AQUA EXCEL 2020

AQUAculture infrastructures for EXCELlence in European fish research towards 2020 — AQUAEXCEL2020

D6.1: Effect of early life water oxygen concentration on experimental outcomes at later life in rainbow trout

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Executive Summary

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

Rationale: 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 and consequently modify brain-related processes such as behavior or stress response. 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'. In these conditions, oxygen consumption and feed intake (which are mutually connected) can also be regulated by early life exposure to low oxygen level. 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 groups before undertaking experiments.
In order to assess the above objectives, a cohort of rainbow trout larvae were exposed during 17 days (just after hatching) to hypoxia conditions or kept in normoxia conditions. Later fish from both experimental groups were studied in two research infrastructures at INRA/PEIMA (France) and at WU (the Netherlands).
Long-term effects on adaptation and stress functions were studied in PEIMA. Specifically, 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 for corticotrope axis, acute hypoxia for behaviour test, 24h hypoxia challenge for gill functions).
Assessments whether early life hypoxia has long term effects on feed intake, feed efficiency and metabolic parameters related to oxygen use were performed by WU in collaboration with INRA/NuMeA. Parameters related to these functions were studied in fish receiving two contrasted diets, i.e. high-dietary oxygen demand (DOD) or low-DOD diets. It is expected that when fed to satiation, diets contrasting in DOD will result in a similar oxygen consumption, but different feed intake. However, no information is available on possible effects of early-life hypoxia on these parameters.

Main Results:

Adaptation and stress: Early-life chronic hypoxia did not affect later in life (juvenile stage) basal activity of the corticotrope axis (assessed through plasma cortisol levels) nor gill functions (assessed through expression of specific genes related to these functions). However in juvenile fish exposed to challenging conditions, some effects of early hypoxia were observed: This includes i) a minor effect on plasma cortisol response after an acute stress during the recovery phase ii) significant changes in expressions of genes involved in some gill functions (i.e. osmoregulation and acid-base balance) in fish exposed to 24h hypoxia challenge.

Feed intake and oxygen consumption: The assessments of feed intake, feed efficiency and metabolic parameters related to oxygen consumption in juvenile fish exposed or not to early-life hypoxia showed:
- There is an effect of early life hypoxia on oxygen consumption an on maximum feed intake in later life in juvenile rainbow trout;
- The impact of early life hypoxia on feed intake and oxygen consumption is dependent upon dietary oxygen demand (DOD): these parameters increased in fish fed with high-DOD.

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Table of contents

1. Introduction and rationale

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 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 infrastructure.

The aims of the present study were to assess in rainbow trout to which extend early-life challenging factor (i.e. hypoxia) can negatively affect performance in juveniles later in life. These issues have been approached through two complementary questions: 1) Are biological functions involved in adaptation and stress of juveniles response modified by early chronic hypoxia? 2) Does chronic hypoxia applied during early life modify feed intake, feed efficiency and metabolic parameters related to oxygen use in later life?

These studies were developed on two research infrastructures (INRA/PEIMA and WUR) but all fish used were issued from the same cohort exposed during larvae stage to hypoxia in INRA/PEIMA.

2. Exposure to chronic hypoxic stimulus during early life affects ability to cope with changing environment in later life.

2.1. Introduction

Phenotypic plasticity is defined as the individual's capacity to change its phenotype in response to environmental cues in order to increase its fitness in a given environment. Thus, stressful situations will affect individual ability to cope with their environment and this capability may be genetic or acquired during early life stages (Vindas *et al.*, 2017). Mammalian literature suggests that it is possible to prepare individuals to cope with future challenging environment through environmental programming during early life (ex. Champagne *et al.*, 2008). In fish, such issue has been recently studied by Vindas *et al.* (2017) and these authors showed that salmon which experienced unpredictable chronic stress during early life stage display a higher growth rate during the challenging developmental periods of their life cycle and also had lower hypothalamic catecholaminergic serotoninergic response to acute stress.

Long-term effects of early exposure to chronic hypoxia have also been recently studied in fish: Cadiz *et al.* (2017) showed in sea bass that such early exposure modified long-term regulation of haemoglobin gene expression, thus suggesting possible effect on physiological responses to hypoxia. Moreover, in zebrafish, early hypoxia during embryogenesis leads to increased hypoxia tolerance as larvae. Overall, these first studies support the hypothesis of long-term physiological consequences of early-life exposure to hypoxia. The main objective of the present study was to clarify such long-term consequences by analysing physiological and behavioural ability to cope with a challenging situation. More precisely, we studied i) response of the HPI axis to acute stress ii) response of the gill functions to acute hypoxia exposure iii) behavioural response to exposure to hypoxia.

2.2. Materials and method

2.2.1. Fish and experimental set-up in INRA/PEIMA (Sizun, France)

Experimental research performed in this study was in accordance to the guiding principles for the use and care of laboratory animals and in compliance with the French and European regulations on animal welfare. Experiments were conducted within INRA-PEIMA (Sizun, France) facilities that have authorization for animal experimentation (C29-277-02) and were approved by the Local Animal Care and Ethics Committee provided by the French legislation under the official license N°74. The project's agreement number is: APAFIS#7508-2016110211489713

Figure 1 summarizes the experimental protocol. Fertilized eggs obtained from male and female rainbow trout (autumn-spawning strain) were incubated in standard conditions until hatching. Two days after hatching, half of the larvae were exposed to hypoxia during 17 days. Such chronic hypoxia conditions were obtained by adding nitrogen in the water which supplies rearing tanks. Such procedure allowed us to have between 3.4 and 4.4 mg/l O2 levels. These levels were continuously measured during the 17 days period. The other half of the larvae were reared during these 17 days under normoxia conditions (10-11mg/l). At the end of the experimental period (resorption stage), the 2 groups were fed and reared under normoxia conditions. Some fish were sampled at the end of the hypoxia stress and 3 weeks after and first feeding to measure a direct and mid-term effects of chronic stress on hypoxic and glucose metabolism-related genes in alevins. Two months later, part of the fish was transferred to WUR experimental installation (see section 3) whereas the remainders were kept in PEIMA.

From 162 days (~5 months) after hypoxia treatment, control and hypoxia exposed juvenile trout were assessed for long term effect of early hypoxia stress on growth and on ability to cope with acute environmental challenges. This included the following challenges:

(1) Exposure to acute confinement stress 162 and 163 days after hypoxia. In order to assess cortisol response to acute stress (Hypothalamo-Pituitary-Interrenal axis responsiveness), part of the fish was immediately sampled and the other part was exposed to 4 minutes confinement stress. They were netted and transfered to a bucket where they were kept 4 minutes at high density (200kg/m^3). Thereafter, fish were transferred back to the recovery tank and further sampled 1h, 3h and 6h after confinement stress.

(2) Exposure to 24h hypoxia 162 days after hypoxia. A group of juvenile trout was exposed to hypoxia conditions during 24h (8-9mg/l O_2 before challenge and 3-4mg/l O_2 during challenge). This was achieved by increasing fish density from 14-16kg/m^3 to 50-60 kg/m^3 (reduction of the water level in experimental tanks) and by reduction of the water flow in the tanks from 2 to 1 renewal/h. Such procedure led to increases in CO_2 and NH_4 levels which stayed at non-toxic levels (CO_2 < 20 mg/l and NH_4<0.5 mg/l). Such hypoxia challenge was used to assess responses to this acute stressor of various physiological parameters including osmoregulation, immunity, oxidative stress and HPI axis responsiveness. Fish were sampled before and after the 24h hypoxia challenge.

(3) Behavioural measurement in the "hypoxia test" 176 days after hypoxia. The objective of hypoxia test was to assess escape from a hypoxic to a normoxic compartment according to the life history. This hypoxia test was adapted from Ferrari and al. (2015) with some modifications. Briefly, the experiments were carried out with two identical circular tanks (A and B, 70liters) attached to each other via a transparent pipe. Each tank had its own water circulation with its own oxygen measurement. 28-32 fish were placed in the tank A (density 20-25kg/m3). After an acclimation period of 30 min, progressive hypoxia was supplied into the tank A using water bulled with nitrogen. At the end of the experiment (90 min) mean oxygen was 3.2 ± 0.8mg/l (N=12). The second tank B was supplied with water containing 9-10mg/l O_2. Due to a dominance problem, each fish entering in tank B was caught. Escape activity was measured in 6 groups of 'control' fish and 6 groups of 'early hypoxia' fish.

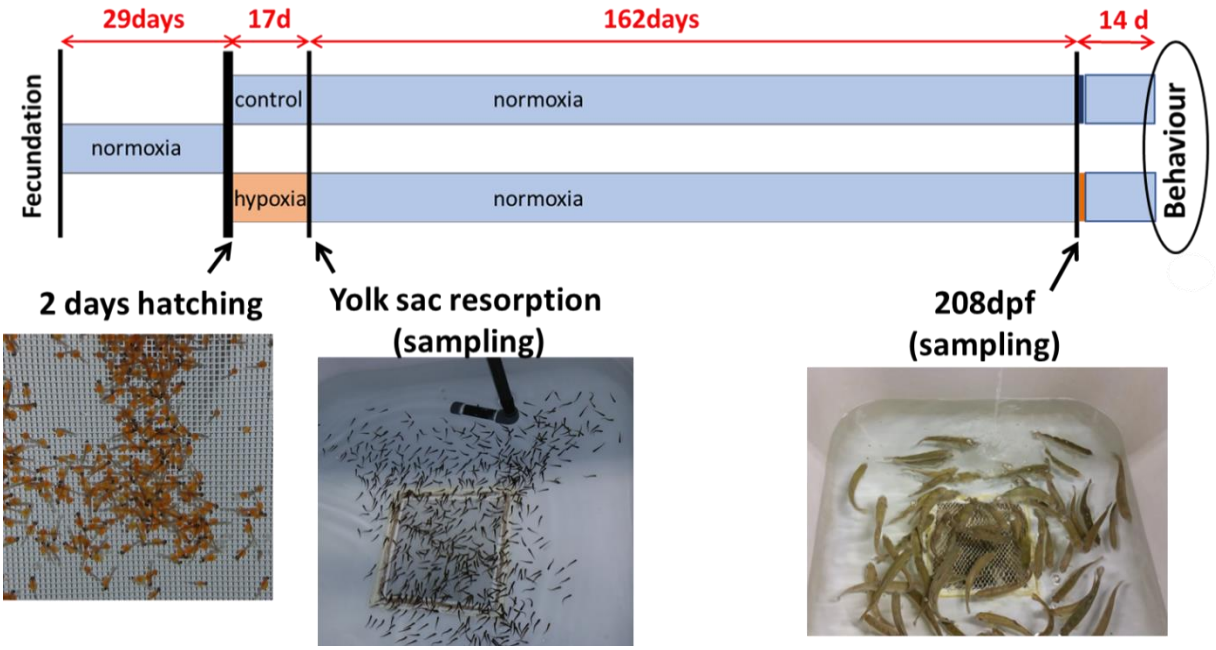

Figure 1 Scheme summarizing the experimental protocol.

2.2.2. Blood analysis and plasma biochemistry

Blood was sampled from the dorsal aorta using lithium-heparinized needles. pH was directly measured in blood with i-STAT Handheld (Abbott, France). Haematocrit was obtained using ammonia-heparinized microcapillary tubes and a Hettich haematocrit-200 centrifuge. Plasma was collected after centrifugation and stored at -80°C.

Ions in plasma

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)

Complement activity in plasma

The plasma complement activity was performed by hemolytic assay with rabbit red blood cells (RRBC, Biomérieux, France). The protocol was adapted from Yano (1992) and Danion *et al.* (2012) with modifications. Buffer used to measure complement was DGHB (Dextrose Gelatin Hepes Buffer) with EGTA and Mg (Moreno-Indias *et al.*, 2012) (4.2mM HEPES, 59mM NaCl, 10mM $MgCl_2$, 2.08% glucose, 0.08% gelatin, 10mM EGTA, pH 7). Briefly, different dilutions of rainbow trout plasma were mixed with 50µl of 2% RRBC suspension (total volume per well = 150µl). Plate was incubated for 60min at 20°C then centrifuged at 1000g for 5min at 4°C. 75µl

of supernatant from each well was transferred with 75µl of PBS into another plate. Absorbance was measured at 414nm with Synergy2 (Biotek, France). Complement activity was expressed as the 50% lysis dilution calculated by linear regression**.**

Lysozyme activity in plasma

Plasma lysozyme activity was determined using turbimetric assay (Douxfils *et al.*, 2012). Briefly, 20µl of rainbow trout plasma was mixed with 150µl 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

Plasma cortisol

Measurement of plasma cortisol. Steroids were measured by LC-MS/MS according to the protocol described in Dufour-Rainfray *et al.* (2015).

2.2.3. Gene expression

Analysis of genes involved in 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.

Analysis of genes involved in intermediary metabolism

Total RNA of whole-body alevins was extracted with Trizol method following the manufacturer instructions (Invitrogen). In alevin samples, 5 pg of luciferase control RNA (Promega) per mg of tissues was added for data normalisation as previously described (Marandel *et al.*, 2012). 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). qPCR assays were carried out as previously performed by Liu and collaborators (Liu *et al.*, 2017). Luciferase and ef1α genes were chosen as reference genes 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).

2.2.4. Statistical analysis

Non parametric tests (Mann-Whitney, Kruskal-Wallis ANOVA and post-hoc multiple comparisons tests) were used with low sample sizes (n=3-6) and when the test of normality of residues or equal variance failed. Else biochemistry and gene expression were analyzed using one or two-way ANOVA.

2.3. Results

2.3.1. Effect of chronic hypoxia exposure on larvae stage

Rainbow trout larvae were exposed or not to hypoxia conditions between hatching and yolk sac resorption. Various parameters were analyzed at the end of the experimental period in both hypoxia and control groups.

Survival and growth

As shown in table 1, we did not observe at the end of the hypoxia exposure period any significant effect of chronic hypoxia exposure on mortality nor on growth performances.

Table 1 Effect of hypoxia stress during 17 days (between hatching and yolk sac resorption) on mortality and growth of rainbow trout fry. Values are mean ± SD, N=3 tanks per condition.

	Control	Hypoxia for 17 days	P-value (Mann-Whitney test)
Survival (%)	94.9 ± 4.4	90.5 ± 1.5	0.257
Weight after stress (mg)	93.3 ± 4.6	91.7 ± 2.2	0.827

Expression of genes related to whole body intermediary metabolism.

At the end of hypoxia exposure, RNAs were extracted from whole body of larvae in order to check that chronic hypoxia stress was effective. In this context, we analyzed the mRNA levels for key target genes susceptible to be sensitive to hypoxia (Table 2). Thus, 3 genes known to be sensitive to hypoxia (egln3b, Bnip3-s95, Bnip3-s75) showed expression which was significantly increased in the chronic stress group. This clearly confirmed that the hypoxia treatment was effective during the 17 days experimental period. However, this treatment did not modify expression of glucose-metabolism related genes (which are well known to be also sensitive to the level of oxygen).

Table 2. Direct effects of chronic hypoxia stimuli applied after hatching on mRNA levels of hypoxic and glucose metabolism-related genes in alevins (at the end of stimulus). Values are presented as mean ± SD. One-way ANOVA was used for statistical analysis.

Target gene	Control	Hypoxia	p-value
Markers of hypoxia			
hifab1	0.77± 0.26	0.82± 0.06	0.692
hifab2	0.75± 0.25	0.76± 0.21	0.94
egln3a	1.24± 0.84	1.08± 0.37	0.687
egln3b	0.32± 0.05	1.34± 0.35	***0.002****
Anaerobic glycolysis			
pdk1	0.80± 0.16	0.84± 0.04	0.613
ldhaa	0.42±0.20	0.64±0.17	0.081
ldhab	0.70±0.23	0.77±0.12	0.54
slc16a3a	1.12± 0.46	1.00± 0.49	0.681
slc16a3b	1.26± 0.66	1.43± 0.27	0.583
Glucose transporters			
glut1aa	1.25± 1.03	1.46± 1.39	0.783
glut1ab	0.78± 0.22	0.90± 0.11	0.26
glut1ba	0.78± 0.22	0.90± 0.11	0.26
glut1bb	0.96± 0.27	0.99± 0.12	0.819
glut2a	0.68± 0.26	0.54± 0.10	0.277
glut2b	0.92± 0.39	0.81± 0.06	0.51
glut4a	0.78± 0.12	0.83± 0.14	0.594
glut4b	0.70± 0.13	0.61± 0.10	0.223
Autophagy (marker of hypoxia)			
Bnip3l-s5	1.17±0.25	1.00±0.19	0.239
Bnip3l-s61	1.33±0.27	1.09±0.12	0.082
Bnip3l-s53	1.15±0.28	0.99±0.16	0.28
Bnip3-s95	1.22±0.14	0.97±0.17	***0.035****

Bnip3l-s11	0.94±0.33	1.08±0.23	0.453
Bnip3-s75	2.00±0.56	1.05±0.46	***0.013****

2.3.2. Long term effects of early exposure to hypoxia stress studied in INRA/PEIMA

Following early chronic exposure to hypoxia (17 days between hatching and resorption), fish from both 'early hypoxia' and 'control' groups were kept in normal water during ~5 months. At the end of this period, juveniles were assessed adaptation capacity to cope with an environmental challenging situation Thus, fish from both 'control' and 'early hypoxia' groups were exposed or not to a 24h hypoxia challenge. In addition, before and at the end of this 24h-challenge, fish were acutely confined during 4 minutes in order to analyze the HPI axis response. Finally, other fish were exposed to a behavioral test. The following biological parameters have been analyzed during his experimental period:

- 24h-hypoxia challenge: blood parameters and also gene expression in the gill were measured just before and at the end of the challenge.
- Acute confinement (4 minutes) stress: HPI axis responsiveness to such treatment was assessed by plasma cortisol measure before and 1h, 3h, 6h after the confinement. In addition, gene expressions in head-kidney were analyzed before and 6h after the acute stress.
- Hypoxia challenge: escape behavioral to hypoxia was measured in both 'control' and 'early hypoxia' groups

Growth performance

Growth performances were followed from resorption until 5-6 months breeding of fish kept in normoxia conditions. As indicated in figure 2, we did not observe differences in body weight between fish from the 'control' group and from the 'early hypoxia' group. Then, at the start of the experimental period where we assessed adaptation of the juveniles, the two groups of fish showed similar weight.

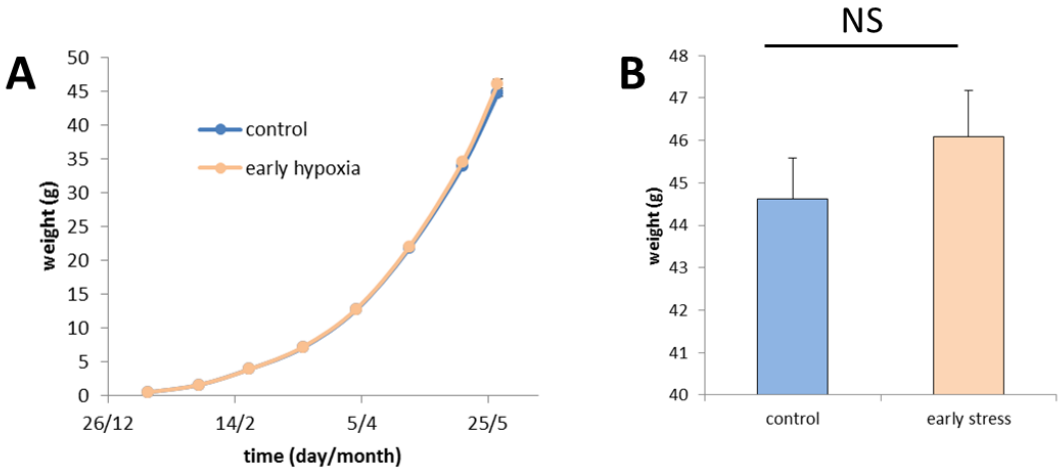

Figure 2: Growth evolution for 6 months N=3 tanks (A) and at the end of the experiment N=144 fish. (B)

Biological responses to a 24h hypoxia challenge

Plasma parameters

After 5-6 months breeding in normoxia conditions, juvenile fish from both 'control' and 'early hypoxia' groups were exposed to hypoxia challenge during 24h. Analysis of plasma parameters as shown in table 3 indicated a significant decrease of lysozyme activity after 24h

challenge. This was also associated with an increase in plasma pH levels whereas no significant effect was observed at the level of the other parameters, i.e. complement, ions and hematocrit levels. Interestingly, 162 days after exposure to early hypoxia, this treatment led in juveniles to a significant decrease in plasma Na+ levels associated with a tendency for a similar effect on plasma Cl- levels. A tendency for a decrease in lysozyme activity was also observed in juveniles exposed to early hypoxia. Finally, we did not observe any interaction between 'early hypoxia' and '24h challenge' suggesting that early hypoxia did not significantly modify the response of our plasma parameters to a 24h challenge.

Table 3. The effects of early hypoxia stress with and without 24 hours challenge of bad water quality on blood parameters.

Blood parameters	Before 24 hours challenge		After 24 hours challenge		P-Value		
	Control	Early hypoxia	Control	Early hypoxia	Early hypoxia	24 hours challenge	interaction
Complement	1062±71	1100±75	1031±55	897±51	0.473	0.075	0.188
Lysozyme	353±21	318±19	307±12	266±14	0.053	**0.009****	0.885
Na^+	141.8±3.2	139±5	141.8±3.2	139.7±2.9	**0.049***	0.741	0.784
Cl^-	118±1.7	116±2	119±1.5	115.1±1.5	0.079	0.86	N/A
Ca^{2+}	2.41±0.05	2.32±0.09	2.42±0.05	2.4±0.04	0.369	0.468	0.639
pH	7.33±0.01	7.3±0.02	7.38±0.01	7.39±0.01	0.517	**0.0003*****	0.175
Hematocrit	51.6±1.7	54.3±1.7	51.5±1.4	51.9±0.6	0.314	0.431	0.424

Complement and lysozyme activities are expressed in U/ml; sodium, chloride and calcium in mM and hematocrit in percentage. Values are presented as mean ± SEM of 8-12 fish. Two-way ANOVA were used for statistical analysis except for chloride. For this parameter, a non-parametric Kruskal-Wallis ANOVA test was used due to a failure to the normality test.

Gill functions

Assessment of the early hypoxia stress and the 24h hypoxia challenge was further analyzed at the level of gills. Various gill functions were studied through the measurement of expression of sets of genes involved in specific functions, including osmoregulation (ion transporter and epithelial permeability), stress and metabolism, cellular function (cell cycle), immunity and hormonal regulation. As shown in table 4, the 24h-hypoxia challenge significantly modified gene expression in several sets of genes: in relation to osmoregulation function, we observed that 8 major genes involved in gill ion transport have their expression modified by the 24h challenge whereas 6 important genes involved in epithelial permeability showed a significantly different expression after the challenge. This 24h-hypoxia had also a significant effect on many of the main genes involved in cell cycle, including caspase, pcna p53, p21, rad51, gadd45. As anticipated, two genes involved in hypoxia stress response also showed significant change (hif1a, egln3) as well as genes involved in metabolism (st1s3, glut1a). Significant effect of the challenge was also observed for genes involved in the immune response (il1b, mx, nod2) and in hormonal regulation (igf1, ghr2, 11bhds2, mr).

When considering the early hypoxia stress as factor, overall no significant effect was observed on any gene expressions measured in the present study. However, for several genes involved in osmoregulation (ion homeostasis and epithelial permeability), cell cycle, stress and metabolism and immunity, significant interactions between early hypoxia stress and 24h challenge were observed: this indicates that for those genes, the early hypoxia exposure has long term significant effects on the acute response to a 24h challenge. Interestingly, we can note that, in most cases, the genes for which such interaction was observed were different from the genes responding to 24h challenge.

In summary, analysis of gene expressions at the level of gill clearly indicated that i) early chronic hypoxia exposure has long term effects on response to an acute hypoxia challenge

and this involves several gill functions ii) a 24h hypoxia challenge has significant effects on all the gill functions analyzed.

Table 4: The effects of early hypoxia stress with and without 24 hours hypoxia challenge on gene expressions in gill. Values are presented as mean ± SD of 5-8 fish. Two-way ANOVA was used for statistical analysis.

		Before 24 hours challenge		After 24 hours challenge		P-value		
		Control	Early hypoxia	Control	Early hypoxia	Early hypoxia	24 hours challenge	Interaction
ions homeostasis	atp1a1a	1.03 ± 0.24	0.95 ± 0.35	0.89 ± 0.22	0.9 ± 0.2	0.755	0.280	0.643
	atp1a1b	1.01 ± 0.16	1.04 ± 0.17	1.05 ± 0.34	0.84 ± 0.08	0.315	0.098	0.181
	atp1a1c	1.01 ± 0.14	1 ± 0.12	1.33 ± 0.21	1.18 ± 0.16	0.279	**0.0002*** **	0.230
	atp1a3	1.19 ± 0.62	1.58 ± 0.52	1.48 ± 0.65	1.07 ± 0.45	0.919	0.574	**0.039***
	at233	1.03 ± 0.28	1.44 ± 0.35	1.46 ± 0.4	1.21 ± 0.23	0.537	0.462	**0.007***
	nbc1	1.03 ± 0.27	1.08 ± 0.33	0.99 ± 0.21	0.79 ± 0.24	0.445	0.090	0.197
	atp6v1b	1.01 ± 0.18	1.09 ± 0.1	1.16 ± 0.22	1.01 ± 0.13	0.563	0.596	0.065
	Ca	1.09 ± 0.47	1.34 ± 0.26	0.87 ± 0.3	0.86 ± 0.24	0.323	**0.0002*** **	0.286
	ca4	1.04 ± 0.29	0.83 ± 0.19	1.88 ± 0.52	1.54 ± 0.69	0.128	**0.0001*** **	0.986
	nhe2	1.08 ± 0.4	1.19 ± 0.24	1.66 ± 0.56	1.25 ± 0.19	0.328	**0.03***	0.059
	nhe3	1.03 ± 0.25	1.16 ± 0.3	1.18 ± 0.18	1.18 ± 0.23	0.473	0.299	0.432
	nkcc1a	1.02 ± 0.23	1.19 ± 0.21	1.37 ± 0.27	1.1 ± 0.29	0.620	0.197	**0.02***
	slc26a6	1.09 ± 0.48	1.03 ± 0.22	1 ± 0.37	0.9 ± 0.35	0.512	0.383	0.869
	cftr1	1.14 ± 0.59	1.58 ± 0.34	2.05 ± 1.04	1.75 ± 0.51	0.476	**0.032***	0.129
	cftr2	1.02 ± 0.2	1.02 ± 0.13	1.79 ± 0.36	1.49 ± 0.34	0.335	**<0.0001*** **	0.158
	slc10a3	1.01 ± 0.18	1.17 ± 0.24	1.15 ± 0.31	0.97 ± 0.26	0.900	0.738	0.077
	atb2b1	1.01 ± 0.13	1.08 ± 0.12	1.24 ± 0.19	1.29 ± 0.23	*0.324*	**0.002** **	0.825
	Ecac	1.03 ± 0.26	1.21 ± 0.22	1 ± 0.25	1.09 ± 0.28	0.138	0.460	0.620
	Rhag	1.02 ± 0.2	1.19 ± 0.18	1.21 ± 0.23	1 ± 0.09	0.810	0.963	**0.005** **
	rhcg1	1.02 ± 0.23	1.14 ± 0.19	1.43 ± 0.29	1.33 ± 0.31	0.916	**0.002** **	0.255
	Ut	1.14 ± 0.54	1.11 ± 0.38	1.5 ± 0.42	1.04 ± 0.55	0.158	0.407	0.207
gill permeability	cldn1	1.03 ± 0.29	1.12 ± 0.16	0.85 ± 0.2	0.78 ± 0.17	0.913	**0.001** **	0.317
	cldn5a	1.02 ± 0.22	1.12 ± 0.14	0.79 ± 0.21	0.85 ± 0.13	0.311	**0.0004*** **	0.787
	cldn7	1.06 ± 0.41	1.28 ± 0.18	1.03 ± 0.33	1.15 ± 0.35	0.137	0.501	0.671
	cldn8c	1.04 ± 0.28	1.4 ± 0.36	1.41 ± 0.45	1.18 ± 0.38	0.641	0.580	**0.032***
	cldn8d	1.02 ± 0.21	1.08 ± 0.18	1.25 ± 0.31	1.05 ± 0.3	0.538	0.279	0.163
	cldn10d	1.02 ± 0.21	1.28 ± 0.22	1.2 ± 0.31	1.17 ± 0.28	0.247	0.705	0.115
	cldn10e	1.03 ± 0.3	1.26 ± 0.4	1.5 ± 0.32	1.24 ± 0.26	0.885	0.069	**0.046***
	cldn12	1.02 ± 0.23	1.26 ± 0.15	1.31 ± 0.33	1.16 ± 0.22	0.656	0.286	**0.033***
	cldn23a	1.02 ± 0.19	1.22 ± 0.18	0.81 ± 0.19	0.83 ± 0.22	0.223	**0.0002*** **	0.203
	cldn27b	1.01 ± 0.14	1.27 ± 0.17	1.29 ± 0.33	1.26 ± 0.34	0.234	0.166	0.129
	cldn28b	1.01 ± 0.15	0.87 ± 0.07	0.54 ± 0.11	0.67 ± 0.18	0.934	**<0.0001*** **	**0.009** **
	cldn30	1.02 ± 0.24	0.97 ± 0.19	0.76 ± 0.2	0.89 ± 0.27	0.632	**0.042***	0.238
	cldn33b	1.16 ± 0.69	1.09 ± 0.46	1.1 ± 0.51	0.64 ± 0.17	0.162	0.156	0.080

	sparc	1.02 ± 0.24	1.22 ± 0.2	1.17 ± 0.24	1 ± 0.18	0.899	0.685	**0.024***
	ocln	1.03 ± 0.26	1.19 ± 0.12	1.35 ± 0.39	1.23 ± 0.25	0.851	0.069	0.162
	zo-1	1.01 ± 0.17	1.2 ± 0.11	1.11 ± 0.26	1.16 ± 0.15	0.071	0.718	0.275
	ag2	1.2 ± 0.79	1.7 ± 1.14	0.95 ± 0.71	1.33 ± 1.32	0.327	0.251	0.893
	muc	1.07 ± 0.46	1.17 ± 0.47	1.29 ± 0.48	1.15 ± 0.43	0.869	0.453	0.443
	vamp8	1.02 ± 0.22	1.15 ± 0.17	1.53 ± 0.33	1.34 ± 0.21	0.784	**0.0003*****	0.078
cell cycle	casp6	1 ± 0.1	1.06 ± 0.12	1.48 ± 0.35	1.33 ± 0.19	0.995	**<0.0001*****	0.270
	casp7	1.05 ± 0.36	1.12 ± 0.14	0.93 ± 0.19	0.92 ± 0.13	0.774	**0.045***	0.622
	casp8	1.01 ± 0.11	1.13 ± 0.13	1.21 ± 0.14	1.23 ± 0.23	0.241	**0.012***	0.319
	casp9	1.02 ± 0.22	1.02 ± 0.13	1.14 ± 0.25	0.99 ± 0.14	0.270	0.468	0.295
	bcl2	1.07 ± 0.39	1.15 ± 0.55	0.94 ± 0.21	0.93 ± 0.19	0.859	0.176	0.737
	vdac2	1.02 ± 0.25	1.28 ± 0.14	1.33 ± 0.31	1.04 ± 0.26	0.877	0.717	**0.004****
	p21	1.04 ± 0.32	1.3 ± 0.38	2.1 ± 1.04	1.62 ± 0.48	0.666	**0.003****	0.109
	p53	1.02 ± 0.19	1.07 ± 0.14	0.94 ± 0.17	0.9 ± 0.15	0.938	**0.037***	0.397
	pcna	1.02 ± 0.2	1.05 ± 0.17	0.59 ± 0.06	0.64 ± 0.14	0.646	**<0.0001*****	0.829
	rad51	1.04 ± 0.33	1.09 ± 0.21	0.78 ± 0.24	0.62 ± 0.22	0.618	**0.0003*****	0.243
	gadd45	1.02 ± 0.23	1.01 ± 0.14	0.93 ± 0.17	0.84 ± 0.13	0.417	**0.039***	0.522
stress and metabolism	gsr	1.03 ± 0.26	1.05 ± 0.18	0.96 ± 0.23	0.86 ± 0.22	0.610	0.112	0.433
	sod1	1.09 ± 0.51	1.08 ± 0.28	0.98 ± 0.27	0.89 ± 0.19	0.977	0.198	0.754
	sod2	1.03 ± 0.29	1.18 ± 0.11	1.11 ± 0.22	1.08 ± 0.22	0.453	0.876	0.248
	gpx1a	1.01 ± 0.12	1.1 ± 0.17	1.15 ± 0.28	1.05 ± 0.23	0.956	0.519	0.196
	gst	1.03 ± 0.27	0.99 ± 0.19	0.93 ± 0.34	0.83 ± 0.19	0.441	0.150	0.695
	cyp1a	1.04 ± 0.3	1.06 ± 0.32	1.23 ± 0.32	1.06 ± 0.43	0.686	0.523	0.504
	st1s3	1.02 ± 0.24	1.18 ± 0.2	0.66 ± 0.25	0.69 ± 0.16	0.410	**<0.0001*****	0.406
	sult1a4	1.02 ± 0.2	0.96 ± 0.22	1.03 ± 0.29	0.99 ± 0.22	0.529	0.821	0.926
	cox4a	1.01 ± 0.18	0.98 ± 0.18	1.02 ± 0.18	0.95 ± 0.16	0.377	0.904	0.790
	cs	1.01 ± 0.18	1.07 ± 0.16	1.16 ± 0.22	0.94 ± 0.18	0.272	0.923	**0.048***
	glut1a	1.03 ± 0.25	1.1 ± 0.33	1.48 ± 0.28	1.15 ± 0.22	0.258	**0.019***	**0.044***
	hif1a	1.02 ± 0.2	1.15 ± 0.19	1.34 ± 0.28	1.25 ± 0.26	0.811	**0.017***	0.184
	hsp47	1.02 ± 0.21	1.2 ± 0.22	0.97 ± 0.17	0.95 ± 0.25	0.278	0.061	0.203
	hsp70	1.09 ± 0.5	0.92 ± 0.18	1.13 ± 0.57	1.23 ± 0.51	0.883	0.292	0.408
	hsp90	1.01 ± 0.17	1.02 ± 0.1	1.01 ± 0.19	1.06 ± 0.21	0.619	0.796	0.730
	egnl3	1.14 ± 0.75	1.46 ± 0.62	7.36 ± 2.47	6.86 ± 3.47	0.776	**<0.0001*****	0.171
Immunity	IgM	1.14 ± 0.68	1.26 ± 0.83	0.96 ± 0.3	0.83 ± 0.3	0.775	0.250	0.410
	IgT	1.11 ± 0.47	1.06 ± 0.63	0.91 ± 0.43	1.04 ± 0.31	0.809	0.501	0.593
	mhc1	1.09 ± 0.41	1.36 ± 0.57	1.54 ± 0.86	1.32 ± 0.41	0.658	0.354	0.254
	mhc2	1.02 ± 0.23	1.09 ± 0.16	1.01 ± 0.39	1.13 ± 0.35	0.349	0.894	0.758
	il1b	1.04 ± 0.32	0.99 ± 0.3	1.52 ± 0.69	1.42 ± 0.41	0.738	**0.004****	0.916
	mx	1.11 ± 0.53	0.93 ± 0.27	0.8 ± 0.28	0.71 ± 0.41	0.349	**0.039***	0.811
	lyz	1.07 ± 0.42	1.03 ± 0.35	1.08 ± 0.46	1.06 ± 0.32	0.801	0.874	0.940
	nod2	1.01 ± 0.15	1 ± 0.24	0.73 ± 0.23	0.69 ± 0.19	0.747	**0.0002*****	0.798
	Inos	1.05 ± 0.55	1.32 ± 0.58	1.56 ± 0.64	0.75 ± 0.45	0.692	0.407	**0.009****
Hormonal	igf1	1.04 ± 0.3	1.14 ± 0.18	1.84 ± 0.33	1.58 ± 0.34	0.598	**<0.0001*****	0.095
	igf2	1.02 ± 0.22	1.1 ± 0.19	1.15 ± 0.22	1.08 ± 0.21	0.939	0.461	0.342
	ghr2	1.02 ± 0.2	1.13 ± 0.13	0.85 ± 0.11	0.84 ± 0.11	0.440	**0.0001*****	0.263
	prlr	1.01 ± 0.14	1.14 ± 0.2	1.2 ± 0.23	1.17 ± 0.22	0.463	0.138	0.270

thra	1.01 ± 0.11	1.14 ± 0.08	1.13 ± 0.2	1.19 ± 0.18	0.078	0.132	0.469
hsd11b2	1.04 ± 0.32	0.92 ± 0.33	0.26 ± 0.19	0.3 ± 0.11	0.894	**<0.0001*** **	0.359
gr2	1.02 ± 0.22	1.1 ± 0.12	1.11 ± 0.19	0.96 ± 0.17	0.607	0.718	0.080
Mr	1.04 ± 0.31	1.13 ± 0.13	0.87 ± 0.06	1 ± 0.09	**0.017***	**0.03***	0.942

Corticotrope axis

Adaptation capacity of the experimental groups was further assessed by analyzing response to acute stress. This trait was characterized by analyzing both plasma cortisol levels and expression of the main gene involved in the cortisol production in the head kidney.

Plasma cortisol levels

In the various experimental situations previously described, plasma cortisol was measured just before (0h) and after (1h, 3h, 6h) the 4 minutes confinement stress. This protocol was applied on fish from the 'control' or 'early hypoxia' groups and also on fish from these two treatments but after the 24h challenge. Thus, plasma cortisol levels were followed in 4 experimental groups (figure 3). As indicated in figure 3 (see letters at bottom of the columns for statistical results), we observed a very clear effect of time with a very significant increase of plasma cortisol levels at 1h and 3h and a return at 6h to levels which are not significantly different from 0h levels.

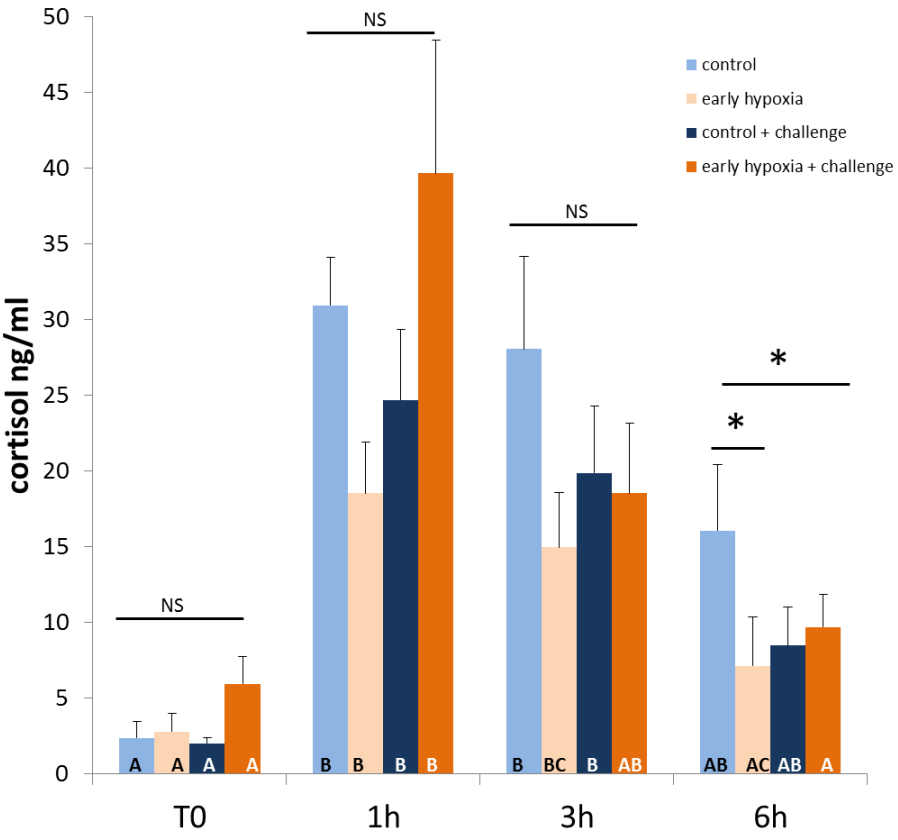

Figure 3: Plasmatic cortisol concentration. Values are presented as mean ± SEM of 9-12 fish. Statistical analysis with Kruskal-Wallis ANOVA test and post-hoc multiple comparisons test, * P<0.05.

At 1h and 3h post-confinement, we did not observe any significant difference between the four groups but at 6h a difference was noted (see stars above the columns). Post-hoc analysis indicated a significant difference between the 'control' and 'early hypoxia' groups not exposed

to 24h-challenge suggesting a different recovery kinetic after acute stress. Overall, there was no significant effect of early hypoxia. However, if we consider only plasma cortisol levels in 'control' and 'early hypoxia' fish not exposed to the 24h-challenge, early hypoxia treatment significantly modified cortisol response. That effect was not observed if we consider cortisol levels in fish after 24h-challenge.

Gene expression in the head kidney

Expression of several genes involved in cortisol synthesis (star, p450, cyp11b2, 11βhsd, 3βhsd) or endocrine regulation of cortisol production (gr1, gr2, mr) was measured just before or 6h after the acute confinement stress in the 4 experimental groups. As indicated in table 5 and 6, the 24h challenge induced a significant increase in expression of p450 and star both before and after acute confinement stress whereas mr expression significantly decreases after the 24h challenge. When considering early hypoxia as stress factor, no significant difference was observed except for p450 which showed a slight but significant increase 6h after acute confinement stress.

Table 5: The effects of early hypoxia stress with and without 24 hours hypoxia challenge on gene expression in the head kidney. Values are presented as mean ± SEM of 9-11 fish. Two-way ANOVA was used for statistical analysis.

without acute stress	Before 24 hours challenge		After 24 hours challenge		P-value		
	Control	Early hypoxia	Control	Early hypoxia	early hypoxia	24 hours challenge	Interaction
11βhsd	1.20 ± 0.2	1.35 ± 0.21	1.12 ± 0.17	1.11 ± 0.11	0.704	0.388	0.687
3βhsd	1.15 ± 0.17	1.20 ± 0.2	1.53 ± 0.27	1.39 ± 0.08	0.922	0.089	0.82
cyp11b2	1.23 ± 0.23	1.06 ± 0.17	1.64 ± 0.28	1.44 ± 0.2	0.442	0.071	0.941
gr1	1.08 ± 0.12	1.15 ± 0.13	0.98 ± 0.09	1.03 ± 0.11	0.487	0.398	0.869
gr2	1.04 ± 0.09	1.17 ± 0.11	1.14 ± 0.12	1.16 ± 0.1	0.496	0.652	0.619
mr	1.04 ± 0.08	1.01 ± 0.12	0.82 ± 0.06	0.8 ± 0.07	0.744	**0.012***	0.982
p450	1.12 ± 0.15	1.17 ± 0.2	1.47 ± 0.25	1.53 ± 0.13	0.642	**0.032***	0.809
star	1.15 ± 0.16	1.28 ± 0.17	1.79 ± 0.3	1.93 ± 0.18	0.573	**0.005****	0.981

Table 6: The effects of early hypoxia stress with and without 24 hours hypoxia challenge and acute stress on gene expression in head kidney. Values are presented as mean ± SEM of 10-12 fish. Two-way ANOVA was used for statistical analysis.

6 hours after acute stress	Before 24 hours challenge		After 24 hours challenge		P-value		
	Control	Early hypoxia	Control	Early hypoxia	early hypoxia	24 hours challenge	Interaction
11βhsd	1.43 ± 0.16	1.13 ± 0.12	1.32 ± 0.15	1.25 ± 0.21	0.231	0.985	0.483
3βhsd	1.49 ± 0.17	1.33 ± 0.12	1.52 ± 0.16	1.87 ± 0.26	0.703	0.165	0.293
cyp11b2	1.42 ± 0.18	1.13 ± 0.14	1.44 ± 0.18	1.82 ± 0.31	0.813	0.107	0.144
gr1	1.09 ± 0.09	1.09 ± 0.07	1 ± 0.08	1.02 ± 0.09	0.934	0.322	0.902
gr2	1.27 ± 0.07	1.15 ± 0.06	1.17 ± 0.08	1.33 ± 0.12	0.784	0.612	0.123
mr	1.11 ± 0.09	1.14 ± 0.08	0.88 ± 0.07	1.02 ± 0.07	0.323	**0.034***	0.468
p450	1.2 ± 0.12	1.42 ± 0.18	1.42 ± 0.15	1.91 ± 0.23	**0.049***	**0.036***	0.449
star	1.84 ± 0.23	1.64 ± 0.19	2.08 ± 0.28	2.45 ± 0.26	0.733	**0.025***	0.277

Behavioral study

Several measures were performed to compare response to "hypoxia test" between 'control' and 'early hypoxia' groups. All these measures presented in the table 7 showed no significant difference between both groups.

Table 7: The effect of early hypoxia stress on fish behavior during "hypoxia test". Values are presented as mean ± SEM, N=6. Mann and Whitney test were used for statistical analysis.

	control	early hypoxia	P-value
Percentage of fish passing from hypoxia (A) to normoxia (B) tanks in 90 min	12.7 ± 2.5	10.3 ± 3.8	0.228
Mean time in min to pass from A to B	37.2 ± 7	41.3 ± 12.2	0.937
Mean O_2 in mg/l at passage from A to B	5 ± 0.5	4.7 ± 0.8	0.937

2.4. Discussion

The main objectives of the present study were to characterize the long term effects of a chronic environmental stress applied on larvae rainbow trout. These issues were developed in the context of improvement of management of experimental fish stocks used in AQUAEXCEL[2020] research infrastructures (RIs). In the present project, assessment of these effects relies on growth performance and on various aspects of physiological and behavioural ability of fish to cope with a challenging situation. By raising such questions, we clearly tackle the issues of modifying phenotypic plasticity in juvenile and adult fish through environmental programming during early life stages. As shown in the results part, we observed several long term effects of such early hypoxia programming. The main question will be to conclude whether these effects results in beneficial or negative consequences for the fish. In the present project, a large experimental protocol has been developed with an early hypoxia stress applied on a large fish population at larvae stage in PEIMA/RI. Then, fish were distributed between WU/RI and PEIMA/RI and at juvenile stage (between 6 months and 15 months of age) analysed for various aspects of their performance. Data obtained in PEIMA and in WU are respectively presented in section 2) and 3).

Chronic (17 days) exposure to hypoxia was performed at larvae stage between hatching and yolk sac resorption. By choosing such early period, we avoided hypoxia effect on first feeding and early growth performance which would certainly lead to indirect long term effects which was not our aim. The chronic hypoxia levels applied on our fish larvae was not too stringent as indicated by similar performances (growth and survival) observed in both hypoxia and control groups after 17 days. However, measurements of expression of metabolism-related genes in larvae after 17 days hypoxia exposure still confirmed the effectivity of this environmental stress which led to increase in egln3 gene expression which has been shown to be a good oxygen-sensitive marker (Liu *et al.*, 2017). Moreover, we also observed induction of expression of two BNIP3 isoforms which mediate mitophagy during hypoxia (Wu *et al.*, 2017). So, overall, these data confirm that the chronic protocol used in the present study led the fish to be exposed to hypoxia conditions.

Stress responses to an acute stressor are an important trait (HPI axis responsiveness) for characterization of coping ability in juvenile fish. The present study allowed us to analyse long term impact of early-life chronic hypoxia on these responses. Our data clearly indicate that 5 months after early-life chronic hypoxia, there was no significant effect of this stressor on basal cortisol levels and on 1h and 3h post-acute stress levels. However, 6h after acute stress, we observed a significant decrease in early-life chronic stress hypoxia suggesting that this early treatment facilitate recovery after acute stress in these juvenile fish. Interestingly, this effect was also associated with a significant effect of this early hypoxia on cytochrome P450 expression in the head-kidney. This enzyme is considered as a rate-limiting step in cortisol steroidogenesis (Payne and Hales, 2004) but it is still difficult to understand how an increase in cytochrome P450 transcript levels may lead to a decrease in plasma cortisol. A similar discrepancy between cortisol response and gene expression is observed in our juvenile trout

exposure again to a 24h hypoxia challenge. This stressor did not modify cortisol response but we observed a significant increase in cytochrome P450 and StAR expression and a decrease in MR expression. Such a discrepancy between HPI axis gene expression (i.e. crf expression in the brain) and cortisol response to acute stress has also been reported by Madaro *et al.* (2015). If we consider now response to acute stress in juvenile trout exposed again to 24h hypoxia, then the early-life chronic hypoxia treatment had no significant effect on cortisol response. Previous studies have been conducted in fish to study long-term effects of early stress on acute cortisol responses: Fish exposed to early stressors such as confinement, air-exposure and unpredictable stress during several days showed variable cortisol responses depending on the fish species and the intensity of the early stress (Auperin and Geslin, 2008; Vindas *et al.*, 2016; Fokos *et al.*, 2017). Thus, our data are in agreement with these previous studies although we did not observe a very strong long-term effect of early-life hypoxia. Whether such mild effect is due to the low intensity of hypoxia and/or to the period of its application would deserve further studies.

Typically, fish respond to hypoxia by a number of morphological, physiological and molecular changes but among all these biological processes, gill functions play an important role in adaptation to hypoxia. This is in line with the multiple adaptation functions located in gill tissue, which include not only respiration and osmoregulation but also acid-base balance, ammonia excretion and Ca absorption, all functions involved in ionic and gas homeostasis. In that context, we logically focus our study on the long term impact of early-life hypoxia on these gill functions which underlie ability of the fish to cope with environmental challenge including hypoxia. Thus, we can summarize our approach by two questions:

Does early-life hypoxia lead to long term effects on gill functions?

Does early-life chronic hypoxia modify later ability of gill functions in response to a new hypoxia challenge?

Measurements of plasma ion levels indicate that early chronic hypoxia has minor negative effects on osmoregulation. Such observation is also confirmed by analysing expression of osmoregulatory genes (i.e. ion transporters, gill permeability, cell cycle) which were not significantly modified by early-life hypoxia. In contrast, exposure of juvenile fish to a new 24h hypoxia challenge led to significant changes in gene expressions. These genes are spread among all the gill functions analysed in the present study, including osmoregulation, immunity, stress metabolism and hormonal regulation of gill functions. These results are in agreement with previous studies of gill transcriptome after hypoxia exposure (Tiedke *et al.*, 2018; van der Meer *et al.*, 2005; Li *et al.*, 2015). Interestingly, statistical analysis of interaction between 'early hypoxia stress' and '24h hypoxia challenge' factors indicate significant effects on several genes: This indicates that responses to '24h hypoxia challenge' were different for fish exposed or not to early-life hypoxia. Such changes were observed on genes involved in the different gill functions (mainly ion transport and gill permeability). Interestingly, these genes are different from those genes significantly modified by 24h hypoxia challenge. Although osmoregulation and acid-base balance seem the main functions modified by early-life hypoxia, it is difficult to conclude from these variable changes (increase or decrease depending on the gene) whether it would result in beneficial or negative effects for fish homeostasis.

3. Effect of water oxygen level during early life stages maximum oxygen uptake and feed intake in rainbow trout.

3.1. 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 conditions (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 is a low 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.
Another factor affecting maximum feed intake regulation under normoxia conditions is dietary composition. Diets differing in dietary oxygen demand (DOD) and fed to satiation result in a different maximum feed intake (Saravanan, 2013). For aerobic metabolism the degree of oxidation is macronutrient depended; fish can for example store dietary fat more efficiently than carbohydrates (Stubbs, 1996). The magnitude of the effect of difference in diet composition on maximum feed intake depends on e.g. the type of dietary energy substrate oxidation and the growth composition of fish. For example, for the onset of fat from fat in the diet, relatively less energy (oxygen) is needed than for the onset of fat from carbohydrates or protein in the diet. Saravanan *et al.* (2012) showed that diets differing in non-protein energy source (starch versus fat) induce a linear decrease in diet induced oxygen demand (DOD) with increasing non protein digestible energy as fat. Since fish have a maximum aerobic capacity, it is expected that when fed to satiation, diets contrasting in DOD will result in a similar oxygen consumption, but different feed intake. This is referred to as the oxystatic concept (Saravanan, 2013). However, no information is available if this effect on maximum feed intake will be similar for low- and high-DOD diets when fed to fish exposed to low oxygen (hypoxia) in early life when compared to fish kept on normoxia conditions in early life.
Early life exposure to low oxygen level is not only expected to affect oxygen uptake capacity it can as well affect the gill physiology and the stress response of fish, e.g. the robustness in relation to stressors as stress demands additional energy for which additional oxygen is required (Sadoul *et al.*, 2015).
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 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 when fed high vs low oxygen demanding diets (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 2 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 of each early life treatment either a high or a low oxygen demanding diet.

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 dietary composition (i.e. dietary oxygen demand (DOD))?

3.2. 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.

3.2.1. 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, 60% oxygen saturation or a high oxygen level, 100% oxygen saturation respectively) for 17 days (see chapter 2.2.1). 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: reference n° 7508 (for more information see above paragraph 2.2). At an age of approximately 3 months rainbow trout were transported from INRA France to the experimental facility of Wageningen University, The Netherlands. Three hundred twenty rainbow trout (*Oncorhynchus mykiss*) were used as the experimental animal for the current study. At the start of the experiment (approximately 13 months after hatching) the fish had a mean weight of 201 ± 8 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 an individual digital water flow meter connected to the tank influent and a faeces collection unit (swirl separator) connected to the tank effluent. Water temperature was 14 ± 1 °C, water flow to each tank was 7 ± 0.5 L min^{-1}. A 12h : 12h (Light : Dark) photoperiod was maintained, with daybreak set at 7:00h.

3.2.2. Experimental design

To assess the effect of early life hypoxia on later life feed intake, growth performance and oxygen consumption a 2 x 2 factorial experiment was designed (Table 1). The first factor was the exposure to two oxygen levels in early life: 60% (Hypoxia) or 100% (Normoxia) oxygen saturation of the water. The second factor was the diet contrasting in oxygen demand: a low oxygen demanding diet (low-DOD) and a high oxygen demanding diet (high-DOD) (Saravanan *et al.*, 2012). To create these two diets, either fat (low-DOD) or starch (high-DOD) was used as non-protein energy source.

At the start of the experiment, fish with a similar oxygen history (normoxia vs hypoxia) were randomly divided over the tanks assigned to the matching oxygen history. Each tank was stocked with 25 fish. Additionally, per oxygen history 10 fish were randomly taken as a start sample for initial body composition. Each treatment was done in triplicate (Table 1). To minimize the influence of tank location, treatments were randomly assigned to a block of four tanks. Oxygen measurements were done per block. The individual tank was the experimental unit. The experiment had a duration of 44 days, during which growth performance, feed intake, oxygen consumption, digestibility and metabolite excretion (TAN, Urea, CO_2 and P) were measured.

Table 1. Experimental design.

Early life history	Normoxia		Hypoxia	
Diet later life	**Low-DOD**	**High-DOD**	**Low-DOD**	**High-DOD**
Replicates	3	3	3	3
Fish per tank	25	25	25	25

3.2.3. Experimental diets and feeding

Two diets, contrasting in DOD (high-DOD vs low-DOD) were formulated by our group and produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) (Table 2). The diets had a similar basal diet, but differed in the ingredients used as non-protein energy source. For the high- and low-DOD diet, starch and fat was used as non-protein energy source respectively. Diets consisted of 2mm extruded sinking pellets. Both diets contained yttrium 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 diets.

Energy source	Low DOD diet	High DOD diet
Ingredient:		
Maize starch (gelatinized)	----	25.00
Rapeseed oil	11.76	----
Wheat	17.05	14.49
Wheat gluten	14.12	12.00
Fish meal (RE>680)	22.53	20.00
Fish oil	1.18	1.00
Soya protein concentrate	14.12	12.00
Pea protein concentrate	14.12	12.00
Lysine HCL	0.12	0.10
DL-methionine	0.47	0.40
Monocalcium phosphate	1.18	1.00
Yttrium oxide	0.01	0.01
Diamol	1.18	1.00
Premix	1.18	1.00
Total	**100.00**	**100.00**
Analysed nutrient content (g/kg feed DM):		
Dry matter (DM, g/kg)	971	942
Crude protein	558	474
Crude fat	178	50

Crude ash	73	63
Total carbohydrates[1)]	191	413
Gross energy (kJ/g)	22.43	20.33

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

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.

3.2.4. Sampling

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

Sampling after 42 days (aerobic and anaerobic nutrient metabolism parameters, INRA NuMeA): After 42 days, three fish per tank were randomly sampled exactly 6 hours after the morning feeding ended. Sampled fish were anaesthetized (1 ml L^{-1} 2-phenoxyethanol), 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 for: 1) haematocrit sampling (2 readings per fish), 2) haemoglobin analyses (200 µl full blood per fish, directly stored at -20 °C) and 3) plasma sampling (remaining blood). For haematocrit determination, samples were centrifuged (10600 rpm, 5 minutes), and subsequently haematocrit reading was done within 15 minutes after centrifugation. 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).
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 immediately frozen in liquid nitrogen and thereafter stored at -80 °C for subsequent analysis.

Sampling after 44 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 in the tanks.

Sampling after 49 days (adaptation function related to behaviour, gill physiology and endocrine responses, INRA LPGP): The day before sampling, animals were not fed. On the sampling day itself, a total of 6 fish per tank were caught: 3 fish per tank were directly sampled for blood (baseline measurements) and tissue samples, while the other 3 fish were first exposed to a stress test before taking blood samples for stress response analysis.
Animals not exposed to the stress test (3 fish per tank) were euthanized by an overdose of anaesthetics (1 ml L^{-1} 2-phenoxyethanol), making sure that they were killed within 1 minute. Thereafter they were individually weighed and blood samples (1 x 1 ml per fish) were taken (baseline). The sampled blood was used for: 1) haematocrit sampling (2 readings per fish) and 2) plasma sampling (remaining blood). For haematocrit, samples were centrifuged (10600 rpm, 5 minutes), and haematocrit reading was done within 15 minutes after centrifugation. Blood for

plasma sampling (2 samples per fish) was centrifuged (3500 rpm, 5 minutes), plasma was sampled and stored in the freezer (-20 °C for 1-2 hours, thereafter they were transported to -80 °C). Centrifugation of the blood was always done within 5 minutes after sampling. After blood sampling, from each fish gills and HPI-axis tissue were sampled. These samples were directly frozen in liquid nitrogen, and thereafter stored at -80 °C.
For the stress test, 3 fish from each tank were pooled within the same treatment, resulting in 9 fish per treatment exposed simultaneously to the stress test. The stress test consisted of a standardized confinement test (4 minutes at 200 kg m^{-3}). After the confinement test, fish were placed back in a tank for recovery. One hour after the stress test, all fish were caught and directly euthanized by an overdose of anaesthetics (1.33 ml L^{-1} 2-phenoxyethanol), making sure that they were killed within 1 minute. Thereafter they were individually weighed and blood samples (1x 1 ml per fish) were taken (stress response). Blood was processed in a similar way as described above for the baseline sampling.

3.2.5. Measurements and analysis

Growth performance. At the start and after 44 days, fish were counted and batch weighed per tank. In addition, fish sampled after 42 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, feces were collected from week 2-6 using the method as described by Amirkolaie *et al.* (2006). Before analyses feces samples were grinded (Retsch ZM 200), and per tank feces collected for the different weeks were pooled. Feces 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 feces 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 from week 2 till week 6 and where performed as described by Saravanan *et al.* (2012). Tanks were measured in blocks (3 blocks of 4 tanks in total) for 2 days per week. 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 have not been analysed yet, due to unforeseen circumstances. These samples will be analysed for dry matter (DM), ash, crude protein (CP), crude fat (CF) and energy.

Gene expression. 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). qPCR assays were carried out as previously performed by Liu and collaborators (Liu *et al.*, 2017). *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).

Blood plasma analysis. Plasma glucose, triglycerides and free fatty acids were analysed with Glucose RTU (BioMerieux), PAP 150 (Biomérieux) and NEFA C (Wako Chemicals GmbH) kits, respectively, according to the recommendations of each manufacturer. Total plasma free amino acid concentrations were determined by the ninhydrin reaction according to the method of Moore with glycine as standard.

Gill physiology- and HPI-axis response measurements. Samples collected at day 49 of the experiment (adaptation function related to behaviour, gill physiology and endocrine responses) were send to INRA LPGP, in France. Due to unforeseen circumstances samples thawed during the transport and could not be analysed.

3.2.6. 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 and oxygen use parameters were analysed for the effect of history, diet and their interaction (two-way ANOVA using PROC GLM of SAS) and was followed by post hoc Tukey test if the interaction effect was significant ($p<0.05$).

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 $kJ/kg^{0.8}/day$	$= FI_{MBW}*(X_{Diet}/1000)*ADC_X$
Energy balance parameters			
Gross energy intake	GE	$kJ/kg^{0.8}/d$	$= FI_{MBW}* GE_{Diet}$
Digestible energy intake	DE	$kJ/kg^{0.8}/d$	$= GE* ADC_{GE}$
Oxygen parameters *(Kaushik, 1980; Saravanan et al., 2012)*			
Oxygen consumption per fish	O_2	$mg/kg^{0.8}/min$	$= ((V_L* \Delta C) + (C_t* \Delta W))/(t * W_{mean})$
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 i^{th} 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).

3.3. Results

3.3.1. Growth performance

History effect. There was a tendency (P<0.10) for an early life oxygen history effect (hypoxia versus normoxia) on growth rate per metabolic weight (GR_{MBW}) and specific growth rate (SGR) showing a tendency for higher growth rates for fish exposed to hypoxia in early life (P<0.10) (Table 4). There was no effect of early life oxygen history on final body weight, survival, absolute growth, and feed conversion ratio (FCR) (table 4).
Diet effect. There was an effect (P<0.0001) of diet (low-DOD versus high-DOD) on FCR, showing lower FCR values for treatments fed the low DOD-diet when compared with the high-DOD diet (Table 4). There was no effect of diet on final body weight, survival, absolute growth, GR_{MBW}, and SGR (Table 4).

Table 4. Growth performance of Rainbow trout fed to apparent satiation with a low-DOD and a high-DOD diet at two different early life oxygen histories: normoxia versus hypoxia (experimental period 44 days; values are presented as mean).

History	Normoxia		Hypoxia			P-values		
Diet	Low-DOD	High-DOD	Low-DOD	High-DOD	SEM	History	Diet	History * Diet
Growth period (d)	44	44	44	44				
No. tanks (n)	3	3	3	3				
No. fish/tank (n)	25	25	25	25				
Initial body weight (g)	196.99	205.89	203.05	197.68		0.8275		
Final body weight (g)	406.06	383.27	419.80	415.18	16.802	0.2113	0.4383	0.6035
Survival (%)	100	97	100	100	1.33	0.3466	0.3466	0.3466
Growth								
Absolute (g/d)	4.75	4.03	4.93	4.94	0.325	0.133	0.3105	0.2893
GR_{MBW} ($g/kg^{0.8}/d$)	13.02	11.11	13.17	13.44	0.663	0.098	0.2504	0.1394
SGR (%/d)	1.64	1.41	1.65	1.69	0.075	0.0908	0.2326	0.1094
FCR	0.92	1.12	0.95	1.11	0.024	0.6855	<0.0001	0.4911

DOD, Dietary Oxygen Demand; SEM, Standard Error of the Mean; GR_{MBW}, growth expressed per metabolic body weight, SGR, Specific Growth Rate, FCR, Feed Conversion Ratio.

3.3.2. Feed intake

History effect. There was an early life oxygen history effect (P<0.05) on feed intake (FI). FI as fed, absolute feed intake (FI_{ABS}), feed intake as percentage of body weight (FI_{PCT}), and feed intake per metabolic body weight (FI_{MBW}) was higher (P<0.05) for the low- and high-DOD diet within the hypoxia history treatment when compared with the normoxia treatment (Table 5).
Diet effect. FI as fed, as percentage of body weight (FI_{PCT}), and per metabolic body weight (FI_{MBW}) was higher (P<0.05) and showed for absolute feed intake (FI_{ABS}) a tendency (P<0.10) for being higher for the high DOD-diet (Table 5).
Interaction effect. There was a tendency (P<0.10) for an early life history * diet effect on FI_{PCT} and FI_{MBW} indicating a tendency for a higher FI in fish fed the high-DOD diet within the hypoxia treatment. There was no difference in FI between fish fed the low- or high-DOD diet within the normoxia treatment and the low-DOD diet within the hypoxia treatment (Table 5).

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

There was an effect (P<0.05) of early life oxygen history on oxygen consumption ($mg/kg^{0.8}/min$). There was no effect of diet (low-DOD versus high-DOD) (P>0.05), but a significant (p<0.01) interaction effect (early life history * diet) on oxygen consumption (Table 5). The post hoc Tukey test showed a higher (P<0.05) oxygen consumption for the high-DOD diet within the hypoxia treatment when compared with the high-DOD diet within the normoxia treatment. No difference was observed in oxygen consumption between the high DOD-diet within the hypoxia treatment and the low-DOD diet within the normoxia and hypoxia treatment. The data show that early life hypoxia treatment affects oxygen consumption ($mg/kg^{0.8}/min$) at apparent satiation feeding level when a high DOD-diet is fed and not when a low DOD-diet is fed.

3.3.4. Dietary oxygen demand

There was no effect of early life oxygen history on dietary oxygen demand 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<0.01) of diet (low-DOD versus high-DOD) on oxygen intake per g DM and per kJ DE intake being higher and lower respectively for the low-DOD diet (Table 5).

Table 5. Feed intake, oxygen consumption and dietary oxygen demand of Rainbow trout fed to apparent satiation with a low-DOD and a high-DOD diet at two different early life oxygen histories: normoxia versus hypoxia (experimental period 44 days; values are presented as mean).

History	Normoxia		Hypoxia			P-values		
Diet	Low-DOD	High-DOD	Low-DOD	High-DOD	SEM	History	Diet	History * Diet
Feed intake (FI)								
FI as fed (g/fish/d)	4.50	4.77	4.80	5.81	0.257	0.0313	0.0368	0.1835
FI_{ABS} (g DM/fish/d)	4.37	4.49	4.66	5.48	0.246	0.0325	0.0910	0.1945
FI_{PCT} (g DM/100g fish/d)	1.54	1.60	1.59	1.91	0.06	0.0152	0.0139	0.0606
FI_{MBW} (g DM/$kg^{0.8}$/d)	11.98	12.40	12.47	14.9	0.487	0.0155	0.0193	0.0727
O_2 consumption and DOD								
mg/$kg^{0.8}$/min	$3.58^{a,b}$	3.43^{a}	$3.55^{a,b}$	3.65^{b}	0.048	0.0452	0.6689	0.0087
mg O_2/g DM intake	416	376	393	351	15.8	0.1334	0.0098	0.9483
mg O_2/kJ DE intake	19.7	22.4	18.7	20.9	0.81	0.1277	0.0032	0.7780

DOD, Dietary Oxygen Demand; SEM, Standard Error of the Mean; FI, feed intake; FI_{ABS}, absolute feed intake; DM, dry matter; FI_{PCT}, feed intake expressed in percentage body weight of fish; FI_{MBW}, feed intake expressed in metabolic body weight; DE, digestible energy.

3.3.5. Apparent digestibility

History effect. There was no effect of early life oxygen history on the digestibility coefficient of dry matter, ash, protein, fat, total carbohydrates and energy (P>0.05) (Table 6).
Diet effect. There was an effect of diet on the digestibility coefficient of dry matter, ash, protein, fat, energy (P<0.001) and total carbohydrates (P<0.01). All digestibility coefficients were higher for the low-DOD diet except for ash and total carbohydrates digestibility which was higher for the high-DOD diet. There was a tendency for a history * diet effect on ash digestibility (P<0.1).

3.3.6. Digestible nutrient intake.

History effect. Early life oxygen history affected digestible ash, protein, fat, total carbohydrates and energy intake (P<0.05) (Table 6). Digestible protein and total carbohydrate intake was higher (P<0.05) for early life hypoxia treated fish when compared with early life normoxia treated fish.

Diet effect. Diet (low-DOD versus high-DOD) affected digestible dry matter- (DM) (P<0.05), ash- (P<0.05), fat (P<0.001), total carbohydrates- (P<0.001) and energy intake (P<0.05) (Table 6). There was a tendency for an effect of diet on protein intake (P<0.10).

Interaction effect. There is a significant interaction (early life oxygen history*diet) effect on ash intake (p=0.05) (Table 6). The post hoc Tukey test showed a higher ash intake for the high-DOD diet in early life hypoxia treated fish when compared with normoxia treated fish. The low- and high-DOD diet treatment in normoxia treated fish had no effect on digestible ash intake and was similar to the low-DOD diet treatment in hypoxia treated fish. There is a significant hypoxia * diet effect on total carbohydrates intake. Total carbohydrates intake was higher in the hypoxia treated fish fed the high-DOD diet when compared with the high-DOD diet treatment in the normoxia treated fish. Total digestible carbohydrate intake was lower but similar for fish fed the low-DOD diet treatment in the normoxia and hypoxia treatment.

Table 6. Apparent digestibility coefficient and digestible nutrient intake of Rainbow trout fed to apparent satiation with a low-DOD and a high-DOD diet at two different early life oxygen histories: normoxia versus hypoxia (experimental period, 44 days; values are presented as mean).

History	Normoxia		Hypoxia			P-values		
Diet	Low-DOD	High-DOD	Low-DOD	High-DOD	SEM	History	Diet	History * Diet
ADC (%)								
DM	85.47	79.47	85.03	79.52	0.465	0.6845	<0.001	0.6190
Ash	52.23	58.86	50.56	59.18	0.444	0.1653	<0.001	0.0549
Protein	94.45	92.51	94.08	92.56	0.209	0.4845	<0.001	0.3224
Fat	95.51	83.49	95.29	83.36	0.845	0.8431	<0.001	0.9584
Total carbohydrates	62.60	67.19	62.24	67.19	0.958	0.8594	0.0011	0.8617
Energy	90.07	82.42	89.78	82.41	0.446	0.7388	<0.001	0.761
DNI (g/kg^0.8/day)								
DM	10.24	9.86	10.6	11.84	0.397	0.3113	0.0182	0.0744
Ash	0.46[a]	0.46[a]	0.46[a]	0.56[b]	0.017	0.0194	0.0214	0.0261
Protein	6.31	5.44	6.54	6.53	0.230	0.0203	0.0905	0.0962
Fat	2.04	0.52	2.11	0.62	0.056	0.1443	<0.001	0.8340
Total carbohydrates	1.43[a]	3.44[b]	1.48[a]	4.13[c]	0.11	0.0102	<0.001	0.0196
Energy (kJ)	252.82	207.84	262.23	249.55	9.023	0.022	0.0127	0.1112

DOD, Dietary Oxygen Demand; SEM, Standard Error of the Mean; ADC, apparent digestibility coefficient; DM, dry matter; DNI, digestible nutrient intake.

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

Later in life, fish were tested with two different diets (High-DOD and Low-DOD) with different history: no hypoxia (NOR) and hypoxia (HYP). mRNA levels for the liver and muscle were analysed for metabolic genes as shown in the Tables 7 and 8. The plasma metabolites were also analysed (Table 9). Although many differences at a molecular level in metabolism were observed linked to the dietary intake (and the difference of the macronutrients composition) in liver, no differences linked to the early hypoxia were detected. In muscle low number of differences linked to the dietary intake was detected (only 1 gene) and two interactions were found. However no difference to the early hypoxia was observed in liver and muscle. Our data suggest that programming linked to the early hypoxia was almost absent in this experiment.

Table 7. The effects of NOR-LOW DOD, HYP-LOW DOD, NOR-HIGH DOD and HYP-HIGH DOD diets on the mRNA levels of lipid metabolism, mitochondrial energy metabolism, amino acid metabolism and glucose metabolism related genes in the liver of rainbow trout. Values are presented as mean ± SD.

Classify	Target gene	NOR-LOW DOD	HYP-LOW DOD	NOR-HIGH DOD	HYP-HIGH DOD	P-value of Two-way ANOVA		
						Hypoxia history	Diets	Interaction
	Luciferase	0.25±0.03	0.25±0.07	0.25±0.04	0.25±0.02	0.764	0.852	0.799
	Hoad	0.77±0.18	0.84±0.37	1.28±0.99	1.24±0.51	0.917	0.027 *	0.772
Lipid metabolism	*Cpt1a*	036±0.44	4.20±9.63	0.20±0.29	0.07±0.04	0.257	0.190	0.226
	Cpt1b	1.14±0.69	1.92±1.62	0.60±0.34	0.68±0.29	0.170	0.006 **	0.263
	D6d(FAD)	0.79±2.00	0.40±0.39	1.16±1.14	2.67±4.81	0.535	0.149	0.294
	Cs	0.71±0.29	0.73±0.37	1.36±0.96	1.42±0.54	0.857	0.002 **	0.929
Mitochondrial	*Qcr2*	0.88±0.28	1.00±0.46	1.16±0.65	1.29±0.51	0.442	0.086	0.978
	Cox2	0.92±0.34	1.01±0.51	1.02±0.28	1.24±0.51	0.269	0.251	0.643
energy metabolism	*Cox4*	0.92±0.24	0.93±0.40	1.25±0.64	1.32±0.48	0.789	0.027 *	0.824
	Atp5a	3.59±1.75	4.46±3.05	5.26±3.56	5.51±2.39	0.551	0.151	0.739
	sdhb	0.93±0.53	1.09±0.58	0.89±0.42	1.30±0.55	0.086	0.611	0.449
Amino acid catabolism	*Gdh1*	0.66±0.38	0.66±0.54	1.04±0.82	1.69±0.95	0.175	0.006 **	0.181
	Gdh2	0.68±0.37	0.68±0.52	1.06±0.78	1.69±0.91	0.175	0.005 **	0.174
	Gdh3	0.92±0.39	0.86±0.46	1.05±0.40	1.34±0.55	0.471	0.053	0.250

	Glut2a	1.00±0.37	1.20±0.75	0.86±0.23	1.06±0.26	0.193	0.359	0.957
	Glut2b	1.00±0.33	1.30±0.85	1.08±0.24	1.21±0.16	0.187	0.967	0.601
Glucose	*Glut1aa*							
Transport	*Glut1ab*	1.90±3.97	18.21±54.52	0.04±0.03	0.13±0.20	0.375	0.282	0.380
	Glut1ba	0.93±0.40	0.76±0.46	1.21±0.67	1.45±0.67	0.864	0.014 *	0.272
	Glut1bb	1.12±0.79	0.75±0.66	0.90±0.49	1.32±1.02	0.918	0.484	0.133
	Gcka	0.58±1.72	0.09±0.12	1.82±1.50	1.85±1.70	0.561	0.001***	0.509
	Gckb	0.79±0.97	0.21±0.28	1.81±1.19	1.60±1.18	0.243	0.001 ***	0.574
Glycolysis	*Pfkla*	0.98±0.40	1.05±0.43	1.05±0.53	1.57±0.97	0.175	0.173	0.282
	Pfklb	1.03±0.39	0.98±0.39	1.03±0.43	1.52±0.80	0.214	0.140	0.139
	Pkl	1.15±0.38	1.14±0.51	1.11±0.43	1.35±0.52	0.479	0.573	0.437
	Pck1	1.31±0.87	1.05±0.75	1.12±1.36	0.78±0.78	0.362	0.480	0.891
	Pck2	0.87±0.49	1.19±0.67	0.99±0.52	1.07±0.58	0.297	0.986	0.531
	Fbp1b1	0.88±0.26	0.94±0.44	1.11±0.44	1.39±0.36	0.186	0.011 *	0.397
	Fbp1b2	1.02±0.58	1.87±2.44	0.78±0.55	0.95±0.40	0.247	0.189	0.438
Gluconeogenesis	*Fbp1a*	1.03±1.76	0.94±1.88	0.61±0.32	0.89±0.67	0.833	0.598	0.680
	G6pca	1.06±0.31	1.36±0.82	0.78±0.14	1.04±0.51	0.112	0.087	0.918
	G6pcb1	0.91±0.53	2.18±2.28	0.58±0.62	0.57±0.29	0.131	0.024 *	0.126
	G6pcb2	0.84±0.81	0.44±0.44	1.37±1.28	1.43±0.68	0.557	0.013 *	0.645

NOR,normoxia; DOD, Dietary Oxygen Demand; HYP, hypoxia.

Table 8. The effects of NOR-LOW DOD, HYP-LOW DOD, NOR-HIGH DOD and HYP-HIGH DOD diets on the mRNA levels of lipid metabolism, mitochondrial energy metabolism, amino acid metabolism, glucose metabolism -related genes in the muscle of rainbow. Values are presented as mean ± SD.

Classify	Target gene	NOR-LOW DOD	HYP-LOW DOD	NOR-HIGH DOD	HYP-HIGH DOD	P-value of Two-way ANOVA		
						Hypoxia history	Diets	Interaction
	Luciferase	0.24±0.04	0.23±0.03	0.25±0.03	0.25±0.03	0.819	0.344	0.588
	Hoad	1.13±0.35	1.70±0.77	1.20±0.73	0.89±0.39	0.528	0.073	0.032 *
Lipid metabolism	*Cpt1a*							
	Cpt1b	1.17±0.98	1.75±1.38	0.55±0.36	0.56±0.28	0.317	0.004 **	0.332
	Cs	1.28±1.01	1.29±0.38	1.35±0.78	1.03±0.20	0.487	0.662	0.475
Mitochondrial	*Qcr2*	1.11±0.58	1.10±0.26	1.22±0.73	0.91±0.20	0.327	0.804	0.373
energy metabolism	*Cox2*	1.05±0.37	1.20±0.28	1.33±0.40	0.96±0.30	0.369	0.838	0.028 *
	Cox4	1.06±0.54	0.97±0.31	1.15±0.70	0.88±0.21	0.260	0.981	0.590
	Atp5a	1.12±0.48	1.13±0.30	1.12±0.62	1.01±0.26	0.740	0.720	0.685
	sdhb	1.48±1.19	1.47±0.60	1.02±0.45	0.96±0.14	0.892	0.050	0.918
Amino acid catabolism	*Gdh1*	1.20±0.68	1.14±0.43	1.20±0.39	1.12±0.27	0.655	0.951	0.946
	Gdh2	1.17±0.80	1.06±0.46	1.19±0.43	1.03±0.30	0.443	0.983	0.901
	Gdh3	1.06±0.47	1.09±0.45	1.24±0.58	1.11±0.34	0.733	0.528	0.602
	Glut1aa							
	Glut1ab	1.66±1.57	1.36±1.27	2.29±1.57	1.87±1.67	0.481	0.271	0.904
Glucose	*Glut1ba*	1.07±0.49	0.90±0.59	1.09±0.53	1.54±0.69	0.483	0.094	0.115
transport	*Glut1bb*	1.15±0.59	1.00±0.38	1.48±0.88	1.20±0.53	0.312	0.227	0.756
	Glut4a	0.89±0.28	0.94±0.46	1.14±0.69	1.01±0.50	0.812	0.342	0.594
	Glut4b	1.00±0.23	1.06±0.37	1.15±0.53	1.11±0.39	0.919	0.453	0.697
	Hk1	1.09±0.47	0.91±0.37	0.93±030	1.12±0.33	0.964	0.816	0.146
	Pkmaa	1.61±1.22	1.12±0.47	1.25±0.41	1.11±0.43	0.204	0.458	0.478
Glycolysis	*Pkmab*	0.94±0.38	1.12±0.45	0.93±0.36	0.98±0.37	0.378	0.584	0.621
	Pkmba	1.15±0.77	1.00±0.33	1.03±0.23	1.00±0.28	0.544	0.681	0.718
	Pkmbb	1.14±0.68	0.99±0.21	1.06±0.23	1.03±0.29	0.489	0.881	0.669

NOR,normoxia; DOD, Dietary Oxygen Demand; HYP, hypoxia.

Table 9 The effects of NOR-LOW DOD, HYP-HIGH DOD, NOR-HIGH DOD and HYP-LOW DOD diets on the contents of glucose (g/L), triglycerides (g/L), free amino acids (µmol/mL) and free fatty acids (mmol/L) in the plasma of rainbow trout. Values are presented as mean ± SD.

Plasma metabolite	NOR-LOW DOD	HYP-LOW DOD	NOR-HIGH DOD	HYP-HIGH DOD	P-value of Two-way ANOVA		
					History	Diets	Interaction
Glucose	0.94±0.14	0.94±0.19	0.85±0.13	1.57±1.44	0.150	0.275	0.151
Triglycerides	8.35±5.53	7.30±4.77	5.60±2.20	6.05±2.46	0.826	0.145	0.579
Free amino acids	2.67±0.75	2.32±0.67	2.67±0.50	2.53±0.67	0.262	0.620	0.622
Free fatty acids	0.41±0.30	0.40±0.27	0.33±0.23	0.34±0.10	0.944	0.383	0.901

NOR,normoxia; DOD, Dietary Oxygen Demand; HYP, hypoxia.

3.4. Discussion

Chronic (17 days) early life exposure of Rainbow trout larvae to hypoxia, resulted in a change in expression of oxygen sensitive genes directly after exposure, confirming that a hypoxia stressor was applied to the animals. However no effect of early life hypoxia was observed on metabolic gene expression and plasma metabolites in later life (approximately 15 months post hypoxia treatment), suggesting an absence of programming linked to early life hypoxia. Nevertheless, our results show that feed intake was affected by early life hypoxia. Fish exposed to early life hypoxia, showed under normoxia conditions a higher maximum feed intake in later life (13-15 months post hatching), when compared with fish kept at normoxic oxygen conditions. The observed effect of diet composition (low-DOD versus high-DOD) on feed intake was expected and confirmed that diets differing in DOD result in a different maximal feed intake as was previously shown by Saravanan (2013).
The results of this study showed also that oxygen consumption (mg/kg$^{0.8}$/min) was affected by early life hypoxia. Interestingly, an interaction effect (history * diet) was found for oxygen consumption showing that fish exposed to hypoxia in early life and fed a high DOD diet (starch) in later life showed a higher oxygen consumption *(*mg/kg$^{0.8}$/min*)* when compared with fish reared continuously under normoxic conditions. The results also showed that oxygen consumption by fish was not affected by diet composition which is in line with the oxystatic theory (Saravanan, 2013).
Although it was hypothesized that the increased oxygen consumption capacity of trout exposed to early hypoxia was the result of adjusted gene functions, analysis of gene expression in later life was not able to detect this. Since feed intake and oxygen consumption were affected by early life hypoxia this might indicate that measurements on gene expression were not sensitive or specific enough. This emphasizes the importance of multi-factors analyses and the search for other more sensitive or specific bio-indicators.
This emphasizes the importance of proper management practices before the actual experiments are undertaken.

In conclusion, the results of this study show:

- there is an effect of early life hypoxia on oxygen consumption in later life;
- there is an effect of early life hypoxia on maximum feed intake in later life;
- impact of early life hypoxia on feed intake and oxygen consumption is dependent upon dietary oxygen demand (DOD).

4. General conclusion

The aim of the present study was to assess in rainbow trout the long-term consequences of an early-life chronic hypoxia. When considering results obtained by partners WU and INRA

and presented in part 2 (INRA) and 3 (WU), we can conclude that early-life chronic hypoxia affect performances of juvenile fish later in life. In the present study, performances were taken in a broad sense and several biological traits have been investigated, including adaptation, stress response, behaviour response, growth potential, feed intake and feed efficiency, oxygen consumption. When considering these various traits, the following conclusions can be drawn:

1) Early-life chronic hypoxia affects oxygen consumption and maximum feed intake in later life measured in normoxia conditions at juvenile stage.

2) Early-life chronic hypoxia affects later in life specific parameters in juvenile fish exposed to challenging conditions: this includes i) cortisol response in the recovery phase after an acute stress ii) gill functions in fish exposed to 24h hypoxia challenge iii) feed intake and oxygen consumption in fish fed a high DOD diet.
Thus, long-term effects of early-life hypoxia can be observed on some traits in fish kept in normal condition or on other traits in fish exposed to a new challenge.

3) Effects of early-life hypoxia on physiological parameters were not necessarily supported by change of expression of related genes (ex. HPI axis responsiveness and main genes of the corticotrope axis; oxygen consumption and metabolic genes in muscle or liver). However, for other functions, such as those located in gills, analysis of expression of a set of specific genes appeared to be more relevant and informative than just measuring plasma parameters. This illustrates the importance to develop a multi-factors approach when assessing biological functions.

4) Despite the above long-term effects of early-life hypoxia in juvenile fish, it is still difficult to conclude whether they will be overall beneficial or detrimental for the juvenile fish. It was impossible to conclude from the effects on gill functions whereas effects on oxygen consumption and feed intake would be beneficial although no clear effects on growth were observed. 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.

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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
HPI axis: hypothalamo-pituitary-interrenal axis
P450: Cytochrome P450
SGR: specific growth rate

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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*
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	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*
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