (**A**) and muscle (**B**) of rainbow trout juveniles fed with HP, LP and HPR diets at the first feeding (p_{liver}=0.662, p_{muscle}=0.002; Anova test). Data were presented as mean ± SD (n=6), Values with different letters are significantly different (p < 0.05). HP diet: without carbohydrates. LP diet: with carbohydrates. HPR: fish fed HP diet every other day.

Table 1. Formulation and proximate composition of the two experimental diets

Ingredients g/100g diet	HP	LP
Fish meal[a]	94.0	52.0
Fish oil[b]	2.0	14.0
Starch[c]	0.0	30.0
Vitamin mix[d]	1.0	1.0
Mineral mix[e]	1.0	1.0
Alginate	2.0	2.0
Proximate composition		
Dry matter (DM,% diet)	94.58	91.50
Crude protein (% DM)	69.25	41.77
Crude lipid (% DM)	9.51	16.27
Gross energy (kJ g^{-1} DM)	20.82	22.59
Ash (% DM)	18.18	10.82
Starch (% DM)	0.31	23.79

HP, high protein diet; LP, low protein diet.[a] Sopropeche, Boulogne-sur-Mer, France; [b] North Sea fish oil, France, Sopropeche; [c] Gelatinized corn starch (Roquette, Lestrem, France); [d] Suppplied the following (kg^{-1} diet): DL-α-tocopherol acetate 60 IU, sodium menadione bisulphate 5 mg, retinyl acetate 15,000 IU, cholecalciferol 3000 IU, thiamine 15 mg, riboflavin 30 mg, pyridoxine 15 mg, vitamin B_{12} 0.05 mg, nicotinic acid 175mg, folic acid 500 mg, inositol 1000 mg, biotin 2.5 mg, calcium panthothenate 50 mg, choline chloride 2000 mg. [e] supplying 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 and sodium chloride 0.4 g.

Table 2. List of the primers used for qRT-PCR to analyze the expressions of genes involved in glucose metabolism

Genes	Forward primers	Reverse primers
luciferase	5′-CATTCTTCGCCAAAAGCACTCTG-3′	5-AGCCCATATCCTTGTCGTATCCC-3′
ef1α	5′-TCCTCTTGGTCGTTTCGCTG-3′	5-ACCCGAGGGACATCCTGTG-3′
glut2a	5′-GACAGGCACTCTAACCCTAG-3′	5′CTTCCTGCGTCTCTGTACTG-3′
glut2b	5′-CTATCAGAGAACGGTACAGGG-3′	5′CAGGAAGGATGACACCACG-3′
glut4a	5′-CATCTTTGCAGTGCTCCTTG-3′	5′CAGCTCTGTACTCTGCTTGC-3′
glut4b	5′-TCGGCTTTGGCTTCCAATATG-3′	5′GTTTGCTGAAGGTGTTGGAG-3′
hk1	5′-CTGGGACGCTGAAGACCAGA-3′	5′-CGGTGCTGCATACCTCCTTG-3′
hk2	5′-GGGACACCGAGAACAAGGG-3′	5′-TCCCTTTGTCATCCTGTGCT-3′
pkmaa	5′-ACATTGCCCCCTACAGTTAC-3′	5′-AAGTGGAAATGAATGGGACGT-3′
pkmab	5′-TGCTGAGGGCAGTGACGTA-3′	5′-AGCTCCTCAAACAGCTGTCTG-3′
pkmba	5′-CAAGCCTGCCAACGATGTC-3′	5′-CAAGGAACAAGCACAACACG-3′
pkmbb	5′-CAACTGTGACGAGAAGCACC-3′	5′-GAGCCCAGAGTACCACCATT-3′
gcka	5′-CTGCCCACCTACGTCTGT-3′	5′-GTCATGGCGTCCTCAGAGAT-3′
gckb	5′-TCTGTGCTAGAGACAGCCC-3′	5′-CATTTTGACGCTGGACTCCT-3′
pfkla	5′-GATCCCTGCCACCATCAGTA-3′	5′-GTAACCACAGTAGCCTCCCA-3′
pfklb	5′-AGTGCTCGCTGTAAGGTCTT-3′	5′-GTGATCCGGCCTTTCTGAAC-3′
pklr	5′-CCATCGTCGCGGTAACAAGA-3′	5′-GCCCCTGGCCTTTCCTATGT-3′
pfkmaa	5′-GTCAGTCTGTCCGGTAACCA-3′	5′-ATCTGGAGGGTTGATGTGGG-3′
pfkmab	5′-TCAGCGGAGGAGGCTAATC-3′	5′-GACTCTGTGCAGTAGTCGTG-3′
pfkmba	5′-CTGGGCATGAAAAGGCGAT-3′	5′-GTCTTCTTGATGATGTGCTCCA-3′
pfkmbb	5′-CGGTCGTATCTTTGCCAACATG-3′	5′-TGTCCATTTCCACAGTGTCATATT-3′
pck1	5′-ACAGGGTGAGGCAGATGTAGG -3′	5′-CTAGTCTGTGGAGGTCTAAGGGC -3′
pck2	5′-ACAATGAGATGATGTGACTGCA-3′	5′-TGCTCCATCACCTACAACCT-3′
fbp1b1	5′-CTCTCAAGAACCTCTACAGCCT-3′	5′-TCAGTTCTCCCGTTCCCTTC-3′
fbp1b2	5′-ATCAGCAGGAATAGGTCGCG-3′	5′-CCTCCTCCAGCACGAATCTC-3′
fbp1a	5′-GACAGAGGACGACCCGTG-3′	5′-GTACTGACCGGGTCCAACAT -3′

g6pca	5′- GATGGCTTGACGTTCTCCT-3′	5′- AGATCCAGGAGAGTCCTCC-3′
g6pcb1	5′-AGGGACAGTTCGAAAATGGAG-3′	5′-CCAGAGAGGGAAGAAGATGAAGA-3′
g6pcb2	5′-CCTGCGGAACACCTTCTTTG-3′	5′-TCAATTTGTGGCGCTGATGAG-3′
ldhaa	5′-GTGTTTCTCAGCGTTCCCTG-3′	5′-GTTACAGAAGGGCACACAG-3′
ldhab	5′-GTGTTCCTCAGTGTGCCATG-3′	5′-TTGCTGATAAATTAACCCTCCG-3′
slc16a3a	5′-TAGTGATGTCAAGGCACCAGAT-3′	5′-CACTCCGAACTCCCTGATCAAC-3′
slc16a3b	5′-GAGTTGCAGGCTGTAGACC-3′	5′-GCTCACCACAAACACAGGG-3′

*Ef1α: elongation factor 1 alpha; glut***,** *glucose transporter; hk, hexokinase; pkm, pyruvate kinase (muscle) ; gck, glucokinase; pfkl,phosphofructokinase*

(liver) ; pklr, pyruvate kinase (liver and red blood cell); pfkm, phosphofructokinase (muscle); pck, phosphoenol pyruvate carboxykinases (cytosolic pck1

and mitochondrial pck2) ; fbp, fructose 1, 6-bisphosphatase; g6pc, glucose 6-phosphatase ; ldha,Lactate dehydrogenase A ; Slc16a3, solute carrier

family 16 (monocarboxylic acid transporters) member 3.

Table 3. Growth performance of rainbow trout during the complete growth trial: **A**) direct effects of the HP, LP and HPR diets; **B**) effects of the HP, LP and HPR histories. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Parameters	Diets			*p-value*
-**A** -	HP	LP	HPR	
The growth trial 0-4 weeks (stimulus)				
Survival (%)	93.10±0.82	90.95±0.82	90.48±2.06	0.118
Final body weight (g)	0.47± 0.01^{b}	0.67±0.02^{c}	0.26±0.01^{a}	**<0.001**
SGR (% day^{-1})	5.58± 0.11^{b}	6.97±0.11^{c}	3.30± 0.16^{a}	**<0.001**
Feed intake (% day^{-1})	3.70±0.35	3.46±0.16	3.15±0.18	0.087
Feed efficiency	1.37± 0.14^{b}	1.71±0.09^{c}	1.05±0.11^{a}	**0.001**
-**B**-	HP history	LP history	HPR history	
The growth trial 5-19 weeks (commercial diet)				
Survival (%)	99.62±0.66	98.69±0.98	86.15±22.30	0.415
Initial body weight (g)	0.47± 0.01^{b}	0.67±0.02^{c}	0.26±0.01^{a}	**<0.001**
Final body weight (g)	45.33±1.01	47.25±2.64	42.75±2.46	0.111
SGR (% day^{-1})	4.40± 0.01^{b}	4.09± 0.03^{a}	4.91± 0.08^{c}	**<0.001**
Feed intake (% day^{-1})	2.29±0.06	2.22± 0.06	2.68± 0.74	0.419
Feed efficiency	1.18±0.03	1.17±0.02	1.22±0.09	0.460
The growth trial 20-30 weeks (dietary challenge with LP diet)				
Survival (%)	98.33±1.67	99.44± 0.96	98.89±1.92	0.702
Initial body weight (g)	45.33±1.01	47.25±2.64	42.75±2.46	0.111
Final body weight (g)	196.57±9.48	211.84±7.88	195.75±14.95	0.220
SGR (% day^{-1})	1.90± 0.08	1.95±0.09	1.97±0.09	0.621
Feed intake (% day^{-1})	2.31±0.09	2.19±0.06	2.22±0.12	0.334
Feed efficiency	0.93±0.05	0.95±0.06	0.96±0.02	0.747

Table 4. The effects of HP, LP and HPR diets at the first feeding on whole body composition of juvenile rainbow trout at the end of dietary challenge test. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Whole body composition	HP history	LP history	HPR history	*p-value*
Crude protein (%)	16.02±0.14	16.12±0.39	16.32±0.28	0.215
Crude lipid (%)	14.84±0.43	14.26±0.87	14.99±1.82	0.551
Gross energy (kJ g^{-1})	6.91±0.52	6.83±0.40	7.04±0.12	0.661

Table 5. The effects of HP, LP and HPR diets at the first feeding on glucose, triglycerides, free amino acids and free fatty acids in the plasma of juvenile rainbow trout at the end of the challenge. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Plasma metabolite(mmol/L)	HP history	LP history	HPR history	*p-value*
Glucose	8.28±1.99	7.80±2.22	7.09±1.06	0.396
Triglycerides	3.36±1.04	3.98±1.17	3.58±1.08	0.482
Free amino acids	14.32±1.70	14.79±3.48	12.41± 2.32	0.144
Free fatty acids	0.31±0.09	0.32±0.08	0.23±0.05	0.051
Lactate	6.85±0.8	6.55±1.4	7.02±1.7	0.836

Table 6. The direct effects of HP, LP and HPR diets (at first feeding) on gene expressions of rainbow trout alevins (whole body) for proteins involved in glucose metabolism at the end of the challenge. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	HP diet	LP diet	HPR diet	*p-value*
Glucose transport				
glut2a	0.53±0.21[a]	1.20± 0.16[b]	0.42± 0.26[a]	**<0.001**
glut2b	0.71±0.19	0.85± 0.24	0.60±0.21	0.056
glut4a	0.90±0.15	0.99±0.20	0.95±0.42	0.778
glut4b	0.94±0.21	1.13±0.18	0.91±0.48	0.321
Glycolysis				
hk1	0.88±0.27	0.73±0.13	1.13±0.51	0.060
hk2	1.38±0.37	1.17±0.24	1.47±0.67	0.386
pkmaa	0.80± 0.37	0.85±0.21	1.08±0.38	0.192
pkmab	0.96±0.17[ab]	1.10± 0.14[b]	0.81±0.25[a]	**0.015**
pkmba	0.83±0.13[ab]	0.97±0.20[b]	0.69±0.24[a]	**0.023**
pkmbb	0.75±0.15	0.87± 0.23	0.76±0.39	0.601
gcka	0.09±0.09[a]	2.94± 1.42[b]	0.03±0.01[a]	**<0.001**
gckb	0.04±0.07[a]	2.81±1.10[b]	0.01±0.01[a]	**<0.001**
pfkla	1.06±0.30[a]	1.52±0.47[b]	0.77±0.27[a]	**<0.001**
pfklb	0.99± 0.21[ab]	1.20±0.34[b]	0.72±0.24[a]	**0.003**
pfkmaa	1.05± 0.60	1.27±0.31	0.87±0.29	0.148
pfkmab	1.17±0.59	1.27±0.13	0.81±0.37	0.060
pfkmba	0.29±0.25[a]	2.77±3.19[b]	0.10±0.09[a]	**0.009**
pfkmbb	1.08±0.47	1.10±0.13	0.73±0.27	**0.038**
pklr	1.07±0.41[ab]	1.40±0.39[b]	0.78±0.28[a]	**0.006**
Gluconeogenesis				
pck1	0.57± 0.45[a]	0.82± 0.37[ab]	1.39± 0.74[b]	**0.012**
pck2	0.67±0.71	1.42±0.65	0.79±0.62	0.053
fbp1b1	1.06±0.31	0.97±0.20	1.03± 0.46	0.834
fbp1b2	0.97±0.25	1.02±0.25	0.98±0.43	0.946
fbp1a	0.95±0.19[a]	1.23±0.21[b]	0.83± 0.29[a]	**0.004**
g6pca	1.12±0.41	0.78±0.23	1.01±0.33	0.105
g6pcb1	0.87±0.30	1.07± 0.26	0.98±0.47	0.486

g6pcb2	1.02±0.47	0.96±0.59	1.30±0.68	0.449
Pyruvate conversion				
ldhaa	0.93±0.31[a]	1.86±0.60[b]	0.50±0.26[a]	**<0.001**
ldhab	0.83±0.27[a]	1.91±0.61[b]	0.54±0.31[a]	**<0.001**
Lactate transport				
slc16a3a	1.24±0.49	1.04±0.58	0.88±0.64	0.412
slc16a3b	1.19±0.29[ab]	0.83±0.20[a]	1.52±0.63[b]	**0.007**

Table 7. The effects of HP, LP and HPR diet histories on hepatic gene expressions of juvenile rainbow trout fed LP diet (challenge) for proteins involved in glucose metabolism. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target genes	HP history	LP history	HPR history	*p-value*
Glucose transport				
glut2a	1.07±0.19	1.00±0.20	1.07±0.21	0.682
glut2b	1.01±0.10	1.00±0.24	1.13±0.25	0.392
Glycolysis				
gcka	0.93±0.66	0.97±0.57	0.96±0.37	0.991
gckb	1.07±0.43	1.11±0.28	0.98±0.31	0.736
pfkla	1.15±0.43	1.09±0.27	1.03±0.25	0.771
pfklb	1.09±0.41	1.05±0.25	1.20±0.49	0.719
pklr	1.19±0.29	1.14±0.23	1.10±0.18	0.761
Gluconeogenesis				
pck1	1.08±0.40	1.08 ±0.29	0.93± 0.33	0.559
pck2	1.22±1.03	1.19±0.64	0.75±0.74	0.410
fbp1b1	1.33±0.96	1.28±0.50	0.83±0.34	0.213
fbp1b2	0.81±0.37	0.94±0.58	1.19±0.60	0.344
fbp1a	1.07±0.50	1.05±0.33	1.04±0.27	0.983
g6pca	0.99±0.20	1.11±0.28	0.96±0.29	0.455
g6pcb1	1.01±0.52	1.35±0.62	0.91±0.39	0.192
g6pcb2	1.08±1.08	1.15±0.95	0.97±0.94	0.927
Pyruvate conversion				
ldhaa	0.91±0.35	1.18±0.38	1.08±0.33	0.322
ldhab	0.99±0.39	0.86±0.22	1.06±0.25	0.346
Lactate transport				
slc16a3b	1.21±1.19	2.28±3.19	0.95±0.70	0.359

Table 8. The effects of HP, LP and HPR diet histories on muscle gene expressions of juvenile rainbow trout fed LP diet (challenge) for proteins involved in glucose metabolism. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	HP history	LP history	HPR history	*p-value*
Glucose transport				
glut4a	0.80±0.17[a]	1.84±1.28[b]	0.46±0.14[a]	**0.005**
glut4b	1.13±0.37	0.88±0.28	0.92±0.23	0.191
Glycolysis				
hk1	0.95±0.21	0.90±0.28	1.01±0.19	0.612
hk2	0.88±0.21[a]	1.35±0.61[b]	0.96±0.27[ab]	**0.044**
pkmaa	1.01±0.33[ab]	1.38±0.64[b]	0.82±0.25[a]	**0.036**
pkmab	0.88±0.14[a]	1.70±1.14[b]	0.66±0.24[a]	**0.009**
pkmba	0.85±0.14	0.97±0.30	0.94±0.45	0.731
pkmbb	1.12±0.31	0.85±0.39	1.01±0.42	0.343
pfkmaa	0.96±0.29	0.97±0.23	1.03±0.31	0.890
pfkmab	1.13±0.26	1.10±0.57	1.04±0.36	0.903
pfkmba	1.07±0.42	0.96±0.45	1.15±0.49	0.665
pfkmbb	1.13±0.31	1.01± 0.39	0.94±0.31	0.509
Pyruvate conversion				
ldhaa	1.05±0.39	1.04±0.35	0.95±0.36	0.820
ldhab	0.92±0.29	1.08±0.37	0.87±0.33	0.368
Lactate transport				
slc16a3a	0.96±0.73	1.40±1.19	0.72±0.57	0.257
slc16a3b	1.15±1.28	0.83±0.64	1.05±0.68	0.745

Table 9. The effects of HP, LP and HPR diets at the first feeding on global DNA C^mCGG methylation level (%C^mCGG) in the muscle of trout juvenile subjected to a 11-week LP challenge test

Tissue	HP history	LP history	HPR history	*p-value*
Muscle	0.81 ± 0.01^b	0.79 ± 0.02^a	0.81 ± 0.02^b	**0.007**

Figure 1

.

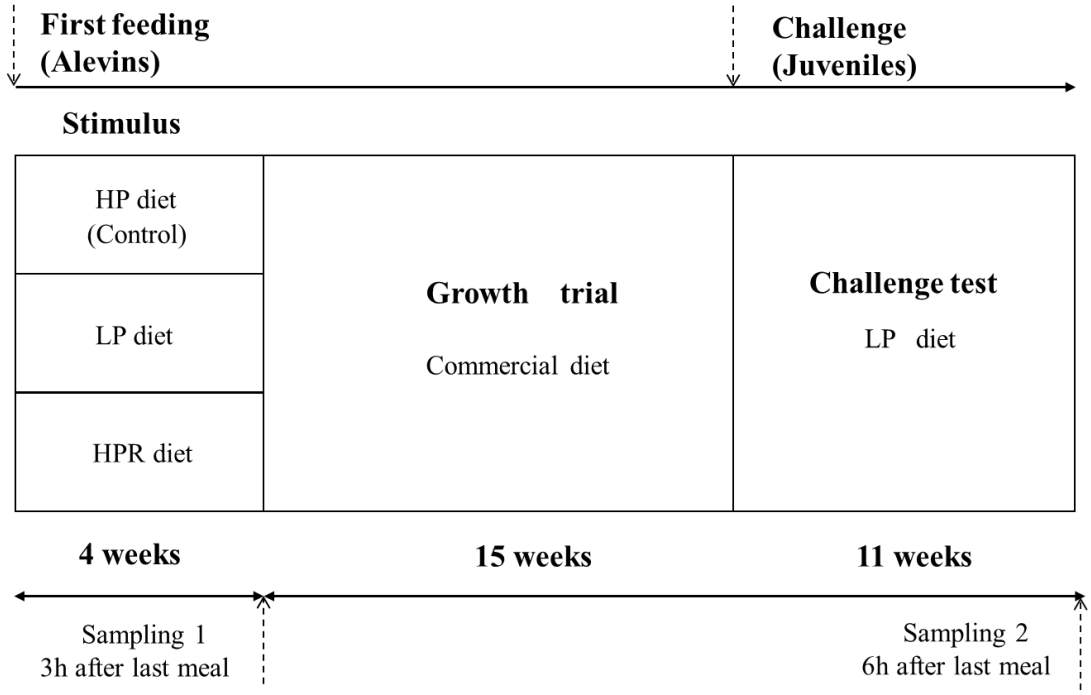

First feeding
(Alevins)
Challenge
(Juveniles)
Stimulus
HP diet
(Control)
LP diet
HPR diet
Growth trial
Commercial diet
Challenge test
LP diet
4 weeks
15 weeks
11 weeks
Sampling 1
3h after last meal
Sampling 2
6h after last meal

Figure 2.

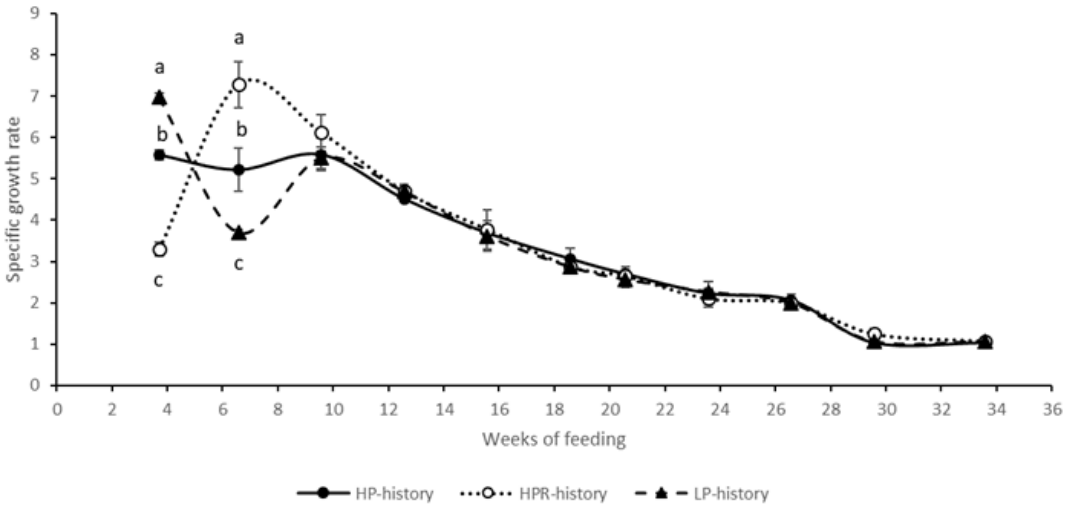
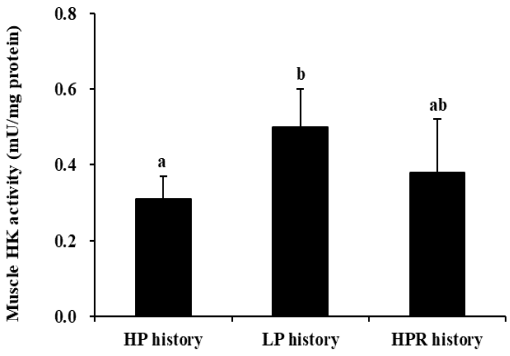
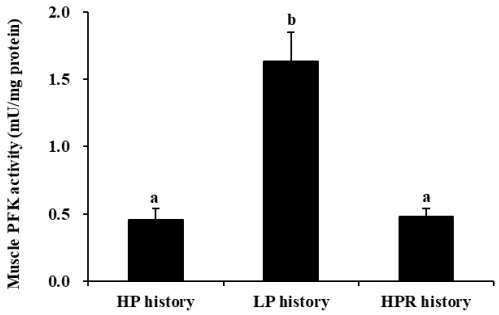

Specific growth rate
Weeks of feeding
HP-history
HPR-history
LP-history
a
b
c

Figure 3.

(A)


Muscle HK activity (mU/mg protein)
0.8
0.6
0.4
0.2
0.0
a
b
ab
HP history
LP history
HPR history

(B)


Muscle PFK activity (mU/mg protein)
2.0
1.5
1.0
0.5
0.0
a
b
a
HP history
LP history
HPR history

(C)

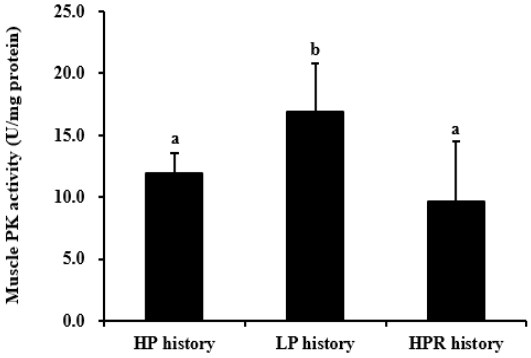
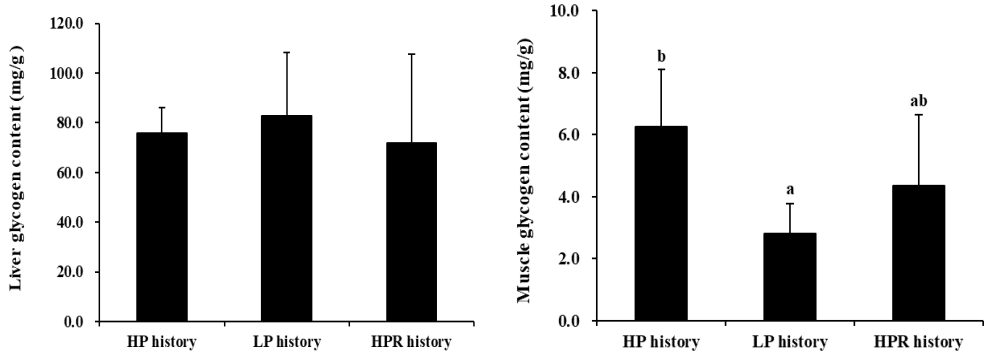

Muscle PK activity (U/mg protein)
25.0
20.0
15.0
10.0
5.0
0.0
a
b
a
HP history
LP history
HPR history

Figure 4.

(A) **(B)**


Liver glycogen content (mg/g)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
HP history
LP history
HPR history
Muscle glycogen content (mg/g)
10.0
8.0
6.0
4.0
2.0
0.0
b
a
ab
HP history
LP history
HPR history

3. EXPOSURE TO HIGH CARBOHYDRATE STIMULI DOES NOT STRONGLY AFFECT LIPID, AMINO ACID AND MITOCHONDRIAL METABOLISM AT A MOLECULAR LEVEL IN LATER LIFE

All the material and all the methods have been presented in chapter 2.

3.1 Results.

3.1.1 Direct effects of the early stimuli

As shown in Table 1, there was no significant effect of LP diet at the first feeding on mRNA levels of the metabolic genes involved in lipid metabolism, amino acid catabolism and mitochondrial metabolism, except one gene (qcr2) which had higher mRNA level in fish fed LP diet compared to those fed HP diet at the first feeding. Moreover, there was no significant effect of HPR diet at the first feeding on gene expressions of the metabolic genes except for 2 genes (hoad and cpt1b) which display lower mRNA levels in fish fed HPR diet compared to those fed HP diet at the first feeding.

3.1.2 Effects at later life.

As shown in Table 2, there was no significant effect of LP and HPR dietary history on mRNA levels of the metabolic genes involved in lipid metabolism, amino acid catabolism and mitochondrial metabolism in trout liver.

As shown in Table 3, significant higher mRNA levels of lipid metabolism gene hoad and mitochondrial energy metabolism gene qcr2 were observed in juvenile fish with LP dietary history compared to those with the HP dietary history. Moreover, there was no significant effect of HPR dietary history on mRNA levels of the metabolic genes involved in lipid metabolism, amino acid catabolism and mitochondrial metabolism in trout muscle.

3.2 Discussion.

At the molecular level, results of this study showed that LP and HPR dietary stimulus significantly influenced the mRNA levels of some lipid metabolism and mitochondrial metabolism genes in trout alevins at the end of the first feeding trial. Indeed, the increase in mRNA level of mitochondrial metabolism gene qcr2 and the decrease in mRNA levels of lipid metabolism genes (hoad and cpt1b) were found in trout alevins subjected to LP and HPR diets compared to those fed HP diet, respectively. These data indicated that both LP and HPR dietary stimuli were well received by trout alevins at the mitochondrial metabolism and lipid metabolism level.

The absence of effects on lipid metabolism, amino acid catabolism and mitochondrial metabolism in liver has been detected in rainbow trout with LP and HPR dietary history. Moreover, dietary LP stimulus at the first feeding had permanent effect on lipid metabolism and mitochondrial energy metabolism in the muscle of juvenile trout. Indeed, higher mRNA levels of muscular lipid metabolism gene hoad and mitochondrial energy metabolism gene qcr2 were detected in juvenile fish with the LP dietary history compared with those with HP dietary history, suggesting that trout may adapt lipid metabolism and mitochondrial energy metabolism in muscle. However, dietary HPR stimulus at the first feeding had no permanent effect on lipid metabolism, amino acid catabolism and mitochondrial energy metabolism in the muscle of juvenile trout.

3.3 Tables/Figures.

Table 1. The direct effects of HP, LP and HPR diets (at first feeding) on gene expressions of rainbow trout alevins (whole body) for proteins involved in lipid, amino acid and microchondrial metabolism at the end of the challenge. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	HP diet	LP diet	HPR diet	p-value
Lipid metabolism				
hoad	1.03± 0.28^{b}	1.22± 0.16^{b}	0.63±0.39^{a}	0.000 *
cpt1a	1.21±0.43	0.83±0.38	0.77±0.42	0.067
cpt1b	1.12±0.31^{b}	0.91±0.35^{ab}	0.65±0.33^{a}	0.018*
d6d	0.93±0.16	0.75±0.10	1.23±0.66	0.052
Amino acid catabolism				
gdh1	1.05±0.22	1.08± 0.22	1.00±0.49	0.857
gdh2	0.96±0.20	1.03±0.09	0.85±0.28	0.200
gdh3	1.00±0.32	1.04±0.13	0.76± 0.30	0.075
ALAT2	0.92±0.30	0.96±0.24	0.79±0.46	0.560
ASAT2	0.95±0.18	1.13±0.22	0.99±0.38	0.364
Mitochondrial energy metabolism				
cs	0.87±0.27	0.94±0.23	0.77±0.39	0.522
qcr2	0.93±0.18^{a}	1.17±0.11^{b}	0.82±0.23^{a}	0.001*
cox2	0.91±0.53	1.30±0.43	1.03±0.74	0.354
cox4	0.95± 0.24	1.05±0.09	0.85±0.38	0.290
atp5a	0.88±0.15	1.06±0.07	0.83±0.36	0.112
sdhb	0.92±0.20	1.11±0.35	1.01±0.38	0.468

Table 2. The effects of HP, LP and HPR diet histories on hepatic gene expressions of juvenile rainbow trout fed LP diet (challenge) for proteins involved in lipid, amino acid and microchondrial metabolism. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	HP history	LP history	HPR history	*p-value*
Lipid metabolism				
hoad	1.04±0.68	1.24±0.44	0.80±0.34	0.195
cpt1a	0.72±0.69^{ab}	0.41±0.31^{a}	1.34±0.94^{b}	0.029*
cpt1b	0.87±0.63	0.81±0.70	1.08±0.72	0.697
d6d	0.78±0.35	1.27±0.94	1.51±1.21	0.283
Amino acid catabolism				
gdh1	1.14±1.04	1.13±0.70	0.78±0.37	0.505
gdh2	1.17±0.59	1.06±0.37	0.82±0.27	0.238
gdh3	1.11±0.37	1.14±0.27	0.96±0.26	0.419
ALAT2	1.09±0.33	1.07±0.35	1.05±0.40	0.976
ASAT 1	1.15±0.49	1.11±0.26	0.92±0.30	0.366
ASAT2	1.25±0.62	1.28±0.41	1.01±0.33	0.428

ASAT3	1.08±0.57	1.18±0.41	0.90±0.23	0.382
Mitochondrial energy metabolism				
cs	1.07±0.45	1.13±0.43	0.88±0.17	0.323
qcr2	1.07±0.50^{ab}	1.43±0.46^{b}	0.78±0.28^{a}	0.013*
cox2	1.12±0.55	1.09±0.32	1.00±0.34	0.815
cox4	0.99±0.46	1.01±0.37	0.77±0.12	0.279
atp5a	1.01±0.44^{ab}	1.29±0.40^{b}	0.77±0.30^{a}	0.028 *
sdhb	1.19±0.41	1.33±0.30	1.19±0.35	0.635

Table 3. The effects of HP, LP and HPR diet histories on muscle gene expressions of juvenile rainbow trout fed LP diet (challenge) for proteins involved in lipid, amino acid and microchondrial metabolism. Data represnt means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	**HP history**	**LP history**	**HPR history**	***p-value***
Lipid metabolism				
hoad	0.77±0.12^{a}	1.87±1.28^{b}	0.65±0.27^{a}	0.004*
cpt1a	0.42±0.15	0.58±0.36	1.24±1.50	0.139
cpt1b	1.23±0.38	1.00±0.74	0.68±0.46	0.124
Amino acid catabolism				
gdh1	0.60±0.14	0.95±0.67	0.86±0.66	0.392
gdh2	1.00±0.46	0.87±0.34	0.90±0.42	0.780
gdh3	0.93±0.38	0.93±0.39	0.99±0.45	0.929
ALAT2	0.81±0.13	1.34±0.78	1.00±0.68	0.183
ASAT 1	1.13±0.17	0.96±0.25	1.12±0.21	0.166
ASAT2	1.02±0.23	1.03±0.26	1.00±0.37	0.983
ASAT3	0.87±0.14	0.87±0.19	0.95±0.43	0.792
Mitochondrial energy metabolism				
cs	0.99±0.33	1.13±0.37	1.09±0.54	0.774
qcr2	0.78±0.30^{a}	1.45±0.84^{b}	0.57±0.18^{a}	0.004 *
cox2	0.79±0.33	0.87±0.30	1.30±1.13	0.274
cox4	0.65±0.17	0.85±0.36	0.84±0.57	0.530
atp5a	0.87±0.14	0.86±0.30	1.04±0.58	0.519
sdhb	0.96±0.16	1.04±0.40	1.07±0.45	0.799

4. EXPOSURE TO EARLY NUTRITIONAL FEEDING RESTRICTION AFFECTS AUTOPHAGY (BUT NOT PROTEOLYSIS) AT A MOLECULAR LEVEL LATER IN LIFE.

All the material and all the methods have been presented in chapter 2.

4.1 Results.

4.1.1 Direct effects of the early stimuli

As shown in Table 4, significant lower mRNA levels of autophagy-related genes bnip3b and bnip3la2 were observed in trout alevins fed LP diet compared to those fed HP diet at the first feeding. Moreover, significant lower mRNA levels of proteolysis-related gene murf2 and autophagy-related gene atg12l were observed in trout alevins fed HPR diet compared to those fed HP diet at the first feeding.

4.1.2 Effects at later life.

As shown in Table 5, there was no significant effect of LP and HPR dietary history on mRNA levels of the metabolic genes involved in proteolysis and autophagy in trout liver.

As shown in Table 6, there was no significant effect of LP dietary history on mRNA levels of the metabolic genes involved in proteolysis and autophagy in trout muscle. Moreover, significant higher mRNA level of autophagy-related gene atg4b was observed in muscle of juvenile fish with HPR dietary history compared to those with HP dietary history.

4.2 Discussion.

At the molecular level, results of this study showed that LP dietary stimulus significantly influenced the mRNA levels of autophagy-related genes in trout alevins at the end of the first feeding trial. Indeed, the decrease in mRNA levels of autophagy-related genes bnip3b and bnip3la2 were observed in trout alevins fed LP diet compared to those fed HP diet. Moreover, HPR dietary stimulus significantly affected the mRNA levels of proteolysis and autophagy genes in trout alevins at the end of the first feeding trial. Indeed, the decrease in mRNA levels of proteolysis-related gene murf2 and autophagy gene atg12l were observed in trout alevins fed HPR diet compared to those fed HP diet. These above data indicated that both LP and HPR dietary stimuli were well received by trout alevins at the proteolysis and autophagy level.

No effects on proteolysis and autophagy in liver has been seen in rainbow trout with LP and HPR dietary history. Moreover, there is also no effect of LP dietary history on proteolysis and autophagy in rainbow trout muscle. In addition, dietary HPR stimulus at the first feeding had permanent effect on autophagy in the muscle of juvenile trout. Indeed, the mRNA level of autophagy-related gene atg4b in the muscle of rainbow trout with HPR dietary history was significantly higher than those with HP dietary history, indicating that the muscle of trout with HPR dietary history may more easily be influenced by autophagy in the long term.

4.3 Tables/Figures.

Table 4. The direct effects of HP, LP and HPR diets (at first feeding) on gene expressions of rainbow trout alevins (whole body) for proteins involved in proteolysis and autophagy at the end of the challenge. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	HP diet	LP diet	HPR diet	*p-value*
Proteolysis				
murf1	1.16±0.96	1.11±0.59	0.96±0.52	0.838
murf2	1.39±0.60[b]	1.03±0.14[ab]	0.90±0.23[a]	0.034*
murf3	1.20±0.59	0.91±0.22	1.01±0.19	0.281
fbx32	1.19±0.57	1.10±0.57	0.59±0.14	0.042
Autophagy				
atg4b	0.99±0.48	1.03±0.18	0.80±0.31	0.362
atg12l	1.17±0.34[b]	1.12±0.17[ab]	0.81±0.28[a]	0.023 *
sqstm1	1.26±0.51[ab]	1.56±0.37[b]	0.87±0.37[a]	0.008*
mul1	0.90±0.44	0.86±0.12	0.79±0.36	0.792
bnip3	1.12±0.71[ab]	0.76±0.31[a]	1.38±0.48[b]	0.059*
bnip3a	1.06±0.43	0.78±0.33	1.71±1.39	0.083
bnip3b	1.04±0.32[b]	0.62±0.18[a]	1.32±0.51[b]	0.001**
bnip3la1	1.21±0.40	1.28±0.32	0.88±0.55	0.138
bnip3la2	1.06±0.29[b]	0.60±0.19[a]	1.27±0.32[b]	0.001**
bnip3lb1	1.11±0.25	1.18±0.12	1.18±0.60	0.913
bnip3lb2	1.08±0.23	1.13±0.12	1.03±0.48	0.803

Table 5. The effects of HP, LP and HPR diet histories on hepatic gene expressions of juvenile rainbow trout fed LP diet (challenge) for proteins involved in proteolysis and autophagy. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	**HP history**	**LP history**	**HPR history**	***p-value***
Proteolysis				
murf1	1.52±0.94	2.75±1.75	2.64±1.43	0.174
murf2	1.43±0.65	2.16±1.29	1.80±1.14	0.390
murf3	1.25±0.41	1.16±0.29	1.21±0.49	0.903
fbx32	0.98±0.39	1.05±0.59	1.11±0.66	0.898
Autophagy				
atg4b	1.06±0.27	1.36±0.41	1.08±0.51	0.258
atg12l	1.22±0.19	1.09±0.20	1.15±0.23	0.487
sqstm1	1.08±0.56	1.28±0.36	1.72±1.02	0.186
mul1	1.02±0.55	0.99±0.21	0.97±0.54	0.972
bnip3	1.11±0.36	1.15±0.46	1.08±0.48	0.949
bnip3a	1.12±0.44	1.26±0.48	1.09±0.45	0.717
bnip3b	1.13±0.54	1.31±0.53	1.05±0.52	0.582
bnip3la1	1.21±0.52	1.18±0.50	1.09±0.51	0.881
bnip3la2	1.11±0.37	1.14±0.39	1.08±0.42	0.961
bnip3lb1	1.12±0.34	1.15±0.22	1.16±0.43	0.964
bnip3lb2	1.08±0.54	1.26±0.50	0.92±0.40	0.345

Table 6. The effects of HP, LP and HPR diet histories on muscle gene expressions of juvenile rainbow trout fed LP diet (challenge) for proteins involved in proteolysis and autophagy. Data represent means ± SD (n = 9 samples per group). Values with different superscripts in the same row are significantly different ($P < 0.05$).

Target gene	HP history	LP history	HPR history	*p-value*
Proteolysis				
murf1	0.94±0.46	1.11±0.43	0.88±0.41	0.500
murf2	1.00±0.31	1.10±0.37	1.15±0.25	0.605
murf3	0.88±0.21	0.81±0.28	1.12±0.33	0.070
fbx32	0.27±0.26	0.56±0.42	1.34±1.68	0.088
Autophagy				
atg4b	0.58±0.26^{a}	0.51±0.19^{a}	1.54±1.16^{b}	0.007*
atg12l	0.73±0.16	0.90±0.38	0.94±0.35	0.317
sqstm1	0.79±0.15	1.22±0.51	1.19±0.38	0.040
mul1	0.79±0.16	0.85±0.39	1.08±0.43	0.185
bnip3	0.77±0.34^{ab}	0.72±0.18^{a}	1.29±0.74^{b}	0.036*
bnip3a	0.92±0.31^{ab}	0.64±0.15^{a}	1.29±0.72^{b}	0.021*
bnip3b	0.89±0.19	0.91±0.29	1.06±0.28	0.344
bnip3la1	0.77±0.25	1.26±0.89	0.83±0.30	0.153
bnip3la2	0.92±0.29	0.88±0.22	1.24±0.47	0.074
bnip3lb1	0.99±0.30^{ab}	0.79±0.22^{a}	1.14±0.30^{b}	0.043*
bnip3lb2	0.90±0.35	0.92±0.25	1.00±0.22	0.738

5. EXPOSURE TO EARLY NUTRITIONAL STIMULI IMPACTS GROWTH-RELATED ACTORS AT A MOLECULAR LEVEL LATER IN LIFE.

All the material and all the methods have been presented in chapter 2.

The knowledge on the effects that changes in first feed availability and composition might induce directly, at short term, or indirectly, at long term, on the muscle growth mechanisms of fish is very limited. We therefore here analysed the effects of such early feeding stimuli on the gene expression of the transcription factors expressed in muscle precursor cells (Pax7) and regulating the different steps of myogenesis, i.e. commitment (Myf5) and activation (Myod) of muscle precursors, and early differentiation/fusion of myoblasts into myotubes (Myog). Myogenic progenitor cells can either activate Myf5 and MyoD and differentiate or proliferate, providing a reserve cell population for muscle growth during development. The daughter cells of the activated precursor cells undergo multiple rounds of division prior to fusion with existing fibres, form new fibres or return to quiescence. We also analysed the effects of early nutritional stimuli on molecular markers of the growth of muscle fibres, which resulted from the balance between hypertrophic (e.g. synthesis of sarcomeric constituents such as myosin and synthesis of extracellular matrix components such as collagen) and atrophic (e.g. degradation of cellular components by lysosome and proteasome) signals.

5.1 Results and discussion

5.1.1 Direct effects of the early stimuli

As shown in Table 1, a dietary restriction at first feeding (HPR stimulus) as here experimented (distribution of HP diet every other day) led in 4 weeks, compared to HP continuous feeding, to a downregulation of the transcript level of myod1b, myod1c, myog, fmlc2 and col1α2 and to an upregulation of the transcript level of Pax7a2. The mRNA level of pax7a1, pax7b, myf5, myod1a, myf6, pcna, fmhc, ctsd and fbx32 in alevins was similar with HPR- and HP-feeding.

A decrease of dietary proteins at the expense of carbohydrates and lipids (LP stimulus) from first feeding onwards led in 4 weeks, compared to HP-feeding, to a downregulation of the transcript level of pax7a1, pax7a2, myod1b and myod1c and to an upregulation of the transcript level of col1α2 and ctsd. The mRNA level of pax7b, myf5, myod1a, myog, myf6, pcna, fmhc, fmlc2 and fbx32 in alevins was similar with in LP- and HP-feeding.

Effects at later life

As shown in Table 2, both HPR- and LP-early feeding histories modify expression of muscle genes in later life, but the consequences of HPR-history and LP-history were not similar.

Compared to juveniles with an HP-history, those with an HPR-history had a lower transcript level of ctsd in white muscle, while those with an LP-history had an higher transcript level of pax7a1, pax7a2, pax7b, myog and ctsd in white muscle. The mRNA level of myf5, myod1a, myod1b, myod1c, myf6, fmhc, fmlc2, col1α2 and fbx32 in juvenile white muscle was not influenced by early nutritional stimuli.

5.2 Discussion

The present study showed that, in rainbow trout, early dietary restriction affected the transcript level of genes regulating important steps in myogenesis and the transcript level of genes involved in the growth of muscle fibres. The upregulation of pax7a1 and pax7a2 gene

expressions and the downregulation of myod1b and myod1c gene expressions in HPR-alevins suggest a higher number of quiescent muscle precursors and a lower number of activated muscle precursors in these fish. The down regulation of myog gene expression suggests a lower early differentiation/fusion of muscle precursors and the down regulation of the gene expression of structural genes a lower hypertrophic growth of muscle fibres, which might in part explain the lowest specific growth rate HPR-alevins during the restriction period. Feeding thereafter a commercial diet ad libitum induced a 4 week-period of compensatory growth, probably linked to changes in transcription of myogenic and/or muscle structural genes, but had consequence at the long term neither on the expression of these genes neither on fish growth.

The present study also showed that the composition of first feed impacted the early expression of myogenic and muscle growth-related genes, as yet demonstrated in sea bass larvae fed diets differing in cholecalciferol level (Alami-Durante et al, 2010) and in rainbow trout alevins fed diets differing in macronutrient composition (Alami-Durante et al., 2014) or methionine level (Fontagné-Dicharry et al., 2017). In present conditions, first feeding with the LP-diet (low protein content, high lipid and carbohydrate contents) led in 4 weeks, compared to first feeding with the HP-diet (high protein content, low lipid and carbohydrate contents), to a downregulation of the gene expression of pax7a1, pax7a2, myod1b and myod1c, suggesting a decrease in the number of quiescent and activated muscle precursor cells; it also led to an upregulation of fmlc2, col1α2 and ctsd gene expressions, suggesting higher synthesis of structural genes and higher proteolysis. Interestingly, first feeding with LP-diet led to long term changes in the white muscle of juveniles, with higher transcript levels of pax7 genes with no change in myod genes, suggesting a higher number of quiescent satellite cells, and higher level of myog, suggesting an improvement of early differentiation/fusion of muscle precursors. Also, the gene expression of ctsd was higher in the muscle of juveniles with LP-history, suggesting higher proteolysis. These results thus confirmed, as initially demonstrated with diets with similar protein content but different lipid/carbohydrate content (Alami-Durante et al., 2014) that the expression of myogenic and muscle growth-related genes in fish juveniles might be programmed by early nutrition.

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5.3 Tables

Table 1. **Direct effects of the early stimuli on the mRNA relative abundance of myogenic and muscle growth-related genes in alevins**

Target genes	HP	HPR	LP	*P-value*
Transcription factors				
pax7a1	1.01±0.05 a	0.90±0.04 a	0.76±0.05 b	**0.0025**
pax7a2	1.01±0.06 b	1.24±0.08 a	0.81±0.07 c	**0.0009**
pax7b	1.01±0.05	1.06±0.03	0.92±0.05	0.0873
myf5	1.03±0.10	0.97±0.10	0.84±0.12	0.4350
myod1a	1.01±0.06	0.82±0.06	0.87±0.05	0.0851
myod1b	1.03±0.08 a	0.72±0.04 b	0.88±0.06 b	**0.0073**
myod1c	1.03±0.08 a	0.78±0.04 b	0.78±0.05 b	**0.0121**
myog	1.05±0.12 a	0.51±0.07 b	0.89±0.04 a	**0.0010**
myf6	1.04±0.11	0.95±0.06	0.96±0.04	0.623
Proliferation				
pcna	1.06±0.13	0.89±0.15	1.20±0.12	0.1800
Structural genes				
fmhc	1.01±0.06	1.13±0.07	1.06±0.07	0.4750
fmlc2	1.01±0.05 a	0.83±0.03 b	1.10±0.06 a	**0.0026**
col1α2	1.04±0.10 b	0.56±0.11 c	1.37±0.07 a	**<0.0001**
Proteolysis				
ctsd	1.04±0.10 b	1.01±0.08 b	1.30±0.04 a	**0.0317**
fbx32	1.14±0.23 ab	0.71±0.07 b	1.27±0.18 a	**0.0088**

Values are means ± SE.

Table 2. Effects the early stimuli on the mRNA relative abundance of myogenic and muscle growth-related genes in the white muscle of juveniles

Target genes	HP-history	HPR-history	LP-history	*P-value*
Transcription factors				
pax7a1	1.16±0.19 b	1.11±0.22 b	3.27±0.69 a	**0.0060**
pax7a2	1.28±0.31 b	1.19±0.37 b	4.98±1.14 a	**0.0066**
pax7b	1.00±0.02 b	0.94±0.04 b	1.42±0.16 a	**0.0073**
myf5	1.09±0.17	1.04±0.07	1.58±0.33	0.4940
myod1a	1.03±0.09	1.01±0.07	0.97±0.08	0.8651
myod1b	1.01±0.04	0.99±0.07	0.92±0.09	0.6192
myod1c	1.06±0.14	0.97±0.08	1.24±0.21	0.6380
myog	1.01±0.04 b	1.09±0.05 b	1.45±0.13 a	**0.0012**
myf6	1.03±0.09	1.06±0.09	1.20±0.14	0.3830
Proliferation				
pcna	1.03±0.09 ab	0.84±0.07 b	1.29±0.14 a	**0.0032**
Structural genes				
fmhc	1.00±0.03	0.97±0.04	0.89±0.10	0.6190
fmlc2	1.01±0.05	1.01±0.06	0.94±0.10	0.7190
col1α2	1.04±0.09	1.31±0.15	1.59±0.24	0.0722
Proteolysis				
ctsd	1.02±0.07 b	0.79±0.06 c	2.28±0.55 a	**<0.0001**
fbx32	1.32±0.29	2.07±0.53	9.42±4.80	0.1620

Values are means ± SE.

6. EXPOSURE TO EARLY NUTRITIONAL STIMULI HAS LONG TERM CONSEQUENCES ON COPING ABILITY OF THE FISH.

Mammalian literature suggests that it is possible to prepare individuals to cope with future challenging environment through environmental programming during early life. Among various factors which can lead to such programming effect; nutrition is perhaps the most important building block for development of the body including brain development and there are now several studies in mammals indicating that food conditions during early-life affected coping style in later life. (Langendhorf and Komdeur, 2018). In fish, it is now well accepted that early nutritional stimuli can permanently modify metabolism of individuals later in life (Panserat et al., 2019). However, there is still a lack of information on other physiological consequences in fish of such early nutritional stimuli, especially on physiology of adaptation and stress. Such long term consequences are all the more possible and chronic nutritional challenges have been shown to modify these physiological responses: Thus, a long-term fasting and refeeding challenge has been shown to modulate stress or ion-osmoregulatory responses in various fish species (Liew et al., 2013; Li et al., 2014; Moyson et al., 2015; Jiang et al., 2017). In this context, the present study aims to analyse long-term effect on adaptation physiology of nutritional challenges applied at first feeding during 4 weeks. These challenges included hyperglucidic-hypoproteic diet or high protein diet restriction and were compared to commercial diet at normal feeding regime. The long-term consequences of these nutritional challenges were studied through analysis of ability to cope with an acute challenge (confinement stress). Such ability was assesses by measuring response of the Hypothalamp-Pituitaty-Interrenal (HPI) axis and gill functions.

6.1 Material and Methods

All the material and all the methods have been presented in chapter 2 except for the final challenge using confinement stress detailed below.

Confinement exposure

Exposure to acute confinement stress: In order to assess cortisol response to acute stress (Hypothalamo-Pituitary-Interrenal axis responsiveness), part of the fish was immediately sampled and the other was exposed to 4 minutes confinement stress: They were netted and transfer to a bucket where they were kept 4 minutes at high density (200kg/m3). Thereafter, fish were transferred back to recovery tank and further sampled 1h, 3h and 6h after confinement stress

Blood analysis and plasma biochemistry

Blood was withdrawn from the dorsal aorta using lithium-heparinized needles. Haematocrit was obtained using ammonia-heparinized microcapillary tubes and a Hettich haematocrit-200 centrifuge. Plasma was collected after centrifugation and stored at -80°C.

Plasma sodium was analysed using flame atomic absorption spectrophotometry (Varian AA240FS, Agilent Technologies, Massy, France). Plasma concentrations of chloride and calcium were measured using colorimetric kits (chloride with a mercuric-thiocyanate method and calcium with Arsenazo III (Biolabo, France)). Absorbance was measured with Synergy2 (Biotek, France).

Plasma lysozyme activity was determined using turbimetric assay (*Douxfils et al 2012 Fish et Shelfish Immunology 32: 1112-1122.*). Briefly, 20µl of rainbow trout plasma was mixed with 160µl of Micrococcus lysodeikticus (Sigma) solution (1.25mg/ml 0.05M sodium phosphate buffer, pH 6.2). Absorbance was measured at 450nm every 3 min during 30 min at 25°C (Synergy2, Biotek, France). Using a standard lysozyme chloride from chicken egg white

(Sigma) in sodium phosphate buffer, the lysozyme concentration in the plasma was expressed in U/ml .
Measurement of plasma cortisol: Steroids were measured by ELISA according to the protocol described by Faught et al. (2016).

Gene expressions
Gill and head-kidney tissues were collected on 12 fish/group just before applying the acute confinement stress. Total RNA from gill and interrenal was extracted using TRIzol reagent, according to the manufacturer's instruction. RNA was quantified by measuring the optical density at 260nm. RNA integrity was checked using the Bioanalyser 2100 Agilent.
Reverse transcriptase was realized from 2µg of RNA at 37°C for 1h using M-MLV reverse transcriptase (Promega). Real time RT-PCR was carried out on a QuantStudio 5 Real-Time PCR system (384-well) with SYBR-Green PCR master Mix (Applied Biosystem) and with BioMark™ HD system using Fluidigm 96x96 Dynamic Array.

Statistical analysis
Comparison was realized between control and 2 stress nutrition performed at first feeding: control vs nutrient restriction and control vs protein restriction. Biochemistry and gene expression were analyzed using student t-test and two-way ANOVA with time and early stress condition as independent variables. For technical reasons, plasmatic cortisol values before acute stress (time zero) were measured separately to values obtained after confinement stress. Consequently, cortisol measured before acute stress was analyzed with student t-test and 2 way ANOVA analysis was performed 1 to 6 hours after acute stress. Non parametric tests (Mann-Whitney, Kruskal-Wallis ANOVA) were used when the test of normality or equal variance of residues failed, this was the case for all plasmatic ions.

6.2 Results

Corticotrope axis
Long-term effects of early nutritional challenges on HPI axis response to acute stress was assessed through measurement of plasma cortisol levels. As shown in figure 1, acute confinement stress induced a very significant increase in plasma cortisol levels in all groups and this effect lasted at least for 6 hours. Early application of a protein restriction regime at first feeding (which is associated with hyperglucidic diet) had no effect on cortisol levels just before the acute stress whereas a significant decrease in cortisol response was observed at 1h, 3h and 6 h after acute stress. On the converse, we did not observed any significant effect of diet restriction at first feeding on plasma cortisol levels before or after the acute confinement stress.

Long-term effects of early nutritional challenges on HPI axis was also assessed through analysis of the main genes involved in cortisol production by the head-kidney (table 1). Interestingly, protein restriction significantly decreased expression of two genes (Star and CYP11A1) involved in cortisol synthesis and one gene involved in cortisol degradation (11βHSD2). On the converse, nutrient restriction has no effect on any expression of the genes analyzed.

Plasma parameters and gills functions

Plasma parameters.
Long-term effects of early nutrient challenges on ability to cope with acute stress was also analysed on plasma parameters. None of the early nutritional challenge (nutrient restriction or protein restriction) has any effect on plasma ions levels or on haematocrit (figures 2, 3, 4 and table 2). However, protein restriction has overall a significant effect on plasma lysozyme activities. This effect is specific to protein restriction stimuli as nutrient restriction at first feeding has no significant effect on lysozyme activity (figure 5).

Gill functions.
Long-term effects of early nutrient stress at first feeding were also assessed on gill functions. Various functions were studied through the measurement of expression of sets of genes involved in specific gill functions including osmoregulation (ion transporter and epithelial permeability), cellular stress, metabolism, cellular function (cell cycle), immunity and hormonal regulation. These gene expressions were measured in gill tissues collected just before the acute confinement stress (table 3). Protein restriction at first feeding has no clear long-term effects on any of these gill functions. However, the situation is more contrasted for nutrient restriction: significant changes were observed in 3 claudin genes (cldn5a, cldn8c, cldn23a) involved in gill permeability. This early nutrient stress has also significant effects on 3 genes involved in oxidative cellular stress and the other functions were not or only marginally modified. However, in the case of hormonal regulation, it is interesting to note that expression of PRL receptor gene is significantly increased.

6.3 Discussion

It is well known that early nutritional challenge could permanently modulate the metabolism of individuals later in life. In this WP, we investigate the programming effects of hyperglucidic-hypoproteic diet or food restriction at first feeding during 4 weeks and the present task aims to study whether such treatments have also long term effects on other physiological functions. Thus, we have been characterizing long term effects of those early nutritional challenges on adaptation function assessed through ability to cope with an acute confinement stress. Several studies have already shown effects of fasting on osmoregulation in fish (Leiw et al., 2013; Li et al., 2014; Sinha et al., 2015) and also on cortisol response to acute stress have also been reported ((Jiang et al., 2017). However, all these consequences have been analysed at the end of the chronic fasting period and long term consequences of such nutritional stress have not been considered so far in fish. A similar approach has been developed to study the long term effects of an early chronic hypoxia applied on trout larvae and this study showed impacts on gill functions related to osmoregulation and acid-base balance and also minor effects on cortisol response to acute confinement stress (see report D6.1). In this context, it is not surprising that early nutritional stressors used in the present study showed significant impacts on HPI axis responsiveness and on gill functions.

The present study clearly showed that a protein restriction (with a hypoproteic-hyperglucidic diet) has significant effects on HPI axis responsiveness assessed through cortisol response or interrenal gene expression: Such diet decreased plasma cortisol response to acute confinement stress and, in agreement with such decrease, we observed lower expression of 2 major genes (Star and CYP11A1/P450SSC) involved in cortisol synthesis at the level of interrenal. Another gene regulating cortisol production by interrenal (11β HSD2) also showed an decreased expression which is more surprising as this gene is involved in cortisol degradation. Interestingly, These consequences appeared to be specific to protein restriction stimuli as a nutrient restriction has no long term effects on HPI axis responsiveness. One possible explanation could be that such nutritional stress might be a too mild stress factor to have long-term effects on this function. In any case, it will be now very interesting to decipher by which mechanism hypoproteinic diet modulate HPI axis at the level of interrenal. Such information would confirm the potential interest of such early nutritional stimuli on reduction of the stress response later in life.

We have been also measuring other physiological parameters related to coping ability. When considering plasma parameters, only lysozyme activity was significantly modified by early protein restriction stimuli which led to an increase of lysozyme activity later in life. Although this was a moderate effect, it suggests that such nutritional early stimuli improved protection against bacteria. Effects of diet have been already observed in rainbow trout after specific

feeding regime: Thus, dietary application of garlic (Nya and Austin, 2011) or use of probiotic feeding regime (Sharifuzzaman and Austin, 2009) increased lysozyme activity but such a long term effect similar to what we observed in the present study has never been reported. This is an interesting information with possible application which should deserve further studies.

Analysis of expression of 91 genes in gill tissue gave us an interesting picture on the effects of early nutrient restriction or protein restriction on gill functions: Protein restriction had barely no significant effects on the gene expressions. However, nutrient restriction appeared to have more significant effects which could be anticipated based on previous studies indicating effects of fasting on gill osmoregulation (Liew et al., 2013; Moyson et al., 2015). In the present study, nutrient restriction had no effects on expression of genes involved in ion transport but still significantly modify 3 claudin genes involved in epithelial permeability. Genes involved in cellular oxidative stress in gill (cat, sod2, gpx1a) also showed changes of their expression following early nutrient restriction. Such long term effect of an early nutritional stimuli is in agreement with effects of 49 days starvation and food restriction which increased cat and gpx activities suggesting oxidative stress in gill cells during this treatment (Bayir et al;, 2011). In this context, it would be interesting to confirm such effects by measuring ROS activity in the gill.

In conclusion, our data clearly indicated that nutrient or protein restrictions applied at first feeding have long term effects on coping ability assessed at the level of HPI axis and gill functions. These effects are moderate but significant: a similar situation have been observed in experiments on long term effects of early hypoxia stress, thus confirming that different kinds of stressors (environmental, nutritional) applied on early stages at first feeding have long term consequences on coping ability of the fish. Whether these long term effects would be beneficial or detrimental is difficult to conclude.

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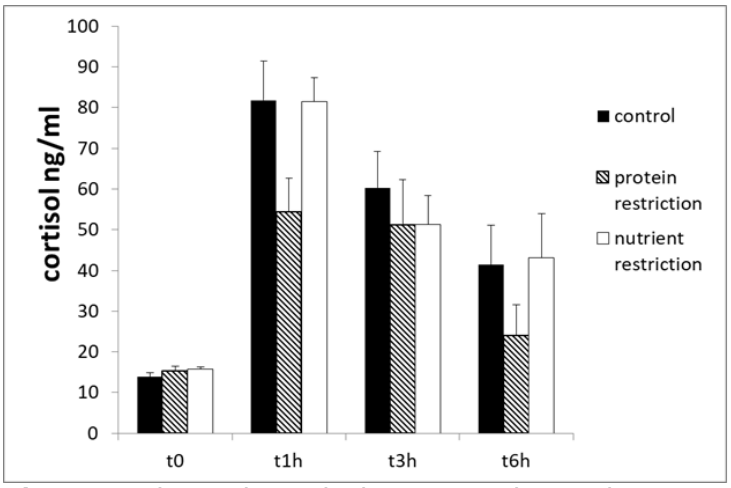
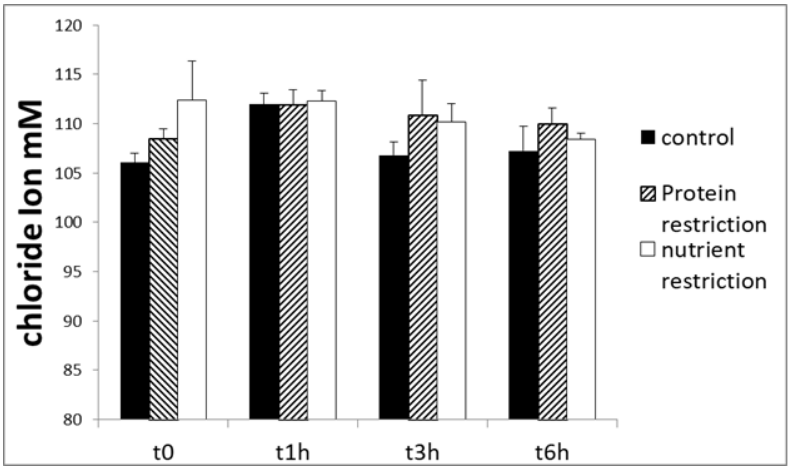
6.4 Tables/Figures



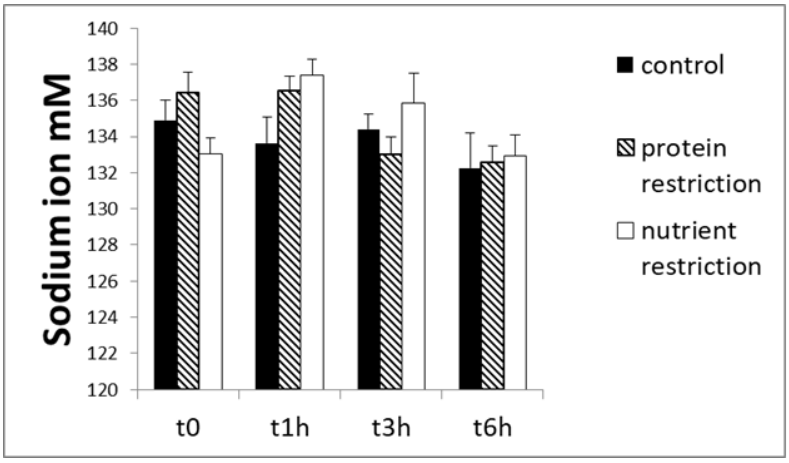
	Protein restriction	Nutrient restriction
Dietary history	*P= 0,017	P=0,71
Time effect	***P= 0,0007	***P=0,00007
Interaction	No	No

Figure 1: Plasmatic cortisol concentration. Values are presented as mean ± SEM of 12 fish. Two ways ANOVA analysis (between t1h and t6h) with time and early stress condition as independent variables. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*



	Protein restriction	Nutrient restriction
Dietary history	P= 0,223	P=0,114
Time effect	**P= 0,0031	***P=0,0004

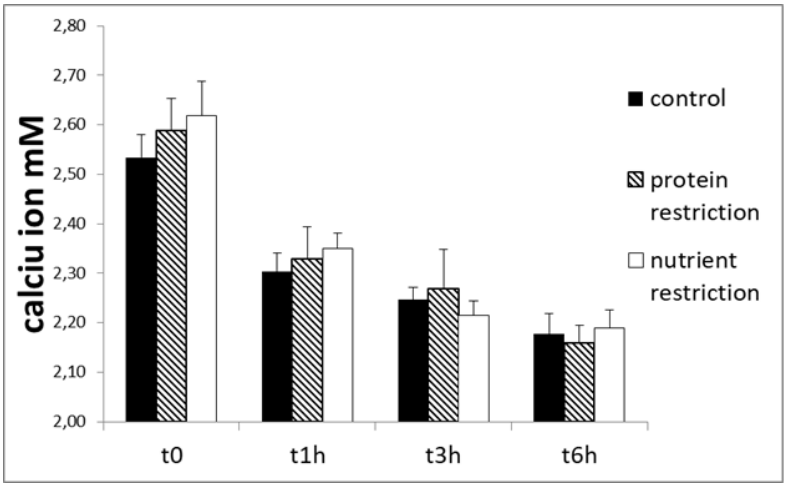
Figure 2: Plasmatic chloride concentration. Values are presented as mean ± SEM of 11-12 fish. Kruskall-Wally test was used for statistical analysis. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*



	Protein restriction	Nutrient restriction
Dietary history	P= 0,657	P=0,654
Time effect	P= 0,08	P=0,211

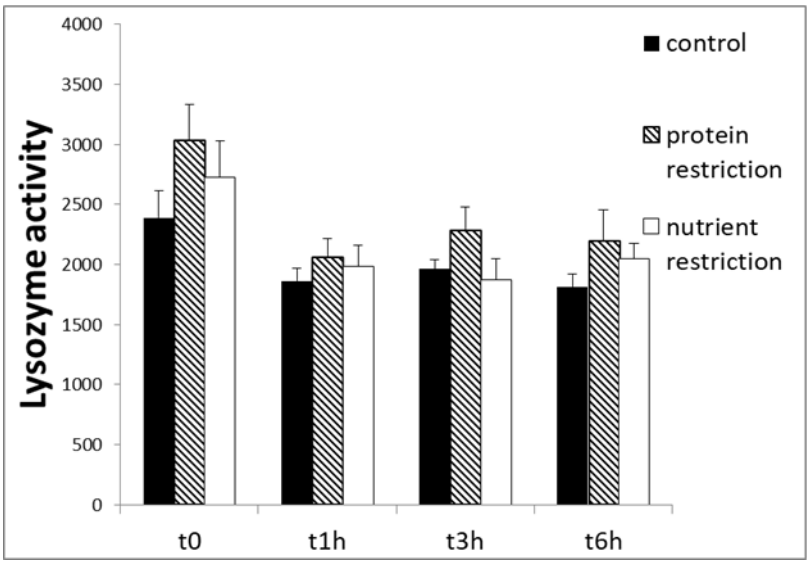
Figure 3: Plasmatic sodium concentration. Values are presented as mean ± SEM of 11-12 fish. Kruskall-Wally test was used for statistical analysis. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*

Calcium



	Protein restriction	Nutrient restriction
Dietary history	P= 0,843	P=0,958
Time effect	***P< 0,0001	***P< 0,0001

Figure 4: Plasmatic calcium concentration. Values are presented as mean ± SEM of 11-12 fish. Kruskall-Wally test was used for statistical analysis. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*



	Protein restriction	Nutrient restriction
Dietary history	**P= 0,009	P=0,317
Time effect	**P= 0,001	**P=0,003
Interaction	No	No

Figure 5: Lysozyme activity in plasma Values are presented as mean ± SEM of 11-12 fish. Kruskall-Wally test was used for statistical analysis. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*

Table 1. The effects of early nutrient stress on gene expression in head kidney. Values are presented as mean ± SEM of 11-12 fish. Student t-test and Mann-Whitney U-test (in italic) were used for statistical analysis. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*

				P-value	
gene	control	protein restriction	nutrient restriction	protein restriction	nutrient restriction
star	1,08±0,1	0,78±0,1	1,28±0,33	**0,048***	0,170
CYP11A1	1,07±0,12	0,76±0,09	1,2±0,11	**0,049***	*0,386*
3βHSD	1,06±0,11	0,87±0,11	1,39±0,13	*0,453*	0,070
CYP11B1	1,06±0,09	0,87±0,13	1,31±0,12	0,252	0,122
GR1	1,01±0,05	1,15±0,07	1,18±0,07	0,129	0,058
GR2	1,04±0,09	0,96±0,07	1,09±0,08	0,495	0,699
MR	1,01±0,05	1,02±0,1	1,03±0,08	*0,908*	0,904
HSD11B2	1,1±0,13	0,72±0,12	1,19±0,12	**0,038***	0,650

Table 2. The effects of early nutrient stress on hematocrit. Values are presented as mean ± SEM of 12 fish. Student t-test was used for statistical analysis. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*

				P-value	
	control	protein restriction	nutrient restriction	protein restriction	nutrient restriction
hematocrit	44,46±1,17	47,5±1,42	47,5±1,27	0,111	0,091

Table 3. The effects of early nutrient stress on gene expression in gills. Values are presented as mean ± SD of 9-12 fish. Mann-Whitney U-test was used for statistical analysis. *Note: Control = HP history; Protein restriction = LP History; Nutrient restriction = HPR history*

					P-value	
	gene	control	protein restriction	nutrient restriction	protein restriction	nutrient restriction
ionic homeostasis	ATP1a1a	1,06±0,35	1,17±0,45	1,14±0,38	0,564	0,686
	ATP1a1b	1,04±0,32	1±0,35	1,16±0,36	0,862	0,644
	ATP1a1c	1±0,07	1,02±0,1	1,02±0,13	0,773	0,686
	ATP1a3	1,07±0,39	1,21±0,27	1,1±0,34	0,204	0,773
	at233	1,03±0,25	0,99±0,17	1,17±0,28	0,862	0,204
	cic2	1,11±0,57	1,4±0,5	1,58±0,66	0,133	0,065
	nkcc1a	1,02±0,24	1,02±0,29	1,17±0,33	0,729	0,184
	nbc1	1,11±0,53	1,33±0,41	1,45±0,44	0,299	0,094
	ATP6v1b	1,01±0,13	1,08±0,11	1,09±0,13	0,149	0,149
	ca2	1,12±0,61	1,12±0,41	1,2±0,34	0,729	0,204
	ca4	1,13±0,53	1,67±0,5	1,36±0,48	**0,021***	0,525
	nhe2	1,02±0,2	1,05±0,12	1,01±0,17	0,525	0,954
	nhe3	1,03±0,26	1,11±0,19	1,26±0,32	0,273	0,106
	slc26a6	1,13±0,54	1,19±0,37	1,39±0,53	0,644	0,273
	cftr1	1,16±0,75	1,05±0,36	1,52±0,75	0,773	0,184
	cftr2	1,01±0,13	0,97±0,22	1,1±0,25	0,773	0,106
	slc10a3	1,01±0,15	1,02±0,19	1,03±0,22	0,817	0,729
	ATP2b1	1±0,09	1,04±0,1	1,06±0,11	0,356	0,326
	ECaC	1,03±0,29	1,18±0,43	1,16±0,53	0,386	0,817
	rhag	1,02±0,23	1,05±0,26	1,01±0,18	0,773	1,000
	rhcg1	1,01±0,17	1,14±0,22	1,21±0,41	0,184	0,248
	rhcg2	1,03±0,25	1,21±0,41	1,16±0,25	0,299	0,166
	ut	1,06±0,39	1,05±0,6	0,98±0,42	0,686	0,908
gill permeability	cldn1	1,01±0,17	1,15±0,17	1,05±0,09	**0,043***	0,686
	cldn5a	1,01±0,19	0,92±0,13	0,81±0,17	0,453	**0,007***
	cldn7	1,02±0,24	1,17±0,18	1,24±0,35	0,094	0,094
	cldn8c	1,08±0,48	1,41±0,52	1,63±0,65	0,065	**0,024***
	cldn8d	1,01±0,16	1,07±0,13	1,08±0,17	0,603	0,453
	cldn10d	1,04±0,32	1,22±0,28	1,22±0,53	0,204	0,603
	cldn10e	1,05±0,32	1,34±0,44	1,24±0,44	0,073	0,273
	cldn12	1,01±0,12	1,05±0,09	1,06±0,13	0,386	0,564
	cldn23a	1±0,1	1,08±0,19	1,14±0,14	0,248	**0,028***
	cldn27b	1,01±0,17	1,06±0,14	1,15±0,28	0,603	0,248
	cldn28b	1,02±0,23	0,94±0,16	0,9±0,13	0,356	0,166
	cldn30	1,03±0,29	1,05±0,25	1,11±0,28	0,729	0,525
	cldn33b	1,07±0,42	1,11±0,59	0,86±0,29	0,686	0,225
	ocln	1,01±0,12	1,06±0,09	1,1±0,13	0,248	0,065
	zo-1	1,01±0,14	1,05±0,15	1,01±0,18	0,564	0,729
	ag2	1,14±0,6	1,38±0,56	1,07±0,65	0,273	0,603

	muc	1,04±0,32	1,2±0,36	0,95±0,36	0,248	0,419
	vamp8	1,01±0,17	0,94±0,13	1,05±0,21	0,248	0,488
cell cycle	casp6	1,02±0,19	1±0,15	1,08±0,17	0,817	0,564
	casp7	1,01±0,16	1,04±0,15	1,08±0,23	0,488	0,225
	casp8	1,03±0,24	0,99±0,22	1,11±0,41	0,773	0,954
	casp9	1,01±0,18	1,05±0,11	1,08±0,17	0,149	0,166
	pcnail	1,03±0,26	1,14±0,22	1,09±0,23	0,184	0,488
	p21	1,02±0,23	1,06±0,28	1,18±0,34	1,000	0,356
	p53	1±0,08	1,06±0,11	0,98±0,1	0,184	0,453
	rad51	1,02±0,21	1,14±0,18	1,2±0,29	0,225	0,083
	gadd45	1,01±0,14	1,05±0,15	1,05±0,13	0,419	0,326
	vdac2	1,02±0,19	1,13±0,12	1,09±0,2	0,119	0,386
	egr1	1,48±1,83	0,64±0,27	1,95±2,65	0,073	0,299
cellular stress	cat	1,01±0,16	1,09±0,1	1,18±0,21	0,273	**0,050***
	gsr	1,01±0,18	1,12±0,19	1,14±0,3	0,356	0,386
	sod1	1,03±0,24	1,08±0,2	1,18±0,19	0,686	0,119
	sod2	1,02±0,21	1,05±0,1	1,16±0,16	0,419	**0,033***
	gpx1a	1,01±0,14	1,23±0,16	1,25±0,12	**0,003***	**0,001***
	gst	1,07±0,42	0,99±0,25	0,91±0,28	0,817	0,356
	cyp1a	1,15±0,26	0,97±0,28	1,21±0,39	0,525	0,644
	st1s3	1,04±0,34	0,95±0,27	1,07±0,4	0,248	0,817
	sult1a4	1,01±0,16	1,2±0,46	1,15±0,4	0,419	0,773
	cox4a	1±0,11	1,05±0,12	1,12±0,17	0,326	0,106
	hif1a	1,01±0,13	1,03±0,1	1,08±0,17	0,729	0,386
	hsp47	1,01±0,17	0,96±0,2	0,86±0,2	0,356	**0,050***
	hsp70	1,1±0,6	1,19±0,57	1,49±0,79	0,488	0,166
metabolism	glut1	1,13±0,57	1,4±0,63	1,4±0,54	0,273	0,273
	glut3	1,05±0,36	0,9±0,13	1,26±0,75	0,273	0,453
	pkm	1,02±0,22	1,11±0,18	1,14±0,16	0,299	0,149
	cs	1±0,09	1,07±0,07	1,1±0,13	0,065	**0,050***
	gys	1,02±0,21	1,04±0,22	1,07±0,23	0,817	0,817
	acly	1,08±0,48	0,98±0,56	0,92±0,58	0,644	0,488
immunity	IgM	1,4±1,41	1,46±1,35	1,05±0,64	0,862	0,908
	IgT	1,85±2,16	2,2±2,73	0,7±0,7	0,773	0,184
	mhc1	1,04±0,26	0,94±0,41	1,05±0,46	0,686	0,603
	mhc2	1,02±0,2	1,02±0,17	0,9±0,16	0,729	0,225
	il1b	1,37±1,48	0,74±0,19	1,71±1,4	0,386	0,094
	mx	1,14±0,66	0,79±0,32	1,18±0,95	0,204	0,564
	lyz-c	1,02±0,23	1,11±0,22	1,02±0,32	0,299	0,773
	lyz-g	1,03±0,29	1,04±0,31	1,02±0,32	0,817	0,453
	nod2	1,02±0,19	0,95±0,12	0,94±0,16	0,488	0,273
	iNOS	2,66±3,65	3,66±1,93	8,05±10,81	0,083	**0,028***
hormonal regulation	igf1	1,01±0,13	0,97±0,2	1,06±0,21	0,644	0,488
	igf2	1,01±0,12	0,99±0,16	1,02±0,18	0,908	0,908
	ghr2	1,01±0,15	0,95±0,14	1,02±0,14	0,419	0,817
	prlr	1,02±0,23	1,12±0,26	1,28±0,34	0,273	**0,024***

	thra	1,01±0,14	1,04±0,11	1,01±0,08	0,773	0,862
	hsd11b2	1,15±0,75	1,47±0,64	1,06±0,38	0,184	0,644
	gr1	1,01±0,16	1,05±0,08	1,01±0,13	0,729	0,686
	gr2	1,01±0,11	1±0,06	0,91±0,13	0,862	0,083
	mr	1,01±0,16	1,02±0,16	0,93±0,15	0,817	0,133
	edn1b	1,07±0,41	1,25±0,3	1,14±0,32	0,248	0,564

7. General Conclusion

The aim of the present study was to assess in rainbow trout the long-term consequences of early feeding (first feeding stage). When considering results obtained by partners INRA-LPGP and INRA-NuMeA and presented in parts 6 (LPGP) and 2-5 (NuMeA), we can conclude that early feeding can affect metabolism and stress response of juvenile fish later in life. When considering these various traits, the following conclusions can be drawn:

1) *Better capacity of glucose metabolism in muscle:* after the first feeding with high level of carbohydrates, in muscle of juvenile fish, higher capacities for glucose metabolism (glycolysis, glycogen) (but not of lipid and protein metabolism) have been observed very clearly. This observation was associated with a lower level of DNA methylation in muscle suggesting the existence of epigenetics mechanisms linked to the intake of a new diet at first feeding. Moreover, expression of myogenic and muscle growth-related genes in fish juveniles might be also programmed by first feeding with low protein/high carbohydrates diet.

2) *Long term effect on autophagy (mitophagy):* when the alevins have been fed 50% less food than the control (energy restriction), higher capacity for autophagy (mitophagy mainly) were detected at the molecular level suggesting that the feed restriction can impact at long term the capacity of the fish to modify the level of autophagy (a main function involved in metabolism, nutrition, growth, health etc…)

3) *Resistance to confinement stress*: our data clearly indicated that nutrient or protein restrictions applied at first feeding have long term effects on coping ability assessed at the level of HPI axis and gill functions. These effects are moderate but significant: a similar situation have been observed in experiments on long term effects of early hypoxia stress, thus confirming that different kinds of stressors (environmental, nutritional) applied on early stages at first feeding have long term consequences on coping ability of the fish.

4) Despite the above long-term effects of early feeding in juvenile fish, it is still difficult to conclude whether they will be overall beneficial or detrimental for the juvenile fish. The multi-functions approach developed in the present study provide us clear information on these long term effects but does not allow us to conclude positively or negatively on fish robustness.

Glossary

AQUAEXCEL^{2020}: AQUAculture Infrastructures for EXCELlence in European Fish Research towards 20

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Annex 1: Check list

Deliverable Check list (to be checked by the “Deliverable leader”)

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time		*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW		*If not please inform the Management Team with justification*
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	I am using the AQUAEXCEL^{2020} deliverable template (title page, styles etc)		*Available in “Useful Documents” on the collaborative workspace*
The draft is ready			
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	The deliverable has been reviewed by all contributors (authors)		*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified		
	I have sent the final version to the WP Leader, to the 2nd Reviewer and to the Project coordinator (cc to the project manager) for approval		*Send the final draft to your WPLeader, the 2nd Reviewer and the coordinator with cc to the project manager on the 1st day of the due month and leave 2 weeks for feedback. Inform the reviewers of the changes (if any) you have made to address their comments. Once validated by the 2 reviewers and the coordinator, send the final version to the Project Manager who will then submit it to the EC.*