AQUA EXCEL 2020

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D6.8: Early life stage and environment effect on FI (WU)

Authors: *Roel Maas, Ep Eding, Stephane Panserat, Patrick Prunet, Isabelle Leguen, Johan Schrama*

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Executive Summary

Objectives: Identification of long-lasting effects of low oxygen concentration (hypoxia) in very early stages (larval) on performances of juvenile rainbow trout under varying oxygen levels.

Rationale: Previous studies in mammals and birds but also recently in fish have acknowledged that early life is a critical period for developmental plasticity. Exposure to stressful events during early life stages may affect the metabolic phenotype and a stimulus received in early life can modulate metabolism in later life, a process which is called 'metabolic programming'. In this context exposure to low oxygen levels in early life might alter oxygen consumption and feed intake in later life. 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 later performance at the juvenile or adult stages. Information on such long-lasting effects is important to ensure proper management of experimental fish groups before undertaking experiments.
In order to assess the effect of early life hypoxia on later life performance a cohort of rainbow trout yolk-sac larvae were exposed for 17 days (starting two days after hatching) to hypoxia- or normoxia conditions. In later life fish from both early life treatment groups were assessed for carry over effects on feed intake, feed efficiency and metabolic markers and parameters related to oxygen use. These performance parameters in later life were quantified for five declining oxygen levels using six replicates per early life treatment (hypoxia, normoxia). It was expected that fish exposed to early life hypoxia will show a higher feed intake when fed to satiation under varying available oxygen levels. In addition, it was expected that the point where the available oxygen becomes limiting for feed intake is at lower levels for the hypoxia treatment group when compared to the normoxia treatment group. However, no information is available on possible effects of early-life hypoxia on these parameters.

Main Results:

Feed intake and oxygen consumption: The assessments of feed intake, feed efficiency and metabolic parameters related to oxygen consumption in juvenile fish exposed life or not to early-life hypoxia under varying oxygen levels showed:
- there is an significant effect of early life hypoxia on growth performance in later life in juvenile rainbow trout;
- decreasing levels of available oxygen reduced feed intake, this effect was independent of early life history;
- there is no effect of early life hypoxia on feed intake, oxygen consumption and dietary oxygen demand in later life in juvenile rainbow trout;
- no effect in the molecular data showing that programming of energy metabolism linked to the early hypoxia treatment in yolk sac larvae of rainbow trout was not effective in this experiment when trout are challenged with different oxygen levels in later life.

Authors/Teams involved:

WU: Roel Maas
Ep Eding
Milan Jagt
Johan Schrama

INRA/NuMeA: Stephane Panserat

INRA/LPGP: Isabelle Leguen, Patrick Prunet

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1. Introduction and rationale (general)

Excellent management of experimental fish stocks is 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 birds 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 disrupt brain development thus altering brain maturation endpoints and consequently modifying brain-related processes such as behavior or stress response (Auperin et al., 2008; Fokos et al., 2017). It could also affect metabolic phenotype and stimulus received at early life can modulate metabolism in later life, a process which is called 'metabolic programming' (Lui 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. Water quality and particularly O_2 levels are considered as the primary stressors in fish, a situation which is particularly important in early life stages where oxygen depletions are likely to affect survival and the success of larval development (Cadiz et al., 2017 ; Mu et al., 2017). In this context, filling the knowledge gap on the later life consequences of hypoxia exposure during early life stages would improve our understanding regarding the quality of the experimental fish for use later in life in aquaculture research infrastructures.

The aim of the present study was to assess in rainbow trout to which extend early-life challenging factor (i.e. hypoxia) can affect performance in juveniles later in life. These issues have been approached through a research question: Does chronic hypoxia applied during early life modify feed intake, feed efficiency and metabolic parameters related to oxygen use in later life?

The study was performed at the research infrastructure of WUR (The Netherlands). All fish used had the same genetic background and were in INRA/PEIMA (France) exposed during early life (yolk-sac stage) to either hypoxia or normoxia conditions.

2. Effect of early life hypoxia on growth, feed intake and oxygen consumption of rainbow trout in later life at varying oxygen levels

2.1. Introduction

Experiments in aquaculture research infrastructures (RIs) are often compromised by sub-maximal feed intake (FI) and growth in control groups. If maximal feed intake is not achieved, conclusions on several research questions cannot be drawn, and the industry relevance of the results become questionable.
This experiment aims to study the factors and mechanisms which affect voluntary feed intake regulation of fish in later life. The effects of factors such as nutrition, environment and physiological factors on voluntary feed intake regulation are less extensively researched in fish when compared with warm blooded animals. However, it is well known that under hypoxia conditions (oxygen is limiting maximum feed intake) oxygen availability determines feed intake (Tran Duy et al., 2012; Saravanan, 2013). Under these conditions, oxygen consumption and feed intake are mutually connected. However, even under normoxia (oxygen level in the water is not limiting) there are several factors which might play a role in feed intake regulation and determine maximum feed intake.

One of these factors which might play a role in determining maximum feed intake in later life is the oxygen level fish are exposed to in early life. It is our hypothesis that a low oxygen level in early life adjusts gene functions (epigenetics) which are responsible for an increased oxygen uptake capacity of fish in later life. A higher oxygen uptake capacity means a potential for a higher maximum feed intake when this characteristic remains in later life. Increased feed intake capacity is expected to increase nutrient metabolism (e.g. lipid metabolism, mitochondrial energy metabolism, amino acid catabolism, glucose transport, glycolysis and gluconeogenesis) and blood plasma levels of e.g. glucose, triglycerides, free amino acids and free fatty acids.
Dissolved oxygen (DO) levels are called hypoxic when FI is limited by the DO level. There is no tangible value for DO at which hypoxia occurs, as this is dependent on many variables, such as species, metabolic needs and dietary values. When DO is not limiting FI, circumstances are called normoxic. DO can be limiting for FI as a fish will reduce its feed intake in order to prevent exceeding its maximum aerobic capacity. The concentration at which oxygen becomes limiting for feed intake is termed incipient dissolved oxygen (iDO). However, no information is available if the effect on maximum feed intake will be similar for fish exposed to low oxygen (hypoxia) in early life when compared to fish kept on normoxia conditions in early life. It is also unclear if the effect of varying oxygen levels on feed intake is different for fish with a hypoxia history- compared to fish kept on normoxia conditions in early life, i.e., do fish with an hypoxia history have a higher tolerance for hypoxia and have an iDO at a lower DO level?
In conclusion, identification of potential long-lasting effects of low oxygen concentrations (hypoxia) in very early (larval) rearing phases on future fish performance is important, to ensure proper management of experimental groups before the actual growth and nutrition experiments are undertaken. Information on the effect of low oxygen levels in early life on the maximum oxygen uptake and feed intake in later life under varying oxygen levels is not available. Therefore, this experiment is related to feed intake regulation and assesses weather early life hypoxia affects:

- growth potential, feed intake (FI), feed efficiency and metabolic parameters related to oxygen use under varying oxygen levels (WU);
- aerobic and anaerobic nutrient metabolism parameters in relation to oxygen use and

diet composition (INRA NuMeA);

In order to assess the above main objectives a 2 x 5 (with repeated measures) factorial experiment was designed to determine the impact of early life history (exposure to hypoxia or normoxia) on later life apparent voluntary feed intake when feeding fish under varying available oxygen levels (5 levels).

The following research questions were formulated:
(1) Does early life hypoxia affect oxygen consumption in later life?
(2) Does early life hypoxia change feed intake in later life?
(3) Is the impact of early life hypoxia on feed intake and oxygen consumption dependent upon the available DO level (i.e., shift in iDO)?

2.2. Material and methods

This experiment was performed in the experimental facilities of WU in accordance with the Dutch law on the use of experimental animals and was approved by the Central Animal Experiments Committee (CCD) (project number 2017 W.0037). Fish were kept and handled in agreement with the current EU-legislation on handling experimental animals.

2.2.1. Fish and Housing

Early life history. Two days post hatching (dph) fish were exposed to normoxic or hypoxic conditions (water with a low oxygen level, 30-40% oxygen saturation or a high oxygen level, 90-100% oxygen saturation respectively) for 17 days. Afterwards the fish were kept under similar conditions and fed restrictively. The rainbow trout used were produced, cultured, maintained and treated by INRA France, in agreement with the regulations in force and under the formal French approval.
The rainbow trout were transported from INRA France to the experimental facility of Wageningen University, The Netherlands. Fish were fed a commercial diet (ME-1.0 MP Presta, Skretting, France). The applied feeding level was 12.5g feed $kg^{-0.8}day^{-1}$. Two weeks prior to the start of the experiment fish (229 and 233 fish of the hypoxia and normoxia treatment respectively) were transferred to the 12 experimental tanks to adapt to the new experimental environment and were fed a commercial diet (T-1P Optiline, Skretting, France). During the last two weeks, the feeding level per tank (between 5-8 g feed $kg^{-0.8}day^{-1}$) was adapted based on the mean initial bodyweight. This allowed to correct for differences in mean body weight and to equalize the mean initial weights at the start of the experiment. Three hundred eighty rainbow trout (*Oncorhynchus mykiss*) were used as experimental animal (190 fish per treatment, 10 for initial body composition and 30 fish/tank) for the current study. At the start of the experiment the fish had a mean weight of 38.6±0.26 (SD) g.

Housing. Fish were housed in 12 glass tanks (200L each) of the metabolic research unit (MRU) of Wageningen University. The tanks were covered with a floating lid to prevent gas exchange with the air. All tanks were connected to the same water recirculation system (RAS). Each tank was facilitated with a digital water flow meter connected to the tank influent and a faeces collection unit (swirl separator) connected to the tank effluent. Water temperature was 15 ± 1 °C, water flow to each tank was 4 - 7 ± 0.1 L min^{-1}, depending on the intended available oxygen level. A 12h : 12h (Light : Dark) photoperiod was maintained, with daybreak set at 7:00h.

2.2.2. Experimental design

To assess the effect of early life hypoxia on later life growth performance, nutrient digestibility, body composition and the nitrogen- and energy balance for the whole experimental period (using varying levels of available oxygen), two treatments were compared: Hypoxia vs. Normoxia. For the assessment of the effect of early life hypoxia on later life feed intake and oxygen consumption a 2 x 5 factorial design was used (Table 1).

The first factor was the exposure to two oxygen saturation levels in early life: 30-40% oxygen saturation (Hypoxia) or 90 - 100% oxygen saturation (Normoxia). The second factor was the available DO level in later life, using 5 graded levels over time (repeated measures). The applied available DO levels were indicated as 100%, 90%, 80%, 70% and 60%. The oxygen concentration in the water inflow of the 12 tanks were equal for all 5 DO levels, with the exception of the 100% level where oxygen injection was used to elevate the oxygen concentration to approximately 11.3 mg O_2/L in the water inflow. The 7 L min^{-1} water inflow per tank without pure oxygen was the 90% DO level treatment. The steps from 90% to 60% were realized by decreasing the water inflow per tank with 1 L min^{-1} per step. After a period of 2-weeks the fish were gradually exposed to the different DO levels by reducing the flow rate to the 12 tanks as previously described. Flow rates and thus available oxygen levels were fixed for 2 days before being decreased to the next level. When the tanks were supplied with water at a flow rate of 4 L min^{-1} water supply to the tanks started to fluctuate due to clogging of the water inlet. After 2 days at 4 L min^{-1}, the flow was increased to 7 L min^{-1} and oxygen was again injected in the tank inlet water. The 12 tanks ran on these settings for 4 days, after which the recirculating aquaculture system (RAS) where the tanks were part of was switched to a partly flow through system in order to prevent fouling of the tank water inlets. The system was kept on the 100% level for 9 more days before the levels were decreased again in the same fashion as described above. The system was kept at the minimum level of 60% for 7 days. After this period, available DO levels were increased again to 100% level, stayed there for two days and were thereafter as before gradually decreased to 60%. After 4 days on the 60% level, oxygen levels were increased to 100% again. The 100% level was maintained for 2 days after which the experiment was ended. Figure 1 gives an overview of the moment and exposure time to each available oxygen level treatment over time.

At the start of the experiment, fish with a similar early life oxygen history (normoxia vs hypoxia) were randomly divided over the tanks assigned to the matching oxygen history. Each tank was stocked with 30 fish. Additionally, per oxygen history 10 fish were randomly taken as a start sample for initial body composition. Each treatment was done with 6 replicate tanks (Table 1). To minimize the influence of tank location, treatments were randomly assigned to a block of two tanks, the tank treatments of the tanks 1-6 mirrored the tanks 7-12. Oxygen measurements were done continuously over all 12 tanks. The individual tank was the experimental unit. The experiment had a duration of 63 days, during which growth performance, feed intake, oxygen consumption, digestibility and metabolite excretion (Total Ammonia Nitrogen (TAN), Urea, CO_2 and P) were measured. During the experiment 54 fish were sampled for plasma, liver and white muscle tissue for gene expression. Nine fish from each early life history treatment were sampled at the 100% (day 15), 80% (day 55) and 60% (day 61) oxygen level.

Table 1. Experimental design. In early life treated normoxia and hypoxia treated fish (6 replicates per early life treatment, 30 fish per tank) were exposed in later life to 5 available oxygen level treatments (100%, water flow 7 L/min with oxygen injection; 90%, flow 7 L/min; 80%, flow 6 L/min; 70%, flow 5 L/min; 60% flow, 4 L/min).

Early life treatment	Available oxygen level treatment				
	100%	**90%**	**80%**	**70%**	**60%**
Normoxia (6 replicates)	80.5[1)]	70.7	60.6	50.5	40.4
Hypoxia (6 replicates)					

1) Values per treatment represent the absolute amount of oxygen available per minute (mg O_2/min) per available oxygen level treatment.

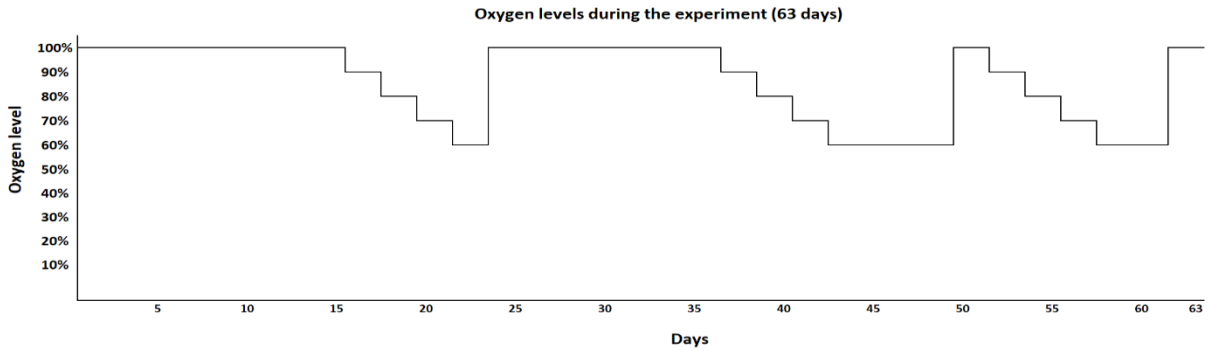

Figure 1. Overview of the exposure moment and exposure duration (2 days) to each available oxygen level treatment during the experimental period.

2.2.3. Experimental diet and feeding

The experimental diet used was high in dietary oxygen demand (high DOD) and contained high levels of starch as non-protein energy source. This challenging diet was used in order to magnify the potential effect of the treatments on feed intake. The diet was formulated by our group and produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) (Table 2). The Diet consisted of 2mm extruded floating pellets. Yttrium in the diet has been as inert marker to determine apparent digestibility. Before feeding, feed fines were removed by sieving. Weekly a sample of 100 gram per diet was collected for later analysis.

Table 2. Experimental diet composition.

Energy source	
Ingredient (%):	
Maize starch (gelatinized)	25.00
Wheat	21.49
Wheat gluten	10.00
Fish meal (RE>680)	20.00
Fish oil	1.00
Soya protein concentrate	10.00
Pea protein concentrate	10.00
Lysine HCL	0.10
DL-methionine	0.40
Monocalcium phosphate	1.00
Yttrium oxide	0.01
Premix	1.00
Total	**100.00**
Analysed nutrient content (g/kg feed DM):	
Dry matter (DM, g/kg)	908
Crude protein	416
Crude fat	56
Crude ash	59
Total carbohydrates[1)]	469
Gross energy (kJ/g)	19.8

[1)] calculated as, total carbohydrates = 1000- (crude protein + crude fat + ash).

Fish were acclimatised to the new diet by feeding them 25%, 50% and 75% of the expected satiation feeding level on day 1 (day of stocking the tanks), day 2, and day 3 respectively. From day 4 onwards maximum daily feed intake per tank was recorded. During the experiment, animals were hand-fed twice a day (09.00-10:00h and 16:00-17:00h) to apparent satiation (i.e. ad libitum). Visual observation of uneaten pellets at the bottom of a tank indicated cessation

of feeding. Fifteen minutes after feeding uneaten pellets were collected from the faecal collection units (swirl separators) and the tank bottom, and pellets were counted for leftovers. For each feed moment, feed ration, uneaten pellets and feed remains were registered and used to calculate daily feed intake.

2.2.4. Sampling

Initial sampling (initial body composition, WU): At the start of the experiment, per early life history treatment (Normoxia vs Hypoxia), 10 fish were sampled for initial body composition analysis.

Sampling after 15, 55 & 61 days (aerobic and anaerobic nutrient metabolism parameters, INRA NuMeA): Fish were sampled at available oxygen levels of 100% (day 15), 80% (day 55) and 60% (day 61). Approximately 4-4.5 hours after the last morning feeding, for each treatment 9 fish were sampled (2 treatments, 6 tanks each, 1 or 2 fish per tank). Sampled fish were anaesthetized using 2-phenoxyethanol (1 ml L^{-1}), individually weighed, and blood sampled (caudal vein puncture, using Na-Heparin (Leo Pharma, 5000 UI; 0.1 ml $2ml^{-1}$) as anti-coagulant, 2 x 2 ml blood per fish). The sampled blood was used to collect plasma. Blood for plasma sampling was centrifuged within 5 minutes after sampling (3500 rpm, 5 minutes), thereafter plasma was sampled and stored in the freezer (-20 °C first, thereafter transferred to -80 °C). Blood plasma were analysed for glucose, triglycerides, free amino acids and lactate. Blood plasma analysis was done as additional work and not yet available at the time of reporting. Results are therefore not incorporated in the deliverable.
Directly after blood sampling, fish were euthanized by an overdose of anaesthetics (1 ml L^{-1} 2-phenoxyethanol), followed by decapitation. From each fish, liver (whole) and white muscle tissue (2cm x 2cm, muscle anterior of the dorsal fin) were immediately dissected, weighed and frozen in liquid nitrogen and thereafter stored at -80 °C for subsequent analysis.

Sampling at 63 days (final body composition, WU): The day before sampling, animals were not fed. On the sampling day, fish were batch weighed per tank while anaesthetized (0.25 ml L^{-1} 2-phenoxyethanol). For each tank, randomly 10 fish were taken as a sample for final body composition. The 10 fish were euthanized by an overdose of anaesthetics (1 ml L^{-1} 2-phenoxyethanol), pooled per tank, and stored directly in the freezer (-20 °C). Per tank, remaining fish were placed back into the tanks.

2.2.5. Measurements and analysis

Growth performance. At the start and at 63 days, fish were counted and batch weighed per tank were taken. In addition, fish sampled at 15, 55 and 61 days were individually weighed. From these measurements absolute growth rate (g day^{-1}), metabolic growth rate (GR_{MBW}, g $kg^{-0.8}$ day^{-1}), specific growth rate (SGR, % day^{-1}), feed conversion ratio (FCR) and survival (%) was calculated per tank, using the formula's presented in table 3.

Nutrient digestibility. During the growth period, faeces were collected starting from day 10 using the method as described by Amirkolaie et al. (2006). Before analyses faeces samples were grinded (Retsch ZM 200). The collected faeces were pooled per tank and per available oxygen level (100%; 90% and 80%; 70% and 60%). A weighted sample (according to the amount of days at each available oxygen level) was made (after grinding) and analysed. Faeces samples and feed samples (100 gram $diet^{-1}$ $week^{-1}$) were analysed for dry matter (DM), ash, crude protein (CP), crude fat (CF), energy and yttrium (Y) content, as described by Maas et al. (2018). Carbohydrate content of the feed and faeces was calculated as 1000 – CP – CF – ash. From these data apparent digestibility coefficients (ADC, %) and digestible nutrient intake (g $kg^{-0.8}$ day^{-1}) were calculated, using the formula's presented in table 3.

Oxygen measurements. Oxygen measurements were done continuously and where performed as described by Saravanan et al. (2012). For the oxygen consumption calculations only the data from day 50 till 61 was used. All 12 tanks were measured in one block. Oxygen data was used to calculate oxygen consumption (mg $kg^{-0.8}$ min^{-1}) and dietary oxygen demand (mg O_2 per gram DM intake and mg O_2 per kJ digestible energy (DE) intake using the formula's presented in table 3.

Nitrogen and energy mass balance measurements. Fish samples for initial and final body composition were analysed for dry DM, ash, CP, CF and energy, as described by Maas et al. (2018). Frozen fish samples (-20˚) were ground twice using a meat mincer with a 4.5 mm die and homogenised. The energy and nitrogen (N) balance parameters were calculated per tank and expressed as kJ/$kg^{0.8}$/d and mg N/$kg^{0.8}$/d using the formula's presented in table 3.

Gene expression analysis. Total RNA of liver and white muscle of juveniles were extracted with Trizol method following the manufacturer instructions (Invitrogen). Quality test and the reverse transcription of RNA were performed as previously described by Liu and collaborators (Liu et al., 2017). Primers used for quantitative real-time PCR were previously published (Liu et al., 2017; Marandel et al., 2015; Marandel et al., 2016; Veron et al., 2018). qPCR assays were carried out as previously performed by Liu and collaborators (Liu et al., 2017) and Veron and collaborators (Veron et al., 2018). ***ef1α*** gene was chosen as reference gene for normalisation to investigate the relative mRNA level of target gene by the E-method on Light Cycler software as previously described (Marandel et al., 2012).

Table 3. Calculations.

Fish performance parameters	Symbol	Unit	Equation
Average initial body weight	W_i	g/fish	= B_i / N_i
Average final body weight	W_f	g/fish	= B_f / N_f
Survival	S	%	= N_f/ N_i *100
Geometric mean body weight	W_g	g	= $e^{((\ln Wf + \ln Wi)/2)}$
Mean metabolic body weight	MBW	$kg^{0.8}$	= $(W_g/1000)^{0.8}$
Absolute growth	GR	g/d	= (W_f - W_i)/t
Growth expressed per metabolic body weight	GR_{MBW}	g/$kg^{0.8}$/d	= (W_f - W_i)/MBW/t
Specific Growth Rate	SGR	%/d	= (ln W_f – ln W_i) / t * 100%
Feed conversion ratio	FCR	g DM/g fish	= FI_{ABS} / (W_f - W_i)
Feed intake as fed	FI as fed	g/fish/d	= FI_{Total}/N/t
Absolute feed intake	FI_{ABS}	g DM/fish	= FI_{Total} * DM% /N/t
Feed intake expressed in % body weight of fish	FI_{PCT}	g DM/100g fish/d	= (FI_{ABS}/t)/Wg*100g fish
Feed intake expressed in metabolic body weight	FI_{MBW}	g DM/$kg^{0.8}$/d	= FI_{ABS}/MBW/t
Apparent digestibility			
Apparent digestibility coefficient	ADCx	%	= (1-(AIA_{Diet}/AIA_{Feaces}*X_{Feaces}/X_{Diet}))*100%
Digestible nutrient intake	DNI_X	g or kJ/$kg^{0.8}$/day	= FI_{MBW}*(X_{Diet}/1000)*ADC_X
Nitrogen (N) balance parameters *(Saravanan et al., 2012)*			
Gross N intake	GN	mg N/$kg^{0.8}$/d	= FI_{MBW} * N_{Diet}
Digestible N intake	DN	mg N/$kg^{0.8}$/d	= GN * ADC_N
Faecal N loss	FN	mg N/$kg^{0.8}$/d	= GN - DN
Branchial and urinary N loss	BUN	mg N/$kg^{0.8}$/d	= DN - RN
Retained N	RN	mg N/$kg^{0.8}$/d	=(BW_f * N_f – BW_i * N_i)/ MBW_m*(100/t)
Energy balance parameters *(Saravanan et al., 2012)*			
Gross energy intake	GE	kJ/$kg^{0.8}$/d	= FI_{MBW}* GE_{Diet}
Digestible energy intake	DE	kJ/$kg^{0.8}$/d	= GE* ADC_{GE}
Faecal energy loss	FE	kJ/$kg^{0.8}$/d	= GE - DE
Branchial and urinary energy loss	BUE	kJ/$kg^{0.8}$/d	= (BUN * 24.9)/1000
Metabolizable energy intake	ME	kJ/$kg^{0.8}$/d	= DE - BUE
Retained energy	RE	kJ/$kg^{0.8}$/d	= (BW_f * E_f – BW_i * E_i)/ MBW_m*(100/t)
Heat production	H	kJ/$kg^{0.8}$/d	= ME - RE

Metabolizable energy for maintenance	MEm	kJ/kg$^{0.8}$/d	= ME-RE/0.78 (Tran Duy et al, 2006)
Oxygen parameters *(Kaushik, 1980; Saravanan et al., 2012)*			
Oxygen consumption per fish	O_2	mg/kg$^{0.8}$/min	= ((V_L* ΔC) + (C_t* ΔW))/(t * W_{mean})
Variation in O_2 concentration in outlet between two consecutive measurements	ΔC	mg/l	= (C_i – C_{i-t})
Mean O_2 concentration of inlet minus outlet between two consecutive intervals	C_t	mg/l	= (C_i – C_{i-t}/2)
Average predicted metabolic weight of a fish during the measurement days	W_{mean}	kg$^{0.8}$	=(W_p/1000)$^{0.8}$
Predicted daily body weight of individual fish	W_p	g	= $W_{i\ (1-48)}$+ $DFI_{i(1-48)}$/ FCR_{Tank}
Dietary oxygen demand[1)]	DOD	mg O_2/kJ DE	= O_2 /DE
Oxygen intake per g feed DM[1)]	OI_{DM}	mg O_2/g feed DM	= O_2 / FI_{MBW}

[1)] O_2, in g/kg$^{0.8}$/d; ADC_{GE}, apparent digestibility of gross energy diet; B_i, initial biomass (g/tank); B_f, final biomass (g/tank); d, day; $DFI_{i\ (1-48)}$, daily feed intake per fish per tank (g feed DM/fish); DE, digestible energy intake (kJ/kg$^{0.8}$/d) DM, dry matter; FCR_{Tank}, FCR per tank; FI_{Total}, total feed intake per tank (g feed DM); i, is the ith day of the experiment; N_f, final number of fish (number/tank); N_i, initial number of fish (number/tank); GE_{Diet}, energy content diet (kJ/gfeed DM); t, number of experimental days; V_L= volume of water in the tank (l); ΔW, water flow per unit time (l/min); X= dry matter, ash, protein, fat, total carbohydrates, or energy (in g/kg feed DM or kJ/kg feed DM).

2.2.1. Calculations and statistics

The performed calculations per tank are summarized in table 3. For statistical analyses of growth, feed intake, feed efficiency, digestibility and oxygen use, tank was taken as the experimental unit. All growth performance, digestibility, body composition and N & energy balance were analysed for the effect of oxygen history in early life (hypoxia vs. normoxia) (ANOVA using GLM, SPSS v25.0). Feed intake and oxygen consumption were analysed for the effect of early life history and the effect of varying oxygen levels overtime, and their interaction, using repeated measures. Hereby, the available oxygen level is the repeated measurement (within subject variable) and the early life history effect (hypoxia vs. normoxia) the between subject variable (Repeated measures ANOVA using a GLM, SPSS v25.0).
Regarding the analysis of gene expression, normality of distributions was assessed by Shapiro-Wilk test. Data were analyzed by Two-way *ANOVA* to assess the differences between early life (hypoxia vs normoxia), oxygen levels and interactions. If interactions between oxygen levels and early life were statistically significant, *post-hoc* Tukey test would be used to compare all the groups. Data were analysed with R software (v.3.3.3)/R Commander Package. Treatment effects and interactions were considered statistically significant at p<0.05. Results were presented as means ± SD (n=6 samples per treatment based on non-significant differences between tanks per group).

2.3. Results

2.3.1. Growth performance

Survival of fish during the experiment was above 97% and did not significantly differ between the life history treatment (normoxia vs. hypoxia). At the end of the experiment the average individual fish weight of the hypoxia treatment was higher (P<0.05) when compared with the normoxia treatment: 157.2±5.4 (SD) g for the hypoxia treatment compared to 147.2±6.3 g for the normoxia treatment. The absolute growth, growth expressed per metabolic body weight (GR_{MBW}) and specific growth rate (SGR) were all significantly higher for the hypoxia treatment. A trend (P<0.10) towards a lower feed conversion ratio (FCR) was observed for the hypoxia treatment. FCR in the normoxia treatment was 1.04±0.42, compared to an FCR of 0.99±0.32 in the hypoxia treatment (Table 5).

Table 4. Growth performance of Rainbow trout fed to apparent satiation with two different early life oxygen histories: normoxia versus hypoxia (experimental period 63 days; values are presented as means and standard error of the mean (SEM)).

History	Normoxia	Hypoxia	SEM	P-value
Growth period (d)	63	63	-	-
No. tanks (n)	6	6	-	-
No. fish/tank (n)	30	30	-	-
Initial body weight (g)	38.4	38.8	0.07	0.006
Final body weight (g)	147.2	157.2	2.1	0.015
Survival (%)	98.7	96.7	0.86	0.293
Growth				
Absolute (g/d)	1.73	1.88	0.033	0.012
GR_{MBW} (g/kg$^{0.8}$/d)	13.66	14.45	0.175	0.026
SGR (%d)	2.13	2.22	0.021	0.030
FCR	1.04	0.99	0.012	0.052

GR_{MBW}, growth expressed per metabolic body weight; SGR, Specific Growth Rate; FCR, Feed Conversion Ratio.

2.3.2. Feed intake

Numerically the feed intake of the hypoxia treatment was observed to be higher (1.87±0.082 vs. 1.80±0.077) over all oxygen saturation levels (Table 5). There was no early life oxygen history effect on absolute feed intake (FI_{ABS}), feed intake as percentage of body weight (FI_{PCT}), and feed intake per metabolic body weight (FI_{MBW}). When comparing the FI parameters between the different available oxygen levels, a significant effect of oxygen on the FI was observed for all parameters (FI_{ABS}, FI_{PCT} and FI_{MBW}). Correcting for the different weights of the fish over the experimental period, FI_{MBW} and FI_{PCT} appear to be higher at high saturation levels and decrease at lower levels. There were no interaction effects between the early life history treatment and the oxygen levels in later life (Table 5).

2.3.3. Oxygen consumption (mg/kg$^{0.8}$/min).

There was no effect of early life oxygen history on oxygen consumption (mg/kg$^{0.8}$/min) during the last 11 days of the experiment (Table 5). The available oxygen level did influence the oxygen consumption ($P<0.001$). There were no interaction effects between the life history and the available oxygen levels on oxygen consumption.

2.3.4. Dietary oxygen demand

There was no effect of early life oxygen history on dietary oxygen demand (DOD) in mg O_2/g dry matter (DM) intake and mg O_2/kJ digestible energy (DE) intake ($P>0.05$) (Table 5). There was an effect ($P<0.001$) of available oxygen level, with an increase in mg O_2/g dry matter (DM) intake and mg O_2/kJ digestible energy intake ($P<0.001$). This increase is related to decreasing levels of feed intake.

Table 5. Feed intake, oxygen consumption and dietary oxygen demand of Rainbow trout fed to apparent satiation at five available oxygen levels (repeated measures) and at two different early life oxygen histories: normoxia versus hypoxia (experimental period 63 days; data of the last 11 days were used for the calculation of oxygen consumption; values are presented as mean and pooled standard error of the mean (SEM)).

									P-values		
Feed intake (FI)	**History**	**Total average**	**100%**	**90%**	**80%**	**70%**	**60%**	**SEM**	**History**	**O_2 level**	**int.**
FI as fed (g/fish/d)	Normoxia	1.80	1.72	2.15	2.13	1.98	2.18	0.030	0.68	<0.001	0.475
	Hypoxia	1.87	1.80	2.21	2.15	2.02	2.29				
FI_{ABS} (g DM/fish/d)	Normoxia	1.63	1.56	1.95	1.94	1.79	1.83	0.025	0.68	<0.001	0.475
	Hypoxia	1.70	1.64	2.01	1.95	1.83	1.92				
FI_{PCT} (g DM/100g fish/d)	Normoxia	2.17	2.38	2.15	2.06	1.84	1.84	0.034	0.284	<0.001	0.247
	Hypoxia	2.17	2.42	2.07	1.97	1.77	1.82				
FI_{MBW} (g DM/kg$^{0.8}$/d)	Normoxia	12.94	13.70	13.05	12.65	11.46	11.84	0.163	0.267	<0.001	0.209

	Hypoxia	13.05	14.00	12.89	12.31	11.17	11.58				
O_2 consumption and DOD			**100%**	**90%**	**80%**	**70%**	**60%**				
mg/kg$^{0.8}$/min	Normoxia	3.35	3.33	3.45	3.48	3.41	3.20	0.037	0.606	<0.001	0.335
	Hypoxia	3.23	3.19	3.33	3.33	3.31	3.10				
mg/g DM intake	Normoxia	414	357	381	411	463	465	13.6	0.923	<0.001	0.788
	Hypoxia	416	366	369	428	471	452				
mg/kJ DE intake	Normoxia	20.9	18.03	19.18	20.75	22.87	23.49	0.672	0.818	<0.001	0.639
	Hypoxia	21	18.50	18.65	21.62	23.79	22.83				

FI, Feed Intake; FI_{ABS}, absolute feed intake; DM, Dry Matter; FI_{PCT}, feed intake as expressed in percentage body weight of fish; FI_{MBW}, feed intake expressed per metabolic body weight; DOD, Dietary Oxygen Demand; DE, Digestible Energy.

2.3.5. Body composition

There was an effect of early life oxygen history on the crude protein content (P<0.05), with a higher crude protein content for the fish in the normoxia treatment as early life history. The body dry matter, crude fat, energy, ash, phosphorus and calcium content did not show an effect of early life oxygen history (Table 6).

Table 6. Final body composition of Rainbow trout fed to apparent satiation with two different early life oxygen histories: normoxia versus hypoxia (experimental period, 63 days; values are presented as mean and standard error of the mean (SEM)).

	Initial Normoxia	Initial Hypoxia		Final Normoxia	Final Hypoxia	SEM	P-value
Unit in g/kg							
Dry matter	274	273		267	266	1.1	0.524
Crude protein	147	158		168	165	0.63	0.021
Crude fat	92	91		74	74	1.3	0.900
Energy (kJ/g)	7.4	7.4		6.9	6.9	0.061	0.600
Ash	77	79		80	79	0.20	0.185
Phosphorus	3.9	4.0		3.9	3.9	0.039	0.143
Calcium	4.1	4.2		4.0	4.1	0.074	0.228

2.3.6. Apparent digestibility and digestible nutrient intake

There was no effect of early life oxygen history on the digestibility coefficient of dry matter, ash, protein, fat, total carbohydrates and energy (Table 7). There was no effect of early life oxygen history on the digestible nutrient intake per kg metabolic body weight per day for dry matter, ash, protein, fat, total carbohydrates and energy (P>0.1) (Table 7).

Table 7. Apparent digestibility coefficient (ADC) and digestible nutrient intake (DNI) of Rainbow trout fed to apparent satiation with two different early life oxygen histories: normoxia versus hypoxia (experimental period, 63 days; values are presented as mean and standard error of the mean (SEM)).

	Normoxia	Hypoxia	SEM	P-value
ADC (%)				
DM	85.8	85.8	0.27	0.960
Ash	59.2	58.8	0.27	0.520
Protein	95.9	95.8	0.07	0.323
Fat	90.1	90.0	0.24	0.938
Total carbohydrates	79.1	79.4	0.53	0.831
Energy	88.3	88.1	0.26	0.840
DNI (g/kg$^{0.8}$/day)				
DM	16.0	16.1	0.14	0.660
Ash	0.7	0.7	0.01	0.959
Protein	8.2	8.3	0.09	0.771
Fat	1.0	1.1	0.01	0.723
Total carbohydrates	7.6	7.7	0.06	0.521

Energy	0.4	0.4	0.00	0.729

ADC, Apparent Digestibility Coefficient; DM, Dry Matter; DNI, Digestible Nutrient Intake.

2.3.7. Nitrogen and energy balance

There was no effect of early life oxygen history on the nitrogen (N) and energy (E) balance (both balances (N & E) expressed per kg metabolic body weight per day). Expressing the nitrogen and energy balance on absolute values (mg N/d & kJ/d) results in a trend for significance for nitrogen retention (P=0.071) and energy retention (P=0.068) (Table 8).

Table 8. Nitrogen and energy balance of Rainbow trout fed to apparent satiation with two different early life oxygen histories: normoxia versus hypoxia (experimental period, 63 days; values are presented as mean and standard error of the mean (SEM)).

	Normoxia	Hypoxia	SEM	P-value
Nitrogen balance (mg N/kg$^{0.8}$/d)				
Gross N intake	863	870	9.2	0.722
Faecal N loss	35	37	0.9	0.362
Digestible N intake	828	833	8.5	0.771
Branchial and Urinary N loss	444	436	7.8	0.672
Retained N	384	397	4.4	0.169
Energy balance (kJ/kg$^{0.8}$/d)				
Gross E intake	256	258	2.7	0.722
Faecal E loss	30	31	0.9	0.773
Digestible E intake	226	227	2.0	0.729
Branchial and Urinary E loss	11	11	0.2	0.672
Metabolizable E intake	215	217	1.9	0.676
Retained E	93	97	1.5	0.144
Heat production	122	120	1.4	0.380
Maintenance E	96	92	1.6	0.228

N, Nitrogen; E, energy

2.3.8. Long term effect of the early hypoxia stimulus on metabolic gene expression.

Later in life, fish with different early life history: normoxia and hypoxia were tested with different levels of oxygen (100% ; 80% ; 60%). mRNA levels in the liver and muscle were analysed for metabolic genes as shown in the Table 9 and Table 10 respectively.

Effect of early life hypoxia. No differences linked to the early life hypoxia were detected in liver and muscle (Tables 9 and 10), except for the pk genes in muscle which are (slightly) higher expressed in fish with the early life hypoxia history (Table 10).

Effect of oxygen challenge in later life. The final challenge with different levels of oxygen significantly affected the expression of hypoxia induced markers (elgn3a and b, hf1ab1 and bnip genes) in the liver (Table 9). The expression for these hypoxia marker genes is not so clear in muscle as some are induced (bnip genes) and others are decreased (hifab1 and bnip3l-S53 genes; Table 10). There is down-regulation of the expression for genes coding for proteins involved in mitochondrial metabolism in liver (cs, qcr2, atp5a, cox4 genes; Table 9) and muscle (cs, qcr2, atp5a, cox4 genes; Table 10). In muscle a decrease of amino acid catabolism (gdh1, gdh2 and gdh3 genes in Table 10) was observed but no effect on lipid catabolism. In the liver of fish kept under hypoxia a higher level of expression is observed of the hepatic gluconeogenic g6pcb1 gene (Table 9). Fish kept under hypoxia showed a lower potential for glucose transport (glut1 and glut4 genes) and anaerobic glycolysis (ldh genes) in (Table 10).

Interaction effects. No interactions between the early hypoxia and the levels of oxygen were found for all the metabolic genes in muscle and liver of rainbow trout (Tables 9 and 10) except for the cox2 gene which is either higher expressed either in fish liver with hypoxia history or in fish muscle with normoxia history.

Table 9. The effects of hypoxia test (100%-80%-60%) and hypoxia history (NOR: normoxia; HYP: Hypoxia) on the mRNA levels of lipid metabolism, mitochondrial energy metabolism, amino acid metabolism and glucose metabolism related genes in the liver of rainbow trout. Data are mean ± SD. NE: Not expressed

Classify	Target gene	NOR-100%	NOR-80%	NOR-60%	HYP-100%	HYP-80%	HYP-60%	P-value of Two-way ANOVA		
								Hypoxia history	O_2	Interaction
Lipid metabolism	*Hoad*	1.52±0.72	0.94±0.48	1.00±0.46	0.90±0.65	1.03±0.43	1.05±0.49	0.32	0.45	0.09
	D6d (FAD)	1.43±0.79	0.86±0.38	1.13±0.65	0.91±0.53	0.90±0.49	1.43±0.71	0.38	0.34	0.28
	Cpt1a	NE	NE	NE	NE	NE	NE			
	Cpt1b	NE	NE	NE	NE	NE	NE			
Mitochondrial energy metabolism	*Cs*	1.53±0.73	0.79±0.25	0.75±0.48	1.29±0.69	0.89±0.22	1.04±0.79	0.67	**0.01**	0.37
	Qcr2	1.59±0.84	0.83±0.32	0.75±0.31	1.14±0.53	0.98±0.31	1.00±0.64	0.99	**0.01**	0.10
	Atp5a	1.51±0.76	0.87±0.32	0.89±0.37	1.24±0.56	1.06±0.19	1.01±0.54	0.84	**0.02**	0.28
	Cox4	1.58±0.96	0.89±0.33	0.79±0.33	1.24±0.43	1.00±0.28	0.94±0.45	0.95	**0.006**	0.26
	sdhb	1.32±0.74	1.04±0.38	1.05±0.36	1.10±0.53	1.22±0.30	1.46±1.01	0.44	0.84	0.30
	Cox2	1.35±0.65	0.77±0.28	1.10±0.38	0.83±0.25	1.03±0.31	1.51±0.90	0.75	0.08	**0.02**
Amino acid catabolism	*Gdh1*	1.61±1.68	0.70±0.34	1.06±0.65	0.93±0.43	1.30±0.64	1.34±1.24	0.94	0.53	0.16
	Gdh2	1.31±0.86	0.67±0.26	1.10±0.40	0.84±0.70	0.92±0.48	1.60±1.34	0.71	0.09	0.15
	Gdh3	1.42±0.73	0.92±0.38	1.05±0.42	1.15±0.32	0.93±0.28	1.43±1.03	0.84	0.13	0.26
	Glut2a	1.20±0.84	0.89±0.29	1.04±0.30	0.94±0.24	1.03±0.22	1.37±0.71	0.61	0.35	0.19
	Glutt2b	1.26±1.07	0.89±0.33	1.11±0.35	1.00±0.29	0.99±0.25	1.27±0.64	0.97	0.35	0.52
	Glut1ba	1.32±0.68	0.83±0.43	1.06±0.86	1.28±0.85	0.86±0.65	0.83±0.47	0.66	0.11	0.84
	Glut1aa	NE	NE	NE	NE	NE	NE			
	Glut1ab	NE	NE	NE	NE	NE	NE			
	Glut1bb	NE	NE	NE	NE	NE	NE			
Glycolysis	*Gcka*	1.27±1.31	0.78±0.43	0.97±0.42	0.84±0.46	0.98±0.37	1.06±0.65	0.88	0.81	0.31
	Gckb	0.97±1.14	1.11±0.82	1.19±0.54	0.47±0.25	1.15±0.50	1.60±1.35	0.95	0.07	0.29
	Pfkla	1.16±0.58	0.89±0.36	1.16±0.79	0.91±0.24	1.03±0.35	1.29±0.63	0.93	0.34	0.44
	Pfklb	NE	NE	NE	NE	NE	NE			
	Pkl	NE	NE	NE	NE	NE	NE			
Gluconeogenesis	*Fbp1b1*	1.68±0.83	0.85±0.43	1.09±0.40	0.96±0.75	0.87±0.55	1.24±0.87	0.33	0.13	0.11
	Fbp1b2	0.91±0.39	0.92±0.45	1.14±0.47	0.92±0.53	1.12±0.20	1.56±1.10	0.23	0.07	0.56
	G6pca	1.24±0.86	1.22±0.45	1.34±0.38	0.83±0.46	1.09±0.46	1.72±1.07	0.73	0.07	0.21
	G6pcb1	0.42±0.20	1.36±0.73	1.31±0.63	0.42±0.47	1.20±0.71	1.29±1.09	0.76	**0.0004**	0.93
	Fbp1a	NE	NE	NE	NE	NE	NE			
	G6pcb2	NE	NE	NE	NE	NE	NE			
	Pck1	NE	NE	NE	NE	NE	NE			
	Pck2	0.81±0.81	0.77±0.54	1.17±0.57	1.06±0.77	1.20±0.83	1.64±0.84	0.06	0.12	0.90
Markers of hypoxia	*Hifab2*	1.14±0.68	0.85±0.31	0.82±0.31	0.97±0.30	0.98±0.24	1.25±0.69	0.29	0.67	0.16
	egln3a	0.93±0.60	0.94±0.50	1.60±1.14	0.60±0.18	0.89±0.24	1.92±1.56	0.97	**0.002**	0.53
	egln3b	0.76±0.40	0.84±0.54	2.01±2.36	0.62±0.33	0.69±0.33	1.29±1.05	0.27	**0.02**	0.66
	Hifab1	1.00±0.52	0.94±0.38	1.10±0.31	0.72±0.13	0.97±0.40	1.54±1,01	0.66	**0.03**	0.14
Anaerobic glycolysis	*Pdk1*	1.37±0.58	0.98±0.41	0.96±0.42	0.94±0.23	1.14±0.31	1.26±0.62	0.91	0.82	**0.04**
	Idhaa	NE	NE	NE	NE	NE	NE			
	Idhab	NE	NE	NE	NE	NE	NE			
	Slc16a3a	NE	NE	NE	NE	NE	NE			
	Slc16a3b	NE	NE	NE	NE	NE	NE			
Autophagy	*Bnip3l-s61*	1.14±0.74	0.90±0.45	1.03±0.83	0.77±0.18	0.65±0.47	1.24±0.67	0.44	0.24	0.32
	Bnip3l-s75	0.84±0.50	1.04±0.74	1.20±0.78	0.71±0.15	1.00±0.24	1.29±0.53	0.81	**0.01**	0.79
	Bnip3l-s95	1.28±0.63	0.82±0.21	1.24±0.54	0.94±0.29	0.98±0.20	1.53±0.90	0.79	**0.03**	0.18
	Bnip3l-s5	0.99±0.52	0.94±0.38	1.10±0.31	0.72±0.13	0.97±0.40	1.54±1.01	0.66	**0.03**	0.14
	Bnip3l-s11	1.15±0.57	0.96±0.30	1.07±0.41	0.71±0.22	1.08±0.19	1.57±1.05	0.69	0.09	0.04
	Bnip3l-s11	NE	NE	NE	NE	NE	NE			

Table 10. The effects of hypoxia test (100%-80%-60%) and hypoxia history (NOR: normoxia; HYP: Hyperoxia) on the mRNA levels of lipid metabolism, mitochondrial energy metabolism, amino acid metabolism and glucose metabolism related genes in the muscle of rainbow trout. Data are mean ± SD. NE: Not expressed

Classify	Target gene	NOR-100%	NOR-80%	NOR-60%	HYP-100%	HYP-80%	HYP-60%	P-value of Two-way ANOVA		
								Hypoxia history	**O_2**	**Interaction**
Lipid metabolism	*Hoad*	1.13±0.68	1.03±0.57	1.12±0.66	1.06±0.56	0.67±0.27	0.79±0.60	0.11	0.46	0.72
	Cpt1b	0.84±0.35	1.14±0.60	1.22±0.45	1.12±0.63	0.75±0.20	0.93±0.36	0.26	0.66	0.06
	Cpt1a	NE	NE	NE	NE	NE	NE			
Mitochondrial energy metabolism	*Cs*	1.31±0.53	0.98±0.38	1.06±0.38	1.50±0.47	0.88±0.24	0.90±0.32	0.84	**0.001**	0.40
	Qcr2	1.08±0.48	0.93±0.40	1.06±0.48	1.27±0.42	0.78±0.22	0.80±0.34	0.55	**0.06**	0.23
	Cox4	1.41±0.58	0.89±0.41	0.92±0.36	1.43±0.60	0.70±0.24	0.68±0.26	0.27	**0.00005**	0.65
	Atp5a	1.19±0.47	0.95±0.38	1.05±0.32	1.27±0.40	0.80±0.20	0.86±0.28	0.41	**0.01**	0.47
	sdhb	1.10±0.35	0.96±0.31	1.11±0.29	1.22±0.39	0.91±0.17	0.98±0.32	0.89	0.12	0.50
	Cox2	0.79±0.35	1.07±0.33	1.05±0.20	1.10±0.35	0.84±0.15	1.05±0.26	0.86	0.37	**0.04**
Amino acid catabolism	*Gdh2*	1.38±0.35	1.00±0.37	1.04±0.28	1.41±0.38	0.96±0.27	0.94±0.15	0.76	**0.0002**	0.82
	Gdh3	1.29±0.54	0.83±0.42	0.85±0.27	1.24±0.51	0.82±0.24	0.82±0.17	0.79	**0.001**	0.99
	Gdh1	1.36±0.80	0.76±0.41	0.96±0.45	1.51±0.52	0.87±0.32	0.65±0.29	0.92	**0.0003**	0.32
Glucose transport	*Glut1a*	1.31±0.20	0.69±0.29	0.91±0.34	1.32±0.35	1.05±0.39	0.99±0.42	0.06	**0.001**	0.13
	Glut4a	1.34±0.63	0.88±0.27	1.00±0.48	1.13±0.22	1.02±0.23	0.77±0.32	0.38	**0.02**	0.29
	Glut4b	1.27±0.19	1.02±0.25	0.96±0.40	1.27±0.20	1.14±0.17	0.99±0.20	0.46	**0.003**	0.78
	Glut1b	0.86±0.99	1.17±0.80	0.46±0.45	1.42±1.52	1.83±1.47	0.92±0.61	0.06	0.08	0.96
	Glut1a	NE	NE	NE	NE	NE	NE			
	Glut1b	NE	NE	NE	NE	NE	NE			
Glycolysis	*Pkmab*	1.23±0.45	1.01±0.41	1.09±0.33	1.24±0.29	0.96±0.20	0.91±0.34	0.51	0.08	0.68
	Pkmba	0.97±0.25	0.95±0.24	1.08±0.29	1.10±0.12	1.13±0.21	1.19±0.35	0.04	0.45	0.88
	Pkmbb	1.01±0.30	0.96±0.22	1.12±0.28	1.13±0.13	1.16±0.25	1.23±0.37	0.05	0.39	0.84
	Hk1	1.03±0.35	0.95±0.23	1.11±0.48	1.11±0.20	0.99±0.18	1.08±0.28	0.73	0.46	0.86
	Pkmaa	NE	NE	NE	NE	NE	NE			
Marker of hypoxia	*Hifab1*	1.34±0.51	1.01±0.48	1.02±0.40	1.61±0.57	1.12±0.36	0.95±0.33	0.41	**0.004**	0.54
	Hifab2	NE	NE	NE	NE	NE	NE			
	Egln3a	NE	NE	NE	NE	NE	NE			
	Egln3b	NE	NE	NE	NE	NE	NE			
Anaerobic glycolysis	*Idhaa*	1.14±0.56	0.89±0.34	0.94±0.31	1.48±0.37	0.93±0.45	1.06±0.54	0.15	**0.03**	0.62
	Idhab	0.96±0.25	0.98±0.31	1.12±0.31	1.12±0.16	1.11±0.27	1.28±0.50	0.10	0.24	0.97
	Pdk1	NE	NE	NE	NE	NE	NE			
	Slc16a3a	NE	NE	NE	NE	NE	NE			
	Slc16a3b	NE	NE	NE	NE	NE	NE			
Autophagy	*Bnip3l-s61*	0.84±0.20	1.04±0.18	1.13±0.19	1.02±0.16	1.11±0.27	1.14±0.28	0.27	**0.02**	0.38
	Bnip3l-s53	1.23±0.29	0.92±0.29	1.08±0.40	1.38±0.41	1.01±0.36	0.90±0.14	0.72	**0.006**	0.23
	Bnip3l-s95	1.00±0.15	1.04±0.16	1.03±0.24	1.09±0.17	0.92±0.18	1.08±0.21	0.76	0.57	0.34
	Bnip3l-s75	0.83±0.21	1.10±0.31	1.18±0.22	0.91±0.29	0.96±0.21	1.07±0.34	0.39	**0.02**	0.37
	Bnip3l-s5	NE	NE	NE	NE	NE	NE			
	Bnip3l-s11	NE	NE	NE	NE	NE	NE			

2.4. Discussion

2.4.1. Long term effects of the early hypoxia stimulus on growth performance and oxygen use

Fish exposed to early life hypoxia, showed under varying available oxygen conditions a higher growth in later life, when compared with fish kept at normoxic oxygen conditions. After 63 days, the final average body weight was 157.2±5.4 g for the hypoxia treatment compared to 147.7±6.3 g for the normoxia treatment (initial body weight 38.6±0.26 g). A significant higher absolute growth, GR_{MBW} and SGR and a tendency (P=0.053) toward a significant lower FCR (0.99±0.32 vs 1.04±0.42) for the hypoxia treatment were observed.

It was expected that the FI of the hypoxia treatment would be higher under both early normoxic and hypoxic circumstances and that the iDO would be affected by early life history, therefore, different available oxygen levels were applied. The voluntary feed intake was measured daily for all available oxygen levels tested. Absolute feed intake was numerically higher for the hypoxia treatment for all available oxygen levels, however, there was no effect on feed intake. Due to the higher growth and numerically higher feed intake for the hypoxia treatment, FI_{MBW} and FI_{PCT} were highly comparable with the normoxia treatment. This was unexpected as in the previous AQUAEXCEL2020 study (D6.1, 2019) there was a clear effect of life history on all FI parameters (using the same hypoxia protocol) when feeding under high levels of available oxygen. It should however be noted that the oxygen levels were lowered three times, and that the time exposed to the 90%, 80% and 70% available oxygen level was each time 2 days giving only 6 days on which FI was measured. FI was observed to fluctuate substantially between days. These fluctuations increased the variability in the levels with only 6 days of measurements.

Furthermore, it is possible that the 60% available oxygen level was not low enough to cause a potential difference in hypoxia tolerance between the two early life history treatments. It should be noted there is already a decline in FI_{MBW} from the first decrease in available oxygen from the 100% level to the 90% level. Due to problems with clogging and fouling at low tank inlet flow rates, the two intended lowest levels of available oxygen (50% and 40%) were not reached. Because of the decreasing trend in feed intake, starting from the highest available oxygen level, no iDO level could be detected. Therefore, it cannot be concluded whether the 100% available oxygen level (flow 7 L/min, O_2 in ± 11.3 mg/L) was limiting FI and if there is a difference in iDO between the two life histories. From the results (FI_{MBW}, Table 5) it can be suggested that the 90% available oxygen level and lower available oxygen levels were considered hypoxic conditions.

In line with the previous AQUAEXCEL2020 (D6.1, 2019), early life oxygen history did not affect the ADC (apparent digestibility coefficients). No significant differences were found between the two early life history treatments on the nitrogen and energy balances. A trend towards a higher RE (P=0.068) and RN (P=0.071) was found when expressing the nitrogen and energy balance as absolute values (mg N/d & kJ/d) for the normoxia treatment.

As FI is related to the aerobic capacity of the fish, oxygen consumption was measured throughout the experiment. It was expected that early life hypoxia alters gene expression of rainbow trout in such a way that oxygen uptake capacity increases. However, no effect of early life hypoxia treatment was found on oxygen uptake capacity ($mgO_2/kg^{0.8}/min$) in later life. This contradicts with the findings in the previous AQUAEXCEL2020 (D6.1, 2019) experiment, where a significant higher oxygen consumption was found in the hypoxia treatment. Although no significant difference between early life hypoxia and normoxia treated fish was found in this experiment, the oxygen consumption of the hypoxia treatment was numerically lower for all available oxygen levels.

When comparing oxygen consumption ($mgO_2/kg^{0.8}/min$, Table 5) between available oxygen levels a numerically increase was observed when available oxygen levels decreased from 100% to 90% and from 90% to 80%. This was unexpected and not in line with the numerically in oxygen consumption ($mgO_2/kg^{0.8}/min$) trend observed when oxygen levels decreased further from 80% to 70% and from 70% to 60%. This initial increase can likely be attributed to the fact

that FI was lower during the days the fish were kept at the 100% available oxygen level compared to the days at the 90% available oxygen level. The lower FI at 100% is probably due to daily fluctuation in voluntary FI, which were observed throughout the entire experiment. For the oxygen consumption calculation only the data of the last 11 days of the experiment were used. Consequently, there is only data of two days per available oxygen level.
The DOD (mg/kJ DE intake and mg/g DM intake) was not affected by the early life history. The changes in later life available oxygen level had an effect on the DOD, probably due to the changes in FI.
From the results obtained the clear improvement in growth performance is hard to explain. The numerically higher feed intake and lower oxygen consumption and the tendency for a lower FCR, higher RN (mg N/d) and RE (kJ/d) for the hypoxia treatment, could as a whole have contributed to the observed significant effect of early life history on growth.

2.4.2. Long term effect of the early hypoxia stimulus on metabolic gene expression.

The effect of the final challenge with different levels of oxygen is effective as reflected by the higher expression of hypoxia induced markers (elgn3a and b, hf1ab1 and bnip genes) in liver (Table 9). The type of variation of expression for these hypoxia marker genes is not so clear in muscle as some are induced (bnip genes) and other are decreased linked to the hypoxia (hifab1 and bnip3l-S53 genes; Table 10). Interestingly, but as expected also but here clearly significant, there is down-regulation of the expression for genes coding for proteins involved in mitochondrial metabolism in liver (cs, qcr2, atp5a, cox4 genes; Table 9) and muscle (cs, qcr2, atp5a, cox4 genes ; Table 10). These molecular data clearly show that rainbow trout can adapt to the decrease of oxygen availability by decreasing the capacities of oxidative metabolism. Regarding the potential substrates for oxidative metabolism, there is also (but only in muscle) a decrease of amino acid catabolism (gdh1, gdh2 and gdh3 genes in Table 10) but no effect on lipid catabolism.The glucose metabolism is specific because it can be aerobic or anaerobic. Surprisingly, no regulation by levels of oxygen on the glucose transport and glucose metabolism (aerobic and anaerobic) were observed at a molecular level in liver (Table 9). By contrast in muscle (Table 10), there is lower potential for glucose transport (glut1 and glut4 genes) and anaerobic glycolysis (ldh genes) in fish under hypoxia suggesting a lower adaptation of the fish to the only source of energy which can be available in hypoxia conditions ie the glucose. However, this finding could be also to the lower feed intake (diet with high levels of carbohydrates) in trout under hypoxia. Last point, there is higher level of expression of the hepatic gluconeogenic g6pcb1 gene in fish under hypoxia (Table 9), which can suggest higher capacities of hepatic glucose production in this context. **In conclusion, rainbow trout respond well to the lower levels of oxygen in water by decreasing the potential of oxidative metabolism associated with a significant decrease of amino acid catabolism (at least in muscle), proteins being a major energy source in rainbow trout.**

Regarding the potential long term effect of the early hypoxia status of the fish, no differences linked to the early hypoxia were detected in liver and muscle (Tables 9 and 10), except for the pk genes in muscle which are (slightly) higher expressed in fish with the early hypoxia history. **Globally, the present molecular data shown that programming of energy metabolism linked to the early hypoxia in trout larvae was not effective in this experiment when trout are challenged with different levels of oxygen.**

Finally, no interactions between the early hypoxia and the levels of oxygen were found for all the metabolic genes in muscle and liver of rainbow trout (Tables 9 and 10) except for the cox2 gene which is either higher expressed either in fish liver with hypoxia history or in fish muscle with normoxia history.

In conclusion, the results of this study show there is:

- an effect of early life hypoxia on growth performance;
  an effect of the available oxygen level on FI parameters;
- no effect of early life hypoxia on feed intake and oxygen consumption, nor an interaction with varying levels of available oxygen;
- an response to the lower levels of oxygen in water by decreasing the potential of oxidative metabolism associated with a significant decrease of amino acid catabolism (at least in muscle), proteins being a major energy source in rainbow trout;
- no effect observed in the molecular data showing that programming of energy metabolism linked to the early hypoxia treatment in yolk sac larvae of rainbow trout was not effective in this experiment when trout are challenged with different oxygen levels in later life.

3. General conclusion

The aim of the present study was to assess in rainbow trout exposed in early life to chronic hypoxia the effect of different available oxygen levels in later life on growth performance, feed intake and oxygen consumption. When considering the results obtained, we can conclude that early-life chronic hypoxia affect performances of rainbow trout in later life. In the present study, performances were taken in a broad sense and several biological traits have been investigated, like growth potential, feed intake, feed conversion ratio, and oxygen consumption. When considering these various traits, the following conclusions can be drawn:

- there is an effect of early life hypoxia on growth performance;
- there is an effect of the available oxygen level on feed intake parameters;
- there is no effect of early life hypoxia on feed intake and oxygen consumption, nor an interaction with varying levels of available oxygen;
- there is an response to the lower levels of oxygen in water by decreasing the potential of oxidative metabolism associated with a significant decrease of amino acid catabolism (at least in muscle), proteins being a major energy source in rainbow trout;
- there is no effect observed in the molecular data showing that programming of energy metabolism linked to the early hypoxia treatment in yolk sac larvae of rainbow trout was not effective in this experiment when trout are challenged with different oxygen levels in later life.

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Glossary

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

Definitions

DOD: dietary oxygen demand
FCR: feed conversion ratio
FI: feed intake
SGR: specific growth rate
iDO: incipient dissolved oxygen

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Authors (Partner)	WU: R. Maas, E.H. Eding, J. Schrama INRA NuMeA: S. Panserat INRA LPGP: I. Leguen, P. Prunet			
Responsible Author	**Name**	Ep.Eding	**Email**	Ep.Eding@wur.nl

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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	yes	*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW	yes	*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW	yes	
	The contributors (authors) correspond to those indicated in the DOW	yes	
	The Table of Contents has been validated with the Activity Leader	yes	*Please validate the Table of Content with your Activity Leader before drafting the deliverable*
	I am using the AQUAEXCEL2020 deliverable template (title page, styles etc)	yes	*Available in "Useful Documents" on the collaborative workspace*
The draft is ready			
AFTER	I have written a good summary at the beginning of the Deliverable	yes	*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)	yes	*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	yes	
	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	yes	*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.*