AQUAEXCEL

Aquaculture Infrastructures for Excellence in European Fish Research

Project number: 262336

Combination of CP & CSA
Seventh Framework Programme
Capacities

Deliverable D7.5

Set of methodologies for phenotyping complex trait (responses to nutritional and environmental challenges) in trout isogenic lines

Due date of deliverable: M48

Actual submission date: M48

Start date of the project: March 1st, 2011 **Duration**: 48 months

Organisation name of lead contractor: INRA

Revision: V1

Project co-funded by the European Commission within the Seventh Framework Programme (2007-2013)	
Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

Table of contents

Glossary

AQUAEXCEL: Aquaculture Infrastructures for Excellence in European Fish Research

Acute stress: Brief or short term exposure to a stressor or set of stressors.

HPI axis: hypothalamus-pituitary-interrenal axis which is activated by stress.

Homeostasis: The control over internal processes following changes in, often, external stimuli.

Stress: Events that activate catecholamine secretion and activation of the HPI axis for the synthesis of cortisol.

Feed conversion rate (FCR) = Total feed intake (g) / Biomass increase (g); where the total feed intake and the biomass increase in a tank are calculated over the experimental period.

Specific growth rate (**SGR**) (%/BW/day) = (ln(Wt) – ln(W_0)) * 100/T; where Wt is the average final body weight per tank (g), W_0 is the average initial body weight per tank (g) and T is the number of days of the growth period.

Daily growth index (DGI) = 100*(FBW^1/3 - IBW^1/3)/duration (days)

DM = dry matter (g)

Feed intake (FI): DM (g) / fish

Weight gain (WG) (g) = is the increase of body weight after during nutritional trial.

Summary

Objectives: To provide a set of methodologies for phenotyping complex traits (responses to nutritional and environmental challenges) in trout isogenic lines

Rationale: The effects of nutritional or chronic environmental stressors were studied with a multidisciplinary approach in isogenic trout lines.

Teams involved: WUR, INRA NuMeA, INRA LPGP, INRA PEIMA

Task involved: T7.1.4

Geographical areas covered: all Europe

In the present studies, we aim to use the new phenotyping methods obtained in sub-task 7.1.2 and sub-task 7.1.3 and apply them for characterisation of complex traits such as responses to nutritional or environmental challenges in already existing trout isogenic lines. The major interest in using such lines is that all fish within a line are genetically uniform, which will give us highly standardized experimental conditions by either removing genetic variation (experiments with one line) or partitioning it only among lines (experiments with two or more lines) giving ideal conditions to identify genotype by environment interaction. The work carried out within this sub-task will be presented as 3 independent experiments which will be used as case-studies for assessing i) validity/quality of these new methods ii) benefit of using isogenic lines for phenotyping complex traits.

The first experiment assessed the impact of feed abundance (restricted versus satiation) during first-feeding in rainbow trout (*Oncorhynchus mykiss*) fry on their later life energy metabolism and feed intake using the isogenic heterozygous trout line R23. Furthermore, it was studied whether the impact of nutritional history was dependent on the carbohydrate content of the diet. During the first 3 weeks of exogenous feeding, half of the swim-up fry was fed to satiation and the other half (“restricted”) was fed every other day to satiation. Thereafter both groups of fish were treated identically. At 1 year of age, 12 groups of 18 rainbow trout (mean start weight ≈ 230 g) were housed in the metabolic unit of Wageningen University, according to a 2 by 2 factorial design: two different nutritional histories (“restricted” vs “satiation”) and two diets (isonitrogenous and isoenergetic on digestible nitrogen and digestible energy), differing in the type on non-protein energy (“Fat” vs “Starch”). During the 4-week experimental period, voluntary feed intake and total energy and nitrogen balances were quantified by measuring growth, nutrient digestibility, initial and final proximate body composition. The results of this study showed that feed restriction during first feeding of swim-up trout fry has long-lasting effects on energy partitioning in later life. However, this is dependent on diet composition at later life. Impacts are predominantly related to differences in energy intake, heat production (i.e., maintenance requirements) and fat retention. Protein retention was unaffected. Nutritional history also seemed to alter the response to changes in feeding strategies. Changing from restricted to satiation feeding induced a short-term increased feed intake in trout of the “satiation” history.

The second experiment investigated the "oxystatic" theory which assumes that fish have a maximal/target oxygen consumption level and that if this maximal/target oxygen consumption

is reached, this will determine the feed intake. According to this theory both dietary factors and environmental factors can determine the feed intake of the fish if these factors influence either: 1) the amount of oxygen consumed by the fish; or 2) the oxygen uptake capacity from the water by fish.
To verify this theory, twelve groups of 30 rainbow trout (*Oncorhynchus mykiss*) were housed in 12 metabolic research units (MRU's), according to a 2 by 2 factorial design: (a) 2 different diets with contrast in their cation anion differences (CAD): 200 versus 700 mEqv/kg (chronic nutritional stressor) and (b) 2 levels of dissolved oxygen (DO): normoxia versus low oxygen (hypoxia, chronic environmental stressor). At the end of the 7 week experiment the impact of dietary CAD, water DO level and their interaction on the following main measurements/traits were quantified: growth performance, nutrient digestibility, nitrogen and energy partitioning (MRU is the experimental unit) and metabolic bio-markers at organ/cellular level, which are indicative for osmoregulation and the oxidative status (specific enzyme activities and gene-expression). Oxidative status was measured in a subsample of fish per group subjected and not subjected to a standardized acute stressor. The heterozygous isogenic trout line (R23) was used for phenotyping nutritional and environmental stress effects.
The results showed that trout fed a low CAD diet have a 65% lower requirement of metabolizable energy for maintenance (MEm) when compared with the high CAD diet. However, this lower requirement of MEm did not result in a higher feed intake and growth when compared with high CAD diet. No interaction effect (diet x water oxygen level) was observed for feed intake and growth while this was expected as fish fed the high CAD diet and kept under low oxygen conditions will be affected by both the low water oxygen concentration and the lower oxygen transport capacity of the blood due to an altered acid base balance (blood pH and stomach pH). Diet and acute stress are the main factors affecting most of the innate immune and oxidative stress parameters measured in rainbow trout, although different DO levels considered here display interactive effects with diet, acute stress or both factors. CAD levels has significant effects on osmoregulation (calcium homeostasis and plasma osmolality) but low oxygen levels do not have any effect on those parameters.

In the 3rd experiment, we aimed to assess differences between two divergent isogenic rainbow trout lines for their responses to chronic environmental stress. In this study, a multi-parameter approach was used and fish were exposed during 4 weeks to a poor water quality (main stressful factor: hypoxia). For behaviour study, fish from both groups (control and chronic stress –poor water quality-) were analysed through an emotional reactivity test. Fish were also studied for the reactivity of HPI axis after an acute confinement stress. Finally, osmoregulation parameters associated with gene expression analysis in the gill were also analysed. Behavioral data obtained in the present study confirm previous studies and showed that chronic environmental stress modifies behavioural responses in an emotional reactivity test in both isogenic lines. When analysing HPI axis responsiveness, we observed a clear difference between the 2 isogenic lines with A22 line being less reactive when compared to R23. Despite such differences, we have not been able to observed significant effects of chronic poor water exposure on basal (resting) cortisol levels and on cortisol response to acute confinement stress. In agreement, we also did not find effect of such chronic stressors on expression of key-genes regulating HPI axis. Finally, we also analyzed gene expressions important for gill physiology. This study confirms an effect of environmental chronic stress on candidate genes such as nitric oxide synthase, hypoxia inducible factor or complement C3, all genes known to be activated by hypoxia or pathogen exposure.

Moreover, this analysis also showed that line A22 seems to be more sensible to external ammonia concentrations than R23. However, fish from R23 line showed that they can modulate their response to chronic stress by reducing some pathway of glycolysis. Finally, R23 line showed more difficulties to maintain their calcium homeostasis and osmotic pressure after chronic stress than A22. Overall, using isogenic lines, this study allowed us to confirm the importance of a multi-parameters approach and the relevance of parameters associated with behaviour and gill functions for assessing environmental chronic stress.
In conclusion, these 3 cases studies illustrate the possibility but also the limits of using new phenotyping methods and isogenic lines in order to phenotype complex traits such as growth performance or welfare and health status. Thus, the use of a large sub-set of biochemical, physiological and endocrine parameters associated with the use of metabolic chamber units allowed us to describe phenotypic effects of nutritional stress on growth performance. Concerning phenotypic characterization of chronic environmental stress, our studies lead us to highlight the gill as an important target tissue for impact of poor water quality and to suggest further studies on that tissue to develop non-lethal biomarkers using gill biopsies. The importance of using multi-parameters approach for such characterization was also confirmed in our studies as behavioural and endocrine analysis do not always give consistent results which may depend of environmental and/or genetic parameters.

Introduction and Rationale

The previous two sub-tasks 7.1.2 and 7.1.3 were aimed to provide new phenotyping methods for phenotyping respectively fish health under chronic stress conditions (7.1.2) or fish performance under nutritional stress. These studies were developed on standard fish lines, including sea bream, salmon and trout. In order to characterize such complex traits, genomic approaches have been used, including focused candidate genes, focused PCR-arrays and microarrays. In the present sub-task, we aim to use these new phenotyping methods obtained in sub-task 7.1.2 and sub-task 7.1.3 and apply them for characterisation of complex traits such as responses to nutritional or environmental challenges in already existing trout isogenic lines. The major interest in using such lines is that all fish within a line are genetically uniform. Moreover, by using lines with very divergent phenotypes and genotypes, we will be in a much better position for validating these new phenotyping methods and see whether they can be used on quite different genetic backgrounds.

In the context of these objectives, the work carried out within this sub-task will be presented as 3 independent experiments:

- Experiment 1: Feed restriction during first feeding of rainbow trout affects energy balancers and energy intake in later life.
- Experiment 2: Impact of dietary cation anion difference (CAD) and water oxygen level on voluntary feed intake and energy partitioning.
- Experiment 3: Effect of chronic exposure on poor water quality on rainbow trout using a multi-parameters approach.

Experiment 1: FEED RESTRICTION DURING FIRST FEEDING OF RAINBOW TROUT AFFECTS ENERGY BALANCES AND ENERGY INTAKE IN LATER LIFE.

Partners: WUR, INRA NuMeA, INRA PEIMA

Introduction

In mammals it is well accepted that nutrition in early life can permanently influence metabolism and physiology in later life (e.g. Lucas, 1998). This phenomenon is less well studied in fish, but information on this topic is increasing. E.g. dietary carbohydrate content or fatty acid pattern can induce long-lasting molecular changes related with digestion or metabolism. Similarly in trout, early life exposure to a diet rich in plant ingredients, improved later life acceptance and utilization of a plant based diet (Geurden et al., 2013). Information on feeding level during first-feeding stages in fish on later life energy partitioning and feed intake is scarce. This study assessed the impact of feed abundance (restricted versus

satiation) during first-feeding in rainbow trout (*Oncorhynchus mykiss*) fry on their later life energy metabolism and feed intake. Furthermore it was studied if the impact of nutritional history was dependent on the carbohydrate content of the diet.
We used the isogenic trout line R23 (INRA, France) as experimental fish because: (1) all fish within this line are genetically uniform giving best experimental conditions for measuring the the sensitivity of later life energy partitioning and feed intake to feed abundance in early life, (2) reproducibility of the experimental outcomes is expected to be high when using the same genetic line and experimental set up in future experiments, (3) this line is already successfully phenotyped for the effect of short term (3 weeks) exposure to a plant ingredient based diet in early life on later life acceptance and utilization (Geurden et al., 2013) allowing comparison of fish responses over studies.

Material and methods

At 1 year of age, 12 groups of 18 rainbow trout were housed in the metabolic unit of Wageningen University, according to a 2 by 2 factorial design: two different nutritional histories ("restricted" vs "satiation") and two diets, differing in the type on non-protein energy ("Fat" vs "Starch"). We used an isogenic heterozygous family of rainbow trout produced at INRA according to Quillet et al. (2007). During the first 3 weeks of exogenous feeding, half of the swim-up fry was fed to satiation and the other half ("restricted") was fed every other day to satiation. Thereafter both groups of fish were treated identical. . At about 4 months of age, both groups of trout differing in nutritional history were transferred to Wageningen by car under equal transport stress conditions. Although fish of both groups might have experienced transport stress differently (not measured but not visually observed) it is unlikely that transport stress suppresses epigenetic effects and that it has influenced the results in this experiment. After arrival, fish of both histories were fed restrictively, the same amount of feed until the experimental start. During the experiment fish were fed to satiation, one of two diets: "Fat" or "Starch". Diets (table 1) were similar in digestible protein(DP) to digestible energy (DE) ratio, but differed in fat (52 versus 186 g/kg DM) and carbohydrate content (197 versus 424 g/kg DM). During the 4-week experimental period, voluntary feed intake and total energy and nitrogen balances were quantified by measuring growth, nutrient digestibility (by using settling tanks) and initial and final proximate body composition, as described by Saravanan et al. (2013). Data were analysed by 2-way ANOVA.

Table 1. Experimental diets

Ingredients (%)	Starch diet (%)	Fat diet (%)
Maize starch (gelatinized)	25	-
Rapeseed oil	-	11.76
Wheat	14.50	17.06
Wheat gluten	12.00	14.12
Fish meal (RE>680)	20.00	23.53
Fish oil	1.00	1.18
Soya potein concentrate	12.00	14.12
Pea protein concentrate	12.00	14.12
Lysine HCL	0.10	0.12

DL-methionine	0.40	0.47
Monocalcium phosphate	1.0	1.18
Yttrium oxide	0.01	0.01
Diamol	1.00	1.18
Premix	1.00	1.18

Analysed proximate composition of the diets		
Dry matter (g/kg diet)	931	958
Crude protein (g/kg DM)	461	542
Crude fat (g/kg DM)	52	186
Crude ash (g/kg DM)	64	75
NFE (g/kg DM)	424	197
GE (kJ/gDM)	20.06	23.28

Results & Discussion

One year after the nutritional challenge, fish of both histories had similar weights (P>0.10). At the experimental start, mean body weight was 230 g and body composition was fairly equal. Only the fat content of the “restricted” trout was slightly higher (92 vs 87 g/kg fresh). Mean feed intake (in g/d) over the whole period was similar at all treatments (5.2 g/d; P>0.10). But the first 4 days after being switched to satiation feeding feed intake of “restricted” trout was lower (4.4 vs. 5.2g/d; P<0.05). Growth was only affected by diet, being higher with the “Fat” diet (7.1 vs. 5.7g/d; P<0.001). Consequently feed conversion ratio (FCR) was significantly lower with the “Fat” diet (P<0.001). However, FCR was also affected by a significant interaction (P<0.05), thus the diet effect depended on the nutritional history. With the “Starch” diet FCR was higher for “restricted” trout (0.93 vs. 0.91) while with the “Fat” diet FCR was higher for “satiation” trout (0.76 vs. 0.72).

The interaction effect between nutritional history and diet on FCR was not due to effects on digestibility of nutrients. For all dietary nutrients (dry matter, ash, protein, fat, nitrogen free extract and energy), digestibility was unaffected by nutritional history and the interaction (nutrional history * diet) effect (P>0.10). Due to the differences in ingredient/nutrients composition between both diets, digestibility differed between diets (P<0.01). With the “Fat” diet digestibility was higher for DM (87.3 vs 85.4%), crude protein (96.4 vs. 94.9%), fat (92.9 vs. 97.8%) and energy (89.3 vs. 92.9%). With the “Starch” diet digestibility was higher for ash (45.9 vs. 41.3%) and carbohydrates (89.2 vs 92.9%).

Body composition did not cause the difference in FCR. Diet affected body fat and energy content; being higher at the “Fat” diet (P<0.001). Final body energy, protein and fat content were similar for both nutritional histories. No interaction effect was present (P>0.10), but numerically with the “Starch” diet “restricted fish were fatter (83 vs. 80 g/kg fresh), while with the “Fat” diet the restricted fish were leaner (116 vs. 120 g/kg fresh).

Gross energy (GE) content of the “Fat” diet was higher (20.1 vs 23.3 kJ/g DM). Consequently, GE, digestible energy (DE), and metabolisable energy (ME) intake was higher at the “Fat” diet (P<0.001). Also nutritional history and the interaction effect were significantly influencing DE and ME intake (P<0.05). With the “Starch” diet, DE and ME intake were equal (217 and 206 kJ/kg$^{0.8}$/d) but at the “Fat” diet, fish of the “satiation” history had a higher DE (243 vs. 268 kJ/kg$^{0.8}$/d) and ME intake (231 vs. 255 kJ/kg$^{0.8}$/d). Also for heat production (HP), the diet effect differed between nutritional history (P<0.05). Despite the lower ME intake with

the “Starch” diet, HP was highest with this diet 109 and 103 kJ/kg$^{0.8}$/d for “restricted” and “satiation” trout and for the “Fat” diet 79 and 89 kJ/kg$^{0.8}$/d resp. The interaction effect on HP counteracted the interaction effect on ME intake, as indicated by the absence of the interaction effect on energy retention (ER=ME-HP)($P>0.10$). ER was higher with the “Fat” diet (100 vs 159 kJ/kg$^{0.8}$/d; $P<0.001$), due to both a higher fat (49 kJ/kg$^{0.8}$/d) and protein retention (10 kJ/kg$^{0.8}$/d). Nutritional history also affected the ER ($P<0.05$). The trout “restricted” at first-feeding had a lower ER (125 vs 134 kJ/kg$^{0.8}$/d), which was predominantly caused by a lower fat retention (60 vs 67 kJ/kg$^{0.8}$/d, $P<0.05$). Protein retention was similar for both nutritional histories. The interaction effect on HP opposite to that on ME intake suggests that energy requirements for maintenance might differ between the nutritional histories depending on the diet fed. This was confirmed by the calculated energy requirements for maintenance, having a tendency for a significant interaction effect ($P=0.07$). Maintenance requirement was lowest for “restricted” trout at the “Starch” diet and lowest for “satiation” trout at the “Fat” diet.

Summarizing, fish differing in early life nutritional history (restricted versus satiation) had one year after the nutritional challenge similar feed intake and bodyweight. Only the fat content of restricted trout was slightly higher at the start of the experiment. At the end of the experiment in which we tested the effect two different nutritional histories (“restricted” vs “satiation”) and two diets differing in the type of non-protein energy (“Fat” vs “Starch”) feed intake was similar for all treatments. An observed interaction effect on feed conversion ratio indicated that diet effect depended on nutritional history: FCR was higher for “restricted” trout fed the “Starch” diet and for “satiation” trout when fed the “Fat” diet. Numerically “restricted fish” fed the “Starch” diet were fatter while when fed the “Fat” diet fish they were leaner. Nutritional history and the interaction effect were significantly influencing digestible energy and metabolisable energy intake, being equal for both nutritional histories fed the “Starch” diet but higher at fish with “satiation” history fed the “Fat” diet. The interaction effect on heat production counteracted the interaction effect on metabolisable energy intake, as indicated by the absence of the interaction effect on energy retention (energy retention = metabolisable energy – heat production). Nutritional history also affected the energy retention as fish fed “restricted” at first-feeding had a lower energy retention, which was predominantly caused by a lower fat retention. Protein retention was similar for both nutritional histories. The interaction effect on heat production opposite to that on metabolisable energy intake suggests that energy requirements for maintenance might differ between the nutritional histories depending on the diet fed. This was confirmed by the calculated energy requirements for maintenance, having a tendency for a significant interaction effect. Maintenance requirement was lowest for “restricted” trout at the “Starch” diet and lowest for “satiation” trout at the “Fat” diet.

Conclusions

In conclusion, this study shows that feed restriction during first feeding of swim-up trout fry has long-lasting effects on energy partitioning in later life. However, this is dependent on diet composition at later life. Impacts are predominantly related to differences in energy intake, heat production (i.e., maintenance requirements) and fat retention. Protein retention was unaffected. Nutritional history also seemed to alter the response to changes in feeding strategies. Changing from restricted to satiation feeding induced a short-term increased feed intake in trout of the “satiation” history.

This research is a first start to quantify the effect of nutritional history (restricted versus satiation) on energy metabolism and feed intake in later life using an isogenic trout line. The phenotypic characterisation of this line (R23) for nutritional history (environmental events) combined with the possibility to produce and use these genetic identical fish for future research offers a potential high reproducibility of the results reported here . This smoothens the path for future deeper research elucidating the mechanisms at early life nutrition responsible for energy partitioning in later life.

References

Geurden, I., Borchert, P., Balasubramanian, M.N., Schrama, J.W., Dupont-Nivet, M., Quillet, E., Kaushik, S.J., Panserat, S., Médale, F., 2014. The positive impact of the early-feeding of a plant-based diet on its future acceptance and utilisation in rainbow trout. PLoS ONE, 8 (12), art. no. e83162

Lucas, A., 1998. Programming by early nutrition: an experimental approach. Journal of Nutrition 128(2): S401-S406

Quillet, E., Dorson, M., Le Guillou, S., Benmansour, A., Boudinot, P., 2007. Wide range of susceptibility to rhabdoviruses in homozygous clones of rainbow trout. Fish and Shellfish Immunology 22: 510–519.

Saravanan, S., Geurden, I., Figueiredo-Silva, A.C., Kaushik, S., Verreth, J., Schrama, J.W., 2013. Voluntary feed intake in rainbow trout is regulated by diet-induced differences in oxygen use. Journal of Nutrition 143:781-787.

Experiment 2: IMPACT OF DIETARY CATION ANION DIFFERENCE (CAD) AND WATER OXYGEN LEVEL ON VOLUNTARY FEED INTAKE AND ENERGY PARTITIONING.

Partners: WUR, INRA LPGP, INRA PEIMA

Introduction

This study centers on the hypothesis that fish oxygen consumption is involved in determining the feed intake of fish. This "oxystatic" theory assumes that fish have a maximal/target oxygen consumption level and that if this maximal/target oxygen consumption is reached, this will determine the feed intake. According to this theory both dietary factors and environmental factors can determine the feed intake of the fish if these factors influence either: 1) the amount of oxygen consumed by the fish; 2) the oxygen uptake capacity by fish. Dietary cation anion difference (CAD) has been shown (Saravanan et al., 2013) to alter maintenance requirements. Since energy requirements for maintenance are fully spent on ATP production for sustaining vital processes, alterations in maintenance requirements will also coincide with alterations of oxygen consumption spent on maintenance. In the case of an increased maintenance requirement, in the “oxystatic” theory, it is expected that less feed is consumed for growth in order to prevent an increase in the total oxygen consumption. Thereby it is expected that a higher maintenance would lead to a lower feed intake. However, such information on the impact of dietary CAD on feed intake and oxygen consumptions is lacking. Therefore the part of this experiment related to feed intake regulation tests the following:

(1) whether differences in feed intake between diets with CAD levels (i.e., mineral composition) are related to the oxygen consumption (i.e., confirm the oxystatic theory),

(2) whether the response in feed intake to dietary CAD is similar under both normal and low water oxygen concentrations (water DO levels),

(3) whether metabolic and bio-markers at organ/cellular level (i.e., marker involved in feed intake regulation especially at brain and liver level), which are indicative for the oxidative status, confirm the oxygen consumption data at the whole animal level.

In order to assess the above main objectives the impact of dietary CAD, water DO level and their interaction on the following main measurements/traits are quantified:

(1) daily voluntary feed intake and digestible nutrient intake;
(2) mean oxygen consumption over days/weeks;
(3) within day variation in oxygen consumption;
(4) nitrogen and energy partitioning (i.e., nitrogen and energy balance);
(5) metabolic bio-markers at organ/cellular level, which are indicative for the oxidative status (specific enzyme activities and gene-expression).

The second topic addressed in this research is focusing on tosmoregulation. It addresses the interactive effects between nutrition and environmental conditions (in this study water DO level) on energy/nutrient partitioning andosmoregulation parameters.
In aquaculture, several events common to daily maintenance can act as chronic stressor, hampering fish health and growth. For example water DO levels can reach sub-optimal levels due to sub optimal management, such as too high stocking density, too low water refreshment rate; negatively impacting the immune system, growth, swimming performance osmoregulation and reproduction. A large number of studies have focused on the biological response of fish exposed to acute stress, whereas much less information is available on chronic exposure to stressors.
Long term low oxygen levels are considered as a potential chronic stressor. Dietary CAD has been shown to alter the acid base balance in tilapia (post prandial differences in blood pH and stomach pH - Saravanan et al., 2013) and thus should have also impact on osmoregulatory capacity of the fish. However, information on the impact of CAD on the stress responsiveness/osmoregulation in fish is lacking. It is expected that the effects of dietary CAD are enhanced at low DO levels. Therefore this part of the experiment assesses if there is an interactive effect between dietary CAD level and water DO level on osmoregulation of fish.
In this study, we have investigated the response of an isogenic heterozygous family of rainbow trout (*Oncorhynchus mykiss*) to chronic stressors, (including innate immune response and oxidative stress) parameters. We used the isogenic trout line R23 (INRA, France) as experimental fish because: (1) all fish within this line are genetically uniform avoiding confusion of genetic and environmental effects at individual level, (2) reproducibility of the experimental outcomes is expected to be high when using the same genetic line and experimental set up in future experiments, (3) this line is already successfully phenotyped for the effect of short term (3 weeks) exposure to a plant ingredient based diet in early life on later life acceptance and utilization (Geurden et al., 2013) allowing comparison of fish responses over studies.

As chronic stressors we used a high cation-anion difference (CAD700) in the diet and water DO sub-optimal levels. Effects on osmoregulation were assessed by measurement of plasma osmoregulatory parameters, i.e. plasma ion and osmotic pressure. Therefore, tissue and blood samples will be taken. Then fish were tested for changes in key oxidative stress (glutathione metabolism and lipid peroxidation), and immune response parameters (alternative complement system, peroxidase and lysozyme).

Hypotheses tested:

(1). diets low in dietary electrolyte balance (CAD = Na+ K - Cl, expressed in mEq/kg feed) result in an a lower requirement of metabolizable energy for maintenance (MEm) and in a higher feed intake and growth when compared with the diet high in CAD (see results dissertation Saravanan, 2013),

(2). the assumed lower MEm in fish fed the low CAD diet is explained by the lower energy cost for maintaining the acid base homeostasis in the stomach and blood (based on dissertation Saravanan, 2013),

(3). The assumed higher feed intake for diets fed at satiation and low in CAD is a result of lower oxygen demand (energy) for maintenance leaving more oxygen (oxygen) available for feed intake (scope of feed intake ? scope of growth) (confirms the "oxystatic theory", based on dissertation Saravanan, 2013),

(4). A positive effect on nutrient digestibility is expected for the diet high in CAD due to a higher liquefaction (higher drinking) and/or lower chyme pH (due lower diet pH) (based on dissertation Saravanan, 2013),

(5). An interaction effect (diet x water oxygen level) is expected for feed intake and growth as fish fed the high CAD diet and kept under low oxygen conditions will be affected by both the low water oxygen concentration and the lower oxygen transport capacity of the blood due to an altered acid base balance (blood pH and stomach pH).

(6). An interaction effect (diet x water oxygen) is expected on hydromineral balance due to a reduced ability of fish kept at chronic low oxygen concentration and high CAD levels to mobilize energy for osmoregulation when compared with low oxygen and optimal CAD levels in the diet.

Material and methods

Fish and housing.

We used 370 fish of an isogenic heterozygous family of rainbow trout produced in February 2014 at INRA (France) according to Quillet et al. (2007). This family (R23) is a heterozygote isogenic line, which were produced by crossing two homozygote isogenic lines. At the start of the experiment average weight of the fish was ~ 115 grams. Fish were randomly assigned to one of the twelve glass tanks of the metabolic research unit (MRU) of Wageningen University. Each tank was facilitated with separate automatic probes for detection of water flow and oxygen consumption and was also equipped with faecal collectors for measuring digestibility. Water volume of the tanks was set at 200 L. All tanks were connected to the same recirculating aquaculture system (RAS) and had identical inlet water quality. Daily 28m^3 of system water was refreshed. Water temperature was set at ± 14 ± 1°C. Tanks were in rooms without windows. Photoperiod was maintained at 12 : 12 (Light : Dark) with daybreak set at 07:00.

Experimental design

Twelve groups of 30 rainbow trout (*Oncorhynchus mykiss*) were housed in the metabolic research unit, according to a 2 by 2 factorial design: (a) 2 different diets with contrast in their cation anion differences (CAD): 200 versus 700 mEqv/kg. This difference was created by changing the mineral composition of the diet, and (b) 2 levels of dissolved oxygen (DO):

normoxia versus low oxygen (hypoxia). Each treatment was triplicated. The diet treatments were coded as “CAD200” and “CAD700” and the dissolved oxygen treatments as “normoxia” and “hypoxia” (Table 1). The entire experiment was carried out in the energy metabolism unit (i.e. in 12 metabolic chambers) connected to the recirculating water system (Saravanan, 2013).
Twelve days prior to the start of the experiment 35 fish were randomly assigned to each of the 12 metabolic chambers. From these fish, 30 fish were randomly taken, batch weighed and stocked in their original tank. From all 12 tanks 10 fish were randomly taken for analysis of initial body composition. The 12 tanks are divided into 3 blocks of 4 tanks in each block; tank 1-4, tank 5-8 and tank 9-12. The 4 treatments were assigned randomly within each of 3 blocks to have triplicates for each treatment. The individual tank is the experimental unit. The experiment lasted for 7 weeks during which feed intake, oxygen consumption, digestibility, energy, nitrogen balances and metabolite excretion (TAN, NO_2, NO_3, Urea, CO_2, P) were measured.

At the start of the experiment the water flow of the “normoxia” treatments was kept at 7 L/minute. The water flow for the “hypoxia” treatments was during the first 3 days after the start of the experiment gradually reduced: first to 4 L/min and then depending on the outlet dissolved oxygen value measured the minimal flow was set at 3 L/minute for the hypoxia groups. The flow of the “hypoxia” treatments was adjusted during the experiment in order to maintain the mean outlet daily DO concentration of 4.0 mg/L. The water flow of all tanks at the low DO level was kept equal. In rainbow trout the reported incipient DO is 6.0 mg/L (Pedersen, 1987). Thus a DO level of 4.0 mg/L was expected to be an environmental challenge. The level was not set lower than 4.0 mg/L in order to be sure that fish are able to handle this. In an earlier study (Saravanan et al., 2013) this level was also applied without adverse effects on the health of the fish.

Table 1. Summary of the experimental conditions of the nutritional (CAD200 versus CAD700) and environmental (hypoxia versus normoxia) stress experiment in WU.

Fish species	Data	CAD200 hypoxia	CAD200 normoxia	CAD700 hypoxia	CAD700 normoxia
Rainbow trout	Fish/tank	30	30	30	30
	Replicates	3	3	3	3
	Mean initial weight (g)	114	116	116	115
	Stressors	Environment: Low oxygen	None	Nutritional: Cation Anion Difference & Environment: Low oxygen	Nutritional: Cation Anion Difference
	Duration (weeks)	7	7	7	7

Experimental diets and feeding

Two experimental extruded diets (Table 2) were formulated by our own group and produced by Research Diet Services ((Wijk bij Duurstede, The Netherlands). The diet was a floating 4 mm pellet and contained Yttrium as inert marker to determine digestibility. The two diets were designed to give a contrast in cation anion difference (CAD); 200 versus and 700 mEqv/kg. This difference is created by adding different amounts of Na_2CO_3 and diamol (inert filler also used as inert marker for digestibility studies) in the diets.

During the experiment fish were hand fed one of the two diets (CAD200 or CAD700) to apparent satiation. The fish were fed twice daily at 09.00 hrs and 16.00 hrs. A known quantity of test diet (weighed daily in excess of estimated feed intake) was gradually delivered (in different lots using a standard cup/spoon) to the individual tank for one hour until fish stops feeding. Cessation of feeding was decided by visual observation of uneaten pellets at the tank bottom. Uneaten pellets were collected in the faecal collection and the pellets were counted for leftovers. The daily amount of feed ration, feed waste (uneaten pellets) and feed remains was registered and accounted for in the feed intake calculation.

Table 2. Experimental diets.

Ingredients	CAD200 Diet1	CAD700 Diet 2
Na_2CO_3	0.25	2.90
Diamol	2.75	0.10
Wheat	27.20	27.20
Wheat gluten	13.00	13.00
Fish meal (RE>680)	13.00	13.00
Fish oil	14.00	14.00
Soya protein concentrate	13.00	13.00
Pea protein concentrate	13.00	13.00
Lysine HCL	0.30	0.30
DL-methionine	0.50	0.50
Monocalcium phosphate	1.50	1.50
$CaCO_3$ (krijt)	0.50	0.50
Yttrium oxide	0.01	0.01
Premix trout	1.00	1.00
Total	**100.01**	**100.01**
Analyzed proximate composition of diets:		
Dry matter (g/kg diet)	962	970
Crude protein (g/kg DM)	449	451
Crude fat (g/kg DM)	179	171
Crude ash (g/kg DM)	87	84
NFE (g/kg DM)	286	295
GE (kJ/gDM)	22.47	22.49

Sampling

Initial sampling. At the start of the experiment ten fish were sample to determine initial body composition.
Sampling after six weeks (First Period). On the last two subsequent days of week 6, each day six groups of fish were sampled. Sampling took place after the morning feeding when fish were fed for 1 h to apparent satiation. At 2 h and 5 h after feeding 3 fish (6 in total) were netted, anaestisized (2-phenoxy ethanol; 0.1 ml/L), individual body weight recorded and sampled as fast as feasible for post-prandial blood: first by cardiac puncture (blood sample 1) followed by caudal vein puncture (blood sample 2). There after fish were euthanized by severing the cervical spinal cord and sampled for chyme from the stomach by putting clips before (oesophagus) and after the stomach. Finally each fish was sampled for stomach tissue (after emptying the content, anterior intestine (rinsed with PBS), gill (2^{nd} gill arch), liver, head kidney and heart. Stomach, intestine and sections of the 2^{nd} gill arch (all taken from the same region per tissue) were stored for 24h in fixative at 4^{o}C (10% formalin buffered with PBS). Thereafter the tissues were transferred to 70% ethanol and store at 4^{o}C. Tissue (50-100mg) of the anterior intestine was flash frozen in liquid nitrogen and stored at -80°C until later analysis. Liver and heart were dissected, weighed, split in two subsamples, flash frozen in two and one sample respectively and thereafter stored at -80°C *(analysis by INRA St Pee and Porto).*
Final sampling (Second Period). On the last two days of the experiment each day six experimental groups of fish were sampled. Fish were not fed on the day prior to sampling. An sampling effect on non-sampled groups was prevented by sampling the tanks following the order tanks were installed in the room starting from the room entrance (tank 1, 2…6) and by putting tanks prior to the start of sampling on flow through to prevent water from these tanks flowing to tanks not yet sampled. At sampling fish from each experimental group were divided into 3 sub-groups: (a) Control fish. Three fish per tank (9 fish per treatment) for measuring parameters with minimal/no exposure to acute stress) were netted and sedated/euthanized as quick and smoothly as possible in a lethal 2-phenoxy ethanol solution (1ml/L) where after the fish were weighed individually and sampled for blood and tissues; (b) Stressed fish. Three fish were directly after netting exposed to a standard acute confinement stress (2 minutes at a density of 200kg/m^3) for measuring the cortisol response. After the confinement stress fish were transferred into their original (empty) tank where they 'recovered'. After exactly 1 hour fish were netted and sedated/euthanized as quick and smoothly as possible in a lethal 2-phenoxy ethanol solution (1mg/L). Thereafter, fish were weighed individually and sampled for blood and tissues; (c) Fish for nitrogen and energy mass balance analysis. Remaining fish were euthanized in an overdose of 2-phenoxy ethanol (1 mg/L), batch weighed and counted. Per tank 10 fish were randomly taken and stored in sealed plastic bags at -20°C for later proximate body composition analysis. Control and stressed fish were sampled for blood (heparin and heparin-lithium treated) by caudal and cardiac puncture (control fish only) and tissues of gill, the brain section containing hypothalamus, mid brain, telencephalon, preoptic area, pituitary, head kidney (which includes interrenal cells), intestine (proximal part), heart and liver. Blood was quickly drawn from the heart and caudal vessels. The total time including anaesthesia and blood withdrawal was 4 min for all sampled fish in a given tank. Blood was centrifuged at 3000g for 20 min at 4 ºC, and plasma samples were frozen and stored for metabolite analyses. Prior to tissue collection, fish were killed by cervical section. Tissues were sampled for the molecular analysis of the gill functions. Plasma samples and tissues were stored at -20°C and at -80°C, respectively until analysis (analysis by INRA Rennes).

Real time RT-PCR: Total RNA from gill 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.
cDNA synthesis and real time RT-PCR were performed as described in Lucas et al (2014). Reverse transcriptase was realized from 1.5µg of RNA at 37°C for 1h using M-MLV reverse transcriptase (Promega). Real time RT-PCR was carried out on a StepOnePlus real time PCR system with SYBR-Green PCR master Mix (Applied Biosystem).

Measurements and analysis

At the start and the end of the experiment the fish were weighed and counted. From these measurements, growth, specific growth rate(SGR), FCR and survival was calculated per tank (Table 3).
Faeces collection was identical to the method described by Amirkolaie et al. (2006). Feed and feces were analysed for dry matter (DM), crude protein (CP), crude fat (CF), crude ash (CA), energy (all according to Saravanan et al., 2013) and yttrium (Y) content. Yttrium content was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (van Bussel et al., 2010). Carbohydrate content (Nitrogen Free Extract (NFE)) was calculated as DM–CP-CF-CA. From these data apparent digestibility coefficients (ADC) of nutrients were calculated (Table 3).
The following immune response parameters were determined in plasma: i) Alternative complement pathway (ACH50) which was evaluated as the required trout plasma to have 50% hemolysis of rabbit red blood cells according to Sunyer et al. (1995); ii) Lysozyme, which was determined by a turbidimetric assay as described by Ellis (1990); and iii) Total Peroxidase, measured according to Quade and Roth (1997).
Liver was homogenized in a K-phosphate buffer (pH 7.4, 0.1M) in a 1:15 (p / v) ratio and used to assay oxidative stress parameters. iv) Lipid Peroxidation (LPO). An aliquot of 2.5 µL BHT (2.6-Di-tert-butyl-4-methylphenol) in 4% methanol was added to 150 µl of liver solution. Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS assay (Ohkawa et al., 1979). Liver homogenate was centrifuged at 10,000 g for 20 min at 4 ºC, and the supernatant was isolated for GST quantification at an approximately soluble protein concentration of 0.7 mg . ml-1. v) Glutathione Peroxidase (GPX) was measured according to Mohandas et al. (1984). vi) Glutathione Reductase (GR), the assay was based on reduction of 5,5'-dithiobis (2-nitrobenzoic acid) to reduced glutathione (GSH), generated from an excess of oxidized glutathione (GSSG) as described by Cribb et al. (1989). vii) Total Glutathione (GT) was quantified by the reaction of GSH with DTNB (5.5'-dithio-bis-2-nitrobenzoic acid) as described by Baker et al. (1990). During the reaction the GSSG was concomitantly reduced to GSH, hence enabling the measure of all glutathione. Protein content in homogenates was quantified according to Bradford (1976).
Plasma concentrations of chloride and calcium were measured using colorimetric kits (chloride with a mercuric-thiocyanate method, calcium with Arsenazo III method). Osmotic pressure was analyzed using a freeze-point osmometer.

Calculations and statistical analysis

For quantifying the routes of dietary nitrogen excretion and dietary energy use, the energy and nitrogen balances were measured. This was done according to the methods and calculations as described by Saravanan et al. (2012) (Table 3). Therefore initial and final body composition regarding nitrogen, fat, energy and phosphorus were measured as described in Saravanan et al. (2013).

Table 3. Fish performance, apparent digestibility, nitrogen and energy balance parameter calculations.

Fish performance parameters	Symbol	Unit	Equation
Mean initial body weight	W_i	g	= B_i / N_i
Mean final body weight	W_f	g	= B_f / N_f
Absolute growth	G	g/fish	= W_f - W_i
Absolute feed intake during the exp. period	FI_{ABS}	g DM/fish	= FI_{Total} /N/t
Feed conversion ratio	FCR	g DM/g fish	= FI_{ABS} / G
Specific growth rate	SGR	%W/day	= (ln W_f – ln W_i) / t * 100%
Geometric mean body weight	W_g	g	= $e^{((\ln Wf + \ln Wi)/2)}$
Mean metabolic body weight	MBW_g	$kg^{0.8}$	= $(W_g /1000)^{0.8}$
Relative growth rate on metabolic weight	RGR_m	$g/kg^{0.8}$/day	= G / t / MBW_g
Relative feeding rate on metabolic weight	RFR_m	$gDM/kg^{0.8}$/day	= FI / t / MBW_g
Survival	S	%	= N_f / N_i * 100%
Apparent digestibility			
Apparent digestibility coefficient	ADC_X	%	= (1 – (AIA_{diet} / AIA_{faeces} * X_{faeces} /X_{diet}))*100
Nitrogen balance parameters			
Gross nitrogen intake	GNI	$mg/kg^{0.8}$/day	= FI_{ABS} * CP_{diet} / 6.25 / t /1000 /MBW_g
Digestible nitrogen intake	DNI	$mg/kg^{0.8}$/day	= GNI * ADC_{CP} / 100%
Fecal nitrogen loss	FN	$mg/kg^{0.8}$/day	= GNI – DNI
Retained nitrogen	RN	$mg/kg^{0.8}$/day	= (W_f * CP_{Wf} -W_i *CP_{Wi}) / 6.25 / t /1000/MBW_g
Branchial and urinary nitrogen loss	BUN	$mg/kg^{0.8}$ /day	= DNI – RN
Protein efficiency	PE	%	= (RN*6.25)/(GNI*6.25)*100%
Energy balance parameters			
Gross energy intake	GE	$kJ/kg^{0.8}$/day	= FI_{ABS} * GE_{diet} / t / MBW_g
Digestible energy intake	DE	$kJ/kg^{0.8}$/day	= GE * ADC_{GE} / 100%
Branchial and urinary energy loss [1]	BUE	$kJ/kg^{0.8}$/day	= (BUN * 24.85 kJ/g N)/1000
Metabolisable energy	ME	$kJ/kg^{0.8}$/day	= DE – BUE
Retained energy	RE	$kJ/kg^{0.8}$/day	= (W_f * E_{Wf} – W_i * E_{Wi}) / t / MBW_g
Heat production	H	$kJ/kg^{0.8}$/day	= ME – RE
Energy retained as protein [2]	RE_p	$kJ/kg^{0.8}$/day	= RN * 6.25 * 23.7 kJ/g
Energy retained as fat	RE_f	$kJ/kg^{0.8}$ /day	= RE – RE_p
Metabolisable energy for maintenance [3]	ME_m	$kJ/kg^{0.8}$/day	= ME – RE / 0.75

$AIA_{diet\ or\ faeces}$: acid insoluble ash in diet or faeces (g/kg DM); B_i: Initial biomass per tank (g/tank) ;B_f: Final biomass per tank (g/tank); CA: crude ash; CF: crude fat; CP: crude protein; CP_{Wi}: protein content of initial mean body weight (g/gW_i); CP_{Wf}: protein content of final mean body weight (g/gW_f); CP_{diet}: crude protein content of the diet (g/g feed DM); DM: dry matter; E_{Wf}; energy content of final mean body weight (kJ/g W_f); E_{Wi} energy content of initial mean body weight (kJ/g W_i); FI_{Total}: Total feed given during the exp. period corrected for dead fish and uneaten pellets (g DM/tank);$F_{Rejected}$: Total feed rejected during the exp. period (g DM/tank);GE: gross energy; GE_{diet}: is gross energy content of the diet (kJ/g feed DM); NFE: nitrogen free extract; N_i: initial number of fish (N/tank) N_f: final number of fish (N/Tank); P: phosphorus; t: duration of the experimental period (days);W: bodyweight (g); $X_{diet\ or\ faeces}$: the quantity of a nutrient (DM, CF, CP, NFE, CA) , mineral (P) or energy (GE) in the diet. [1)] 24.85 kJ/g NH_3–N assuming all nitrogen is excreted as NH_3-N (Bureau, 2002); [2)] 23.7 is the energy content of 1 g protein (Brafield, 1985) [3)] kg =0.75.

For statistical analysis in the first experimental period, tank was taken as the experimental unit. All growth performance, body composition, digestibility, nitrogen and energy balance parameters were analysed for the effect of diet, dissolved oxygen and their interaction effect by two-way ANOVA using PROC GLM of SAS. For statistical analysis in the second experimental period, fish was taken as the experimental unit. All parameters were analysed for the effect of diet, dissolved oxygen, acute stress and their interaction effect by two-way

ANOVA. For ions and osmolality, due to an absence of normal distribution, parameters were analysed by one-way ANOVA Kruskall and Wallis using Statistica.

All procedures were carried out according to the Wageningen University Ethics Board for experimentation with animals and current EU legislation on handling of experimental animals.

Results

Growth performance and body composition

The survival percentage, start weight, specific growth rate (SGR), growth, feed intake and feed conversion ratio (FCR) are presented in Table 4. Start weight, specific growth rate (SGR), growth, feed intake and FCR were not significantly affected by CAD but were, except for start weight and FCR, affected by DO treatment (P<0.01). Growth and feed intake were 53% and 55% higher, respectively, in the normoxic treatment when compared to the hypoxic treatment. There was no interaction effect between CAD and DO on growth performance observed.

Table 4. Effect of dietary CAD level and water DO level on performance of trout.

	CAD200 hypoxia	CAD200 Norm.	CAD700 hypoxia	CAD700 Norm.	sem	P-value CAD	DO	Int
No fish stocked/tank	30	30	30	30				
No tanks	3	3	3	3				
Survival, %	98.9	100.0	100.0	100.0				
Start weight, g	114	116	116	115	2.0	ns	ns	ns
SGR, %/d	1.12	1.51	1.12	1.57	0.10	ns	**	ns
Growth, g/d	1.63	2.47	1.66	2.58	0.23	ns	**	ns
Feed intake DM, g/d	1.10	1.73	1.20	1.83	0.17	ns	**	ns
FCR	0.70	0.73	0.74	0.73	0.013	ns	ns	ns

CAD=cation anion differences; DO is dissolved oxygen; sem=pooled standard error of mean; ns=not significant P>0.1; *P<0.05; **P<0.01; ***P<0.001; SGR=specific growth rate; FCR=Feed conversion ratio.

The final whole body compositions of rainbow are presented in Table 5. Dietary CAD had no effect on final body composition. A significant effect of DO was observed for dry matter, fat and energy (P<0.05) but no effect was found on ash, phosphorus and protein body content. The fish kept at normal DO (normoxia) were 9% more fat than fish kept at low DO (hypoxia). Consequently fish in the normoxia treatment had ~ 4.5% more energy deposit per unit of bodyweight when compared to fish in the hypoxia treatment. There was no interaction effect observed between CAD and DO on body composition.

Table 5. Effect of dietary CAD level and water DO level on body composition of trout on fresh basis.

	CAD200 hypoxia	CAD200 Norm.	CAD700 hypoxia	CAD700 Norm.	sem	P-value CAD	DO	Int
Dry matter, g/kg	288	299	286	296	3.2	ns	*	ns
Ash, g/kg	21	21	22	21	0.4	ns	ns	#
Phosphorous, g/kg	3.89	3.96	3.94	3.83	0.09	ns	ns	ns
Protein, g/kg	174	174	174	175	2.2	ns	ns	ns
Fat, g/kg	97	104	92	102	3.0	ns	*	ns
Energy, kJ/g	7.8	8.2	7.6	7.9	0.15	ns	*	ns

CAD=cation anion differences; DO is dissolved oxygen; sem=pooled standard error of mean; ns=not significant P>0.1; *P<0.05; **P<0.01; ***P<0.001

Nutrient digestibility, nitrogen and energy balance

The apparent nutrient and energy digestibility coefficients (ADC) are presented in Table 6 and are used to calculate the nitrogen and energy balance parameters in table 7 and table 8. CAD significantly affected apparent digestibility of dry matter (P<0.001), ash (P<0.001), fat (P<0.001) and energy (P<0.05) but there was no effect observed of CAD on apparent digestibility of protein and carbohydrates.
DO affected apparent digestibility of dry matter (P<0.001), ash (P<0.05), protein (P<0.001), fat (<0.01), carbohydrates (P<0.001) and energy (P<0.01) but not the apparent digestibility of phosphorus. There was no interaction effect observed between CAD and DO on apparent digestibility.

Table 6. Effect of dietary CAD level and water DO level on the apparent nutrient digestibility (%, ADC) of trout.

	CAD200 Hypoxia	CAD200 Norm.	CAD700 hypoxia	CAD700 Norm.	sem	P-value CAD	DO	Int
Dry matter, %	86.0	84.4	87.9	85.7	0.18	***	***	ns
Ash, %	33.0	32.2	54.9	52.0	0.66	***	*	ns
Phosphorous, %	47.7	48.6	42.9	41.9	0.85	***	ns	ns
Protein, %	97.2	96.4	97.3	96.4	0.09	ns	***	ns
Fat, %	97.7	97.3	96.9	96.5	0.09	***	**	ns
Carbohydrates, %	77.1	73.4	77.7	72.7	0.35	ns	***	ns
Energy, %	92.9	91.5	92.7	90.9	0.12	*	***	ns

CAD=cation anion differences; DO is dissolved oxygen; sem=pooled standard error of mean; ns=not significant P>0.1; *P<0.05; **P<0.01; ***P<0.001

CAD treatment did not affect the nitrogen balance parameters and protein efficiency (Table 7). However, DO significantly affected gross nitrogen intake (P<0.01, GE), digestible nitrogen intake (P<0.01, DN), branchial and urinary nitrogen loss (P<0.01, BUN), retained nitrogen (P<0.05, RN) but not protein efficiency. Digestible nitrogen intake and retained nitrogen in growth was 41% and 42 % respectively and is higher in the normoxia treatment when compared to the hypoxia treatment.

Table 7. Effect of dietary CAD level and water DO level on the nitrogen balance of trout.

	CAD200 Hypoxia	CAD200 Norm.	CAD700 hypoxia	CAD700 Norm.	sem	P-value CAD	DO	Int
GN, mg/kg$^{0.8}$/d	373	536	402	569	41.8	ns	**	ns
DN, mg/kg$^{0.8}$/d	362	517	391	548	40.3	ns	**	ns
BUN, mg/kg$^{0.8}$/d	147	218	175	233	13.2	ns	**	ns
RN, mg/kg$^{0.8}$/d	215	299	216	315	27.9	ns	*	ns
Protein efficiency, %	59.4	57.6	55.3	57.2	1.2	#	ns	ns

CAD=cation anion differences; DO is dissolved oxygen; sem=pooled standard error of mean; ns=not significant P>0.1; *P<0.05; **P<0.01; ***P<0.001; GN= gross energy; DN=digestible nitrogen; BUN=branchial and urinary nitrogen loss; RN= retained nitrogen.

The energy balance is presented in Table 8. CAD did not affect the energy balance of trout except for heat production (P<0.01, HP) and maintenance (P<0.001). Heat production (HP) and energy requirement for maintenance was 26% and 65% higher, respectively, on CAD700 diets than on CAD200 diets. The observed difference in heat production between CAD200 and CAD700 treatments within hypoxia and anoxia treatment corresponds, to the observed difference in energy required for maintenance when comparing the same treatments.
DO treatment affected the energy balance significantly except for maintenance. Gross energy and digestible energy intake were 43 % and 40 % higher respectively in the normoxia treatment when compared to the hypoxia treatment. Metabolisable energy (ME), heat production (HP) and retained energy (RE) were 40%, 23% and 54% higher respectively in the normoxia treatment when compared to the hypoxia treatment. DO significantly affected energy retention as fat (P<0.01) and as protein (P<0.05). Fish in the normoxia treatment retained 64% more energy as fat and 42 % more energy as protein when compared with the hypoxia treatment.

Table 8. Effect of dietary CAD level and water DO level on the energy balance of trout.

	CAD200 Hypoxia	CAD200 Norm.	CAD700 hypoxia	CAD700 Norm.	sem	P-value CAD	DO	Int
GE, kJ/kg$^{0.8}$/d	117	168	125	178	13.1	ns	**	ns
DE, kJ/kg$^{0.8}$/d	108	153	116	161	11.9	ns	**	ns
BUE, kJ/kg$^{0.8}$/d	3.6	5.4	4.3	5.8	0.33	ns	**	ns
ME, kJ/kg$^{0.8}$/d	105	148	112	156	11.6	ns	**	ns
HP, kJ/kg$^{0.8}$/d	39	51	52	61	3.0	**	**	ns
RE, kJ/kg$^{0.8}$/d	65	97	60	95	9.2	ns	**	ns
RE as fat, kJ/kg$^{0.8}$/d	33	52	28	48	5.2	ns	**	ns
RE as prot, kJ/kg$^{0.8}$/d	32	44	32	47	4.1	ns	*	ns
Maintenance, kJ/kg$^{0.8}$/d	18	19	32	29	2.2	***	ns	ns

CAD=cation anion differences; DO is dissolved oxygen; sem=pooled standard error of mean; ns=not significant P>0.1; *P<0.05; **P<0.01; ***P<0.001; Maintenance was calculated from RE using a k_g value of 0.75; GE=gross energy intake; DE=digestible energy; BUE=branchial and urinary energy excretion; ME=metabolisable energy; HP=heat production; RE=retained energy.

In summary, CAD treatment (CAD200 versus CAD700) did not affect growth performance and body composition. CAD200 treatment resulted in significantly lower apparent digestibility of dry matter, ash, fat and energy but resulted in a higher apparent digestibility for phosphorus when compared with CAD700. No effect of CAD treatment was observed on apparent digestibility of protein and carbohydrates and on the nitrogen and energy balance parameters except for energy lost as heat production (HP) and spent in maintenance which was significant lower in the CAD 200 treatment. The lower amount of energy spent in maintenance by fish fed CAD200 diets when compared with CAD 700 diets corresponds to the observed difference in heat production within hypoxia and normoxia treatment when comparing the same treatments.
DO treatment only affected growth and feed intake (growth performance) and were 53% and 55% higher respectively in the normoxia treatment when compared with the hypoxia treatment. Fish in the normoxia treatment contained significant more dry matter, 9% more fat and deposited ~ 4.5% more energy per unit of bodyweight when compared to fish in the hypoxia treatment. At normoxia treatment apparent digestibility was significantly lower for dry matter, ash, protein, fat, carbohydrates and energy but not for phosphorus which was unaffected by DO treatment. Nitrogen and energy mass balance components were all significantly higher in the normoxia treatment except for maintenance energy costs which was not affected by DO. Protein efficiency was not affected by DO treatment.

Osmoregulatory responses

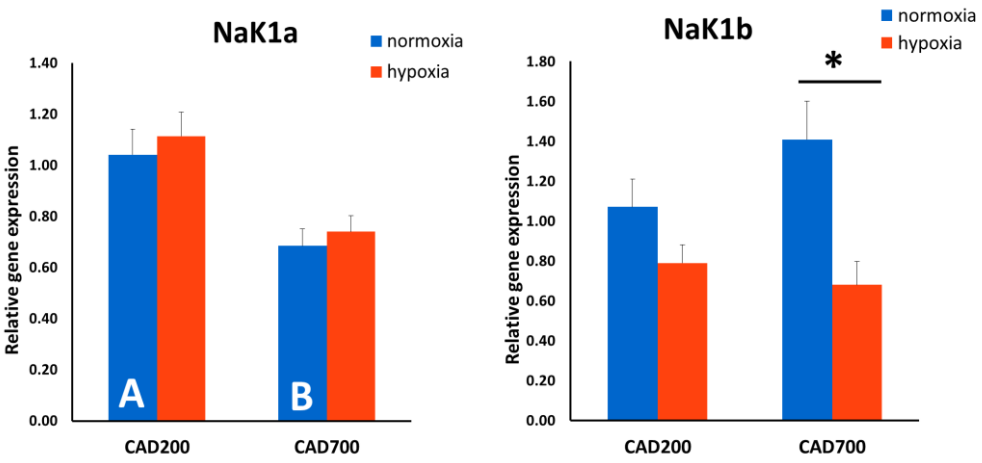
Parameters characteristic of hydromineral balance were measured. Plasmatic concentrations of calcium, chloride and osmolality were not significantly affected by DO treatment. However, plasma calcium and osmotic pressure were affected by CAD treatment (Table 9).
In complement to these plasmatic analysis, we also analysed gill expression of two genes, isoforms α1a and α1b of Na/K-ATPase pump (respectively NaK (1a and1b). In normaxia, a significant decrease in NaK1a was observed in CAD700 treated group but not for NaK1b. However, hypoxia condition significantly reduced NaK1b expression compared to normoxia (figure 1).

Table 9. Effect of dietary CAD level and water DO on the hydromineral balance (calcium, chloride and osmotic pressure) in plasma of rainbow trout.

	CAD200 Hypoxia	CAD200 Norm.	CAD700 hypoxia	CAD700 Norm.	P-value CAD	P-value DO
Calcium mM	2.34±0.05	2.28±0.02	2.4±0.02	2.41±0.04	**	ns
Chloride mM	118.9±2.26	120.6±2.22	115.9±0.88	119.4±1.44	ns	ns
Osmotic pressure mOsmol/l	305.3±1.75	307.6±1.12	309.9±0.98	311.7±1.2	**	ns

CAD=cation anion differences; DO is dissolved oxygen; ns=not significant. *P<0.05; **P<0.01

Figure 1: Effect of dietary CAD lavel and water DO on the expression of NaK1a and NaK1b gene expression in the gill of rainbow trout. Values are mean ± SEM of 8-10 fishes. Mann-Whitney test was used for statistical analysis. * P < 0.05, columns with different capital letter indicated significant differnec at P < 0.05.



Effects on oxydative stress responses and on innate immune parameters.

The effect of Diet (CAD 200 versus CAD700), water DO (Hypoxia versus Normoxia), Stress (No Stress versus Acute Stress) and their interactions on innate immune stress parameters in plasma of rainbow trout are presented in table 9. No effect was observed of Diet, Stress, DO and the interaction between Diet * Stress * DO on ACH50, lysozyme and peroxidase. There was also no effect observed of the interaction Diet * DO on ACH50, Diet * stress on ACH50 and lysozyme and DO * Stress on peroxidase. A significant interaction effect was observed of Diet * DO on ACH50, Diet * Stress on ACH50 and lysozyme and DO * Stress on lysozyme.

Table 10. Effect of Diet (CAD 200 versus CAD700), water DO (Hypoxia versus Normoxia) level and Stress (No Stress versus Acute Stress) and their interactions on innate immune stress parameters in plasma of rainbow trout.

Diet	CAD200		CAD700		
DO	Hypoxia	Normoxia	Hypoxia	Normoxia	P-values

Stress	NS	S	NS	S	NS	S	NS	S	Diet	S	DO	Diet * S * DO	Diet * DO	Diet* S	DO * S
ACH50	**18.4** ±11.0	**20.4** ±6.5	**13.8** ±1.4	**15.8** ±6.8	**8.8** ±3.0	**10.7** ±4.5	**9.1** ±3.2	**13.9** ±5.1	ns	ns	ns	ns	**	*	ns
Lysozyme	**633.3** ±182.9	**596.3** ±150.9	**522.2** ±72.8	**645.4** ±132.1	**544.8** ±178.9	**576.9** ±105.1	**570.4** ±180.8	**815.7** ±415.1	ns	ns	ns	ns	ns	**	*
Peroxidase	**111.2** ±31.3	**119.7** ±14.7	**116.5** ±13.4	**123.0** ±14.6	**123.0** ±19.4	**125.6** ±6.8	**126.4** ±11.7	**101.1** ±37.8	ns	ns	ns	ns	ns	ns	ns

Mean ± SD, CAD=cation anion differences; DO= Dissolved oxygen, NS= no stress, S= acute stress, ns=not significant P>0.1; *P<0.05; **P<0.01
ACH50 plasma units for 50% hemolysis, Lysozyme and Peroxidase in EU . mL plasma

The effect of Diet (CAD 200 versus CAD700), DO (Hypoxia versus Normoxia), Stress (No stress versus Acute Stress) and their interactions on oxidative stress parameters in the liver of rainbow trout. No significant effects of DO on oxidative stress parameters in the liver were observed. A significant effect was observed of Diet on GPX, GR and TG and of Stress on GSH and GSH/GSSH. Interaction effects were observed for
Diet * Stress * DO on GPX, GR and GSSG, for Diet * DO on GR for Diet *Stress on GR, TG,GSSG,GSH and GSH/GSSG and for DO * Stress on GSSG and GSH/GSSG For all other interactions no significant effect was observed on oxidative stress parameters in the liver.

Table 10. Effect of Diet (CAD 200 versus CAD700), water DO (Hypoxia versus Normoxia) level and Stress (No **S**tress versus Acute **S**tress) and their interactions **on oxidative stress parameters in liver of rainbow trout.**

Diet	CAD200				CAD700				P-values						
DO	Hypoxia		Normoxia		Hypoxia		Normoxia								
Stress	NS	S	NS	S	NS	S	NS	S	Diet	S	DO	Diet*S*DO	Diet*DO	Diet*S	DO*S
GPX	**0.6** ±0.1	**0.5** ±0.1	**0.6** ±0.2	**0.5** ±0.2	**0.6** ±0.2	**0.5** ±0.2	**0.4** ±0.1	**0.5** ±0.2	*	ns	ns	*	ns	ns	ns
GR	**2.6** ±0.7	**2.9** ±1.0	**3.6** ±2.0	**4.4** ±1.2	**4.1** ±1.1	**4.1** ±1.7	**3.3** ±0.9	**5.0** ±0.9	*	ns	ns	*	*	*	ns
TG	**0.7** ±0.5	**1.0** ±0.4	**2.0** ±0.5	**1.2** ±0.5	**1.1** ±0.6	**1.7** ±0.5	**0.9** ±0.5	**1.8** ±0.6	*	ns	ns	Ns	ns	*	ns
GSSG	**0.6** ±0.2	**0.4** ±0.2	**0.5** ±0.2	**0.5** ±0.2	**0.6** ±0.2	**0.5** ±0.2	**0.5** ±0.2	**0.6** ±0.1	ns	ns	ns	*	ns	*	*
GSH	**0.6** ±0.1	**0.8** ±0.3	**0.6** ±0.3	**0.8** ±0.2	**0.6** ±0.3	**0.9** ±0.4	**0.7** ±0.2	**0.8** ±0.2	ns	**	ns	Ns	ns	*	ns
GSH/GSSG	**1.2** ±0.4	**1.2** ±0.3	**1.2** ±0.6	**1.6** ±0.5	**1.1** ±0.6	**1.6** ±0.9	**1.5** ±0.5	**1.4** ±0.4	ns	**	ns	Ns	ns	*	*
LPO	**5.6** ±2.3	**4.0** ±1.1	**5.5** ±1.9	**4.7** ±1.3	**6.0** ±2.1	**4.5** ±2.1	**5.1** ±1.1	**6.4** ±2.4	ns	ns	ns	Ns	ns	ns	ns

Mean ± SD, CAD=cation anion differences; DO= Dissolved oxygen, NS= no stress, S= acute stress, ns=not significant P>0.1; *P<0.05; **P<0.01
GPX and GR activities in nmol product . min . mg prot, TG, GSSH, GSH and LPO in nmol product . mg prot, GSH/GSSH ratio

Discussion

In this study we used the isogenic trout line R23 to assess the interactive effects between nutrition (low CAD versus high CAD) and DO (hypoxia versus normoxia) on energy/nutrient partitioning, stress responsiveness and osmoregulation parameters. As all fish within this line are genetically uniform, the experimental conditions allow a good estimation of fish responses on the interactive effect between the environmental factors nutrition (low CAD versus high CAD) and dissolved oxygen (hypoxia versus normoxia).

The results in this study **confirm our hypothesis that diets low in dietary electrolyte balance (CAD = Na+ K - Cl, expressed in mEq/kg feed) result in a significantly lower requirement of metabolizable energy for maintenance (MEm)**. Fish fed the CAD700 diet had 65% higher energy requirement for maintenance when compared with fish fed the CAD200 diet. The observed lower MEm in fish fed the low CAD diet might be explained by the lower energy cost for maintaining the acid base homeostasis in the stomach and blood (Saravanan, 2013). However**, the results do not confirm the hypothesis that fish fed diets low in CAD result in a higher feed intake and growth when compared with the diet high in CAD**. The assumed higher feed intake for diets fed at satiation and low in CAD was based on the assumption of a lower oxygen demand (energy) for maintenance leaving more oxygen (oxygen) available for feed intake (scope of feed intake/ scope of growth) and would confirm the "oxystatic theory" (Saravanan, 2013).

Our results 'confirm' the expected positive effect of high CAD diets on apparent nutrient digestibility. Apparent nutrient digestibility was significantly higher for dry matter and ash in the high CAD treatment. However, apparent digestibility for phosphorus, fat and energy were significant lower for fish fed the high CAD diet. This positive effect on nutrient digestibility in dry matter and ash might to be attributed to a higher liquefaction (higher drinking) and/or lower chyme pH (due to lower diet pH) in fish fed the high CAD diet (Saravanan, 2013). In this study we expected to observe an interaction effect (diet x water oxygen level) for feed intake and growth as fish fed the high CAD diet and kept under low oxygen conditions was expected to be affected by both the low water oxygen concentration and the lower oxygen transport capacity of the blood due to an altered acid base balance (blood pH and stomach pH). However, this effect was not observed.

Analysis of osmoregulatory parameters clearly showed a significant effect on plasma osmotic pressure and calcium levels. Moreover, we also observed significant decrease of expression of isoforms of Na/K-ATPase, a major Na transporter located in gill epithelia. CAD treatment led to an increase in Na_2CO_3 content in the diet (2.75 %). These results should be compared with previous studies indicating that dietary salt feeding (addition of 11% NaCl) in rainbow trout has osmoregulatory effects, i.e. modifications of gill morphology and gill function in rainbow trout. Moreover, Perry and Rivero-Lopez (2012) also observed a significant effect in Chloride uptake but not on Chloride plasma levels in freshwater rainbow trout fed with 11% NaCl, thus confirming the effect of such diet on gill osmoregulation. Our data obtained on an isogenic trout line show some convergences with previous studies: We did not have any effect of CAD on plasma chloride levels but we observed significant changes in NaK1a and NaK1b expression after CAD700 diet or CAD700 plus hypoxia which may be associated with gill remodeling. More detailed analysis of gill morphology and ion transporters expression at the level of gill epithelia would be required to conclude on that hypothesis. In any case, the use of isogenic trout line lead us to observed significant changes in plasma parameters even at rather low salt loading of the diet (2.75%).

Concerning a possible interaction effect (diet x water oxygen levels) on osmoregulation, the present results do not confirm such an hypothesis and CAD /hypoxia treatments does not seem to be acting on plasma osmoregulatory parameters. CAD treatment does affect MEm being higher in the CAD700 treatment when compared to CAD200 treatment (see above). However, to which extend the effects of CAD on osmoregulation have consequences on MEm requires further research.

In this study the alternative complement pathway (ACP) was nutritionally modulated, displaying in general higher values in the animals fed the CAD200 diet, detecting an

interaction effect of diets and DO levels, as well as between diets and acute stress (Table 9). Those differences may be related to the key role of ACP on the defense of the fish, as it neutralizes pathogenic exotoxins and activates proteins that can alert the host to the presence of potential pathogens. As this system contributes to the recruitment of immune cells, the general lower values of fish fed CAD700 may indicate that this group may have a lower immunity status than the group of fish fed the CAD200 diet.

The pattern for lysozyme activity in plasma was more variable; although statically significant differences were observed in the fish fed the CAD700 diet when subjected to acute stress (Table 9). Lysozyme is an important component of the innate immune system which damage cell walls of gram positive bacteria (Jollès & Jollès, 1984). This enzyme released by the leucocytes has been shown to be regulated in fish by several nutritional and environmental conditions (Tort et al. 1998, Torrecillas et al., 2007). In this study, acute stress appears to trigger such a mechanism in fish fed the CAD700 diet, as the plasmatic level of the enzyme was increased in that group. However, total peroxidase activity, which is often used as an indicator of the immunologically active status of circulating leucocytes, did not display statistically significant differences for any of the experimental conditions (Table 9).

Glutatione metabolism, involving several enzymes and metabolites, plays a key role as a protective mechanism against oxidative stress in fish. This study showed that GPx, GR, GSH, GSSG and GT are altered acute stress in liver of trout (Table 10). In particular, fish fed the CAD200 display higher activity levels for GPx. This enzyme reduces lipid hydro-peroxides to their corresponding alcohols using GSH as a co-factor to form oxidized GSSG (Eroglu et al., 2014). The enzyme catalyzing the opposite conversion, GR, is also modulated by dietary conditions, as the activity for this enzyme is increased in fish fed a CAD700. Total glutathione levels (TG) was modulated similarly to GR, displaying higher levels in fish fed the CAD700 diet, particularly in those not subjected to acute stress. A drop in the TG levels signals a greater use of GSH. TG is a tripeptide formed by both GSH and GSSG that works as an electron donor to GPX, as cofactor for GST, and as a direct thiol-based antioxidant. Previous studies in fish suggest that GSH/GSSG ratios may also serve as an indicator of antioxidant state (Eroglu et al., 2014). The GSH/GSSG ratio is used, similarly to TG levels, as biomarkers for oxidative stress conditions in a variety of marine and freshwater organisms. In this study, we observed higher values in the GSH/GSSG ratio of fish exposed to acute stress, displaying an interactive effect with diets and DO levels.

In contrast with the observed changes in glutathione metabolism, lipid peroxidation (LPO), an indirect marker for oxidative stress commonly used in animals, did not display significant differences in the liver of trout under experimental treatments (Table 10).

Conclusions

- **In conclusion,** association of new phenotyping methods using the me**tabolic research unit (MRU) of W**ageningen university associated with the use of isogenic trout lines **allowed us to have a more elaborated view on** the interactive effects between nutrition (low CAD versus high CAD) and DO (hypoxia versus normoxia) on energy/nutrient partition**ing. The main information brought by such approaches**

are:diets low in dietary electrolyte balance (CAD = Na+ K - Cl, expressed in mEq/kg feed) result in a lower requirement of metabolizable energy for maintenance (MEm).

- despite a lower requirement of MEm, fish fed diets low in CAD do not result in a higher feed intake and growth when compared with the diet high in CAD,
- a positive effect on nutrient digestibility for dry matter and ash is expected for the diet high in CAD due to a higher liquefaction (higher drinking) and/or lower chyme pH (due lower diet pH) (based on dissertation Saravanan, 2013). This effect was not observed for phosphorus, fat and energy for fish fed the high CAD diet.
- no interaction effect (diet x water oxygen level) was observed for feed intake and growth while this was expected as fish fed the high CAD diet and kept under low oxygen conditions will be affected by both the low water oxygen concentration and the lower oxygen transport capacity of the blood due to an altered acid base balance (blood pH and stomach pH).
- diet and acute stress are the main factors affecting most of the innate immune and oxidative stress parameters measured in rainbow trout, although different DO levels considered here display interactive effects with diet, acute stress or both factors.
- CAD levels has significant effects on osmoregulation (calcium homeostasis and plasma osmolality) but low oxygen levels do not have any effect on those parameters.

References

- Amirkolaie, A.K., J.A.J. Verreth and J.W. Schrama, 2006. Effect of gelatinization degree and inclusion level of dietary starch on the characteristics of digesta and faeces in Nile tilapia (*Oreochromis niloticus* (L.)). Aquaculture 260: 194–205.
- Baker, M. A., G. J. Cerniglia and A. Zaman, 1990: Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry, 190, 360-365.
- Bradford, M., 1976: A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry, 72, 248–254.
- Brafield, A.E., 1985. Laboratory studies of energy budgets. In: Fish Energetics: New Perspectives, pp. 257-281 (P.Tytler and P. Calow editors). London: Croom Helm.
- van Bussel, W., Kerkhof, F., van Kessel, T., Lamers, H., Nous, D., Verdonk, H., Verhoeven, B., Boer, N. & Toonen, H., 2010. Accurate determination of titanium as titanium dioxide for limited sample size digestibility studies of feed and food matrices by inductively coupled plasma optical emission spectrometry with real-time simultaneous internal standardization. Atomic Spectroscopy: 31, 81–88.
- Cribb, A. E., J. S. Leeder and S. P. Spielberg, 1989: Use of a microplate reader in an assay of glutathione reductase using 5,5'-dithiobis(2-nitrobenzoic acid). Analytical Biochemistry, 183, 195-196.
- Ellis, A., 1990: Lysozyme assays. Techniques in fish immunology, 1, 101-103.
- Eroglu, A., Z. Dogan, E. G. Kanak, G. Atli and M. Canli, 2014: Effects of heavy metals (Cd, Cu, Cr, Pb, Zn) on fish glutathione metabolism. Environmental Science and Pollution Research, 1-9.
- Geurden, I., Borchert, P., Balasubramanian, M.N., Schrama, J.W., Dupont-Nivet, M., Quillet, E., Kaushik, S.J., Panserat, S., Médale, F., 2014. The positive impact of

the early-feeding of a plant-based diet on its future acceptance and utilisation in rainbow trout. PLoS ONE 8 (12), art. no. e83162.

- Jollès, P. and J. Jollès, 1984: What's new in lysozyme research? Molecular and cellular biochemistry, 63, 165-189.
- Mohandas, J., J. J. Marshall, G. G. Duggin, J. S. Horvath and D. J. Tiller, 1984: Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. Biochemical pharmacology, 33, 1801-1807.
- Ohkawa, H., N. Ohishi and K. Yagi, 1979: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry, 95, 351-358 .
- Quade, M. J. and J. A. Roth, 1997: A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology & Immunopathology, 58, 239-248.
- Pedersen, C.L., 1987. Energy budgets for juvenile rainbow trout at various oxygen concentrations. Aquaculture 62: 289-298.
- Perry SF, Rivero-Lopez L (2012). Does the presence of a seawater gill morphology induced by dietary salt loading affect Cl- uptake and acid-base regulation in freshwater rainbow trout Oncorhynchus mykiss. J Fish Biol 80(2): 301-311.
- Perry SF, Rivero-Lopez L, McNeill B, Wilson J (2006). Fooling a freshwater fish: how dietary salt transforms the rainbow trout gill into a seawater gill phenotype. The Journal of experimental biology 209(Pt 23): 4591-4596.
- Quillet, E., Dorson M, Le Guillou, S., Benmansour, A., Boudinot, P., 2007. Wide range of susceptibility to rhabdoviruses in homozygous clones of rainbow trout. Fish and Shellfish Immunology 22: 510–519.
- Saravanan, S., Geurden, I., Figueiredo-Silva, A., Kaushik, S., Haidar, M., Verreth, J., Schrama, J., 2012. Control of voluntary feed intake in fish: a role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. British Journal of Nutrition 108:1519–29.
- Saravanan, S., Geurden, I., Figueiredo-Silva, A.C., Kaushik, S., Verreth, J., Schrama, J., 2013. Voluntary feed intake in rainbow trout is regulated by diet-induced differences in oxygen use. Journal of Nutrition 143:781-787.
- Saravanan, S., 2013. Feed intake and oxygen consumption in fish. PhD-thesis Wageningen University 164 p.
- Sunyer, J. O., E. Gómez, L. Tort, V. Navarro and J. Quesada, 1995: Physiological responses and depression of humoral components of the immune system in gilthead sea bream (*Sparus aurata*) following daily acute stress. Canadian Journal of Fisheries and Aquatic Sciences, 52, 2339-2346.
- Torrecillas, S., A. Makol, M. Caballero, D. Montero, L. Robaina, F. Real, J. Sweetman, L. Tort and M. Izquierdo, 2007: Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. Fish & Shellfish Immunology, 23, 969-981.
- Tort, L., J. Rotllant and L. Rovira, 1998: Immunological suppression in gilthead sea bream (*Sparus aurata*) of the North-West Mediterranean at low temperatures. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 120, 175-179.

Experiment 3: EFFECT OF CHRONIC EXPOSURE TO POOR WATER QUALITY ON RAINBOW TROUT USING A MULTI-PARAMETERS APPROACH.

Partner: INRA LPGP, INRA PEIMA

Introduction

Many studies have been done on the biological response in fish exposed to acute stress, whereas much less information is available on chronic exposure to stressors, particularly in situation where fish are exposed to deterioration of water quality. However, such exposure has been shown to affect food intake and growth in rainbow trout (Person-Le Ruyet et al., 2008) indicating that fish could be chronically stressed. However, markers for such chronic stress status are still lacking in fish.

Within this WP, in sub-task 7.1.3, we have developed a multidisciplinary approach aiming to find accurate and relevant information for characterizing chronic stress status in 3 different fish species (sea bream, trout and salmon). The methodological panel for such multidisciplinary approach includes analysis of behavior, regulation of the HPI (hypothalamus, pituitary, interrenal) axis, physiological and transcriptomic profiles of the organs involved in the stress response (liver, pituitary, brain, head kidney, gills, etc.). Our working hypothesis is that such chronic stress status is a complex situation, that cannot be assessed by only analyzing cortisol, the most commonly measured stress hormone, which may not be elevated in chronically stressed fish due to a negative feedback mechanism of cortisol that causes a down regulation of the HP axis (Pickering and Stewart, 1984; Procarione et al., 1999). In contrast, in the sub-task 7.3, we have shown that a multiple-parameters approach, as indicated above, is necessary to get a better picture of chronic stress and allostatic load in seabream and in trout. However, limits between adaptation or stress responses still need to be further clarified for each fish species and physiological condition.

The present deliverable aims to assess differences between two divergent rainbow trout clonal lines for their responses to chronic environmental stress. For such task, we have been using similar multi-parameters approach as for rainbow trout in sub-task 7.3 (i.e. behavioral, physiological and endocrine parameters). Fish have been exposed during 4 weeks to the same type of chronic stressor, i.e. exposition to deterioration of water quality (mainly hypoxia). The two isogenic lines chosen for this study were previously found to be highly divergent for their ability to grow on a plant-based diet during early-feeding (Geurden et al., 2013). Moreover, these two isogenic lines were found to be highly divergent in their corticosteroid reactivity to acute confinement stress (Sadoul et al., 2015).

Material and methods

Ethical statements

Experiments were conducted within INRA facilities having authorization for animal experimentation (B29-777-02). Fish used in the experiment were reared and handled in strict accordance with French and Europeans policies and guidelines of the INRA PEIMA Institutional Animal Care and Use Committee (which agreement number is B29-777-02).

Fish and chronic stress protocol

Production (Quillet et al. 2007) and breeding of the two isogenic heterozygote rainbow trout lines (A22 and R23) were performed at PEIMA (INRA fish farming in Brittany, France) in running water. These lines were exposed chronically to water of poor quality for 4 weeks (mainly, low oxygen, high ammonia and high CO2 levels). We had four conditions with 3 tanks per condition: A22Control, A22Stressed, R23Control, R23Stressed.

For this experiment, rainbow trout (~ 160-170g) were distributed in twelve tanks to get a density of 50kg/m^3 (59-63 fishes/tank), each line being kept in six tanks . Water renewal in control tank was 6 per hour and it was reduced in stressed tanks to around 0.8-1.4 renewal/h which also mean that water velocity was different between the two conditions. Fishes were fed to satiation with a floating pellet formulated by Aquaculture and Fisheries Group at Wageningen Institute of Animal Sciences (Wageningen, The Netherlands) and produced by Research Diet Service (Wijk bij Duurstede, The Netherlands).

Oxygen was continuously measured in 4 tanks for 4 weeks (one tank per conditions) (Orion equipment). Oxygen was check several times per day in all tanks. N-NH4 was measured every week in at least 4 tanks (Ammonium kit, Hach-Lange, France). CO2 was recorded from the second week 24hours per condition and per week.

Sampling for HPI axis and gill functions

Effect of water quality deterioration was measured on HPI axis and gill functions by sampling fish after 4 weeks in bad water quality. After being euthanized with a lethal dose of phenoxy-ethanol, fishes were weighed. Blood, gill, brain (hypothalamus, mid brain, telencephale, preoptic area), pituitary, interrenal, liver tissue were sampled.

Response of HPI axis was evaluated before and after an acute stress. For acute stress, fishes were confined for 2 min. at 200kg/m^3 then returned to normal condition (lower density). After 1 and 3 hours fishes were euthanized with a lethal dose of phenoxy ethanol and blood was sampled.

Plasma and tissues were stored at -20°C and at-80°C respectively until used.

Behavioral parameters

After 3 hypoxic weeks, 12 fish/group from 8 tanks (2 per experimental group) were analysed through an emotional reactivity test. Fish were individually transferred from their initial tank to another one (Ø=1m^2) and their immediate reaction was analyzed for 20 minutes using EthovisionXT software. Mean and maximum velocity (cm.s^{-1}), total distance travelled (cm), mean turn angle (degree), percentage of time spent in thigmotaxis (close to the wall) and percentage of time spent immobile were recorded.

Response of HPI axis was assessed by measuring plasma cortisol levels measured before and after the emotional reactivity test. Four tanks (one of each experimental group) were dedicated for basal blood cortisol and 12 fish per group were sampled. 30 minutes after the beginning of the acute stress, each fish was sampled for plasma cortisol levels.

Statistical analyses. The analyses were executed using the free software R 2.14.0 (http://cran.r-project.org/). Emotional reactivity data from each fish (12 fish/ treatment/ line) ata were statistically treated using a two-way ANOVA with genetic lines (A22 or R23) and experimental treatment (S or T) as independent variables. Significance level was set at $p<0.05$ and a tendency was considered when $0.05<p<0.1$. Then, post-hoc Tukey tests were performed for the between-treatments comparisons.

Plasmatic parameters

Measurement of plasma cortisol.

Steroids were extracted from 50µl plasma with ethyl acetate/cyclohexane (v/v) and the dissolved in 300 µl assay buffer (0.01M NaH2PO4, 0.01M Na2HPO4, O.9% NaCl, 0.1% gelatin, pH 7.25). Cortisol was assayed according to the method described in Auperin et al. (1997). As plasma cortisol data had no normal distribution (Kolgomorov-Smirnof test), non-parametric tests were used (n = 6). Mann-Whitney and Wilcoxon tests were used to compare experimental groups.

Ions, osmolality and urea

Plasma concentration of chloride, calcium and urea were measured using colorimetric kits (chloride with a mercuric-thiocyanate method, calcium with Arsenazo III method and urea with urease (Biolabo, France)). Osmotic pressure was analyzed using a freeze-point osmometer.

Real time RT-PCR

Total RNA from gill, brain, pituitary, interrenal, liver 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.
cDNA synthesis and real time RT-PCR were performed as described in Lucas et al (2014). Reverse transcriptase was realized from 1.5µg of RNA at 37°C for 1h using M-MLV reverse transcriptase (Promega). Real time RT-PCR was carried out on a StepOnePlus real time PCR system with SYBR-Green PCR master Mix (Applied Biosystem).

Statistical analysis

Results are presented as mean +/- S.E.M. Differences among values were assessed using various statistical analysis method indicated in the legend of each graph.

Results

Low water quality experiment: Water quality during experiment and fish growth

Oxygen and CO2 levels in control tanks were 8-10mg/l and 4-6mg/l respectively. Water renewal reduction, in stressed tanks, allowed to obtain O2 and CO2 concentrations between 3-6mg/l and 8-12mg/l respectively. Ammonia concentration was around 0.1 in control tanks and 0.3mg/l in stressed tanks. Mean temperature decreased from 14 to 10 °C during 4 weeks of experiment.
After 4 weeks of experiment, sampled fishes were weighed. Growth was significantly decreased in stressed trout compared to control for both isogenic lines.

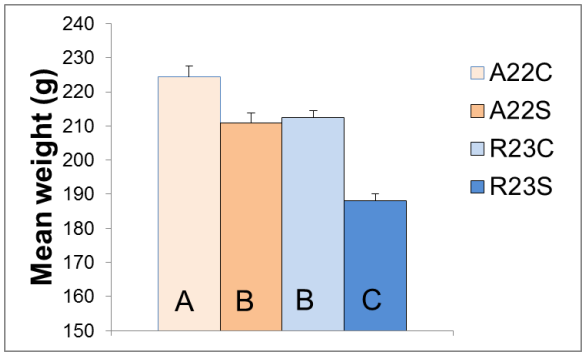


Figure 1: Mean weights of fishes after 4 weeks in bad water quality. Values are mean ± SEM of 57 fishes. Student t-test was used for statistical analysis. Column with different letters were statistically different with p<0.005. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

Behaviour

For mean velocity (Figure 2) and maximum velocity, distance travelled and percentage of time spent immobile, no significant differences appeared between treatments and isogenic lines.

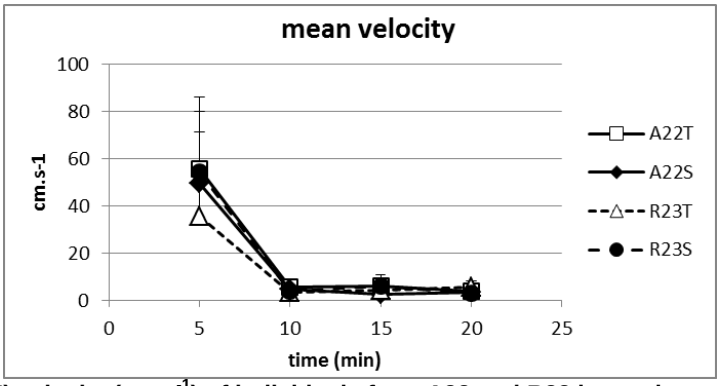


Figure 2. Mean (±SEM) velocity (cm.s^{-1}) of individuals from A22 and R23 isogenic trout lines, chronically stressed by hypoxia (S) or not (T), isolated in a novel tank for 20 minutes (n=12/treatment).

We observed a tendency for the effect of stress on thigmotaxis during the first 5-minute interval of the test (F(1,30)=2.99, p=0.09)) and during the 5-10-minute interval (F(1,30)=3.36, p=0.07)) but a significant effect was observed during the 15-20-minute interval (F(1,30)=5.34, p<0.05)), where time spent in thigmotaxis was higher in stressed groups than in control groups (cf. Figure 3).

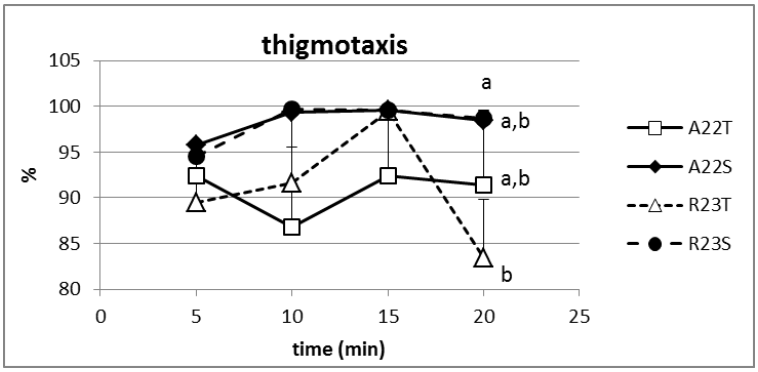


Figure 3. Mean (±SEM) percentage of time spent in thigmotaxis (against the wall) of individuals from A22 and R23 isogenic trout lines, chronically stressed by hypoxia (S) or not (T), isolated in a novel tank for 20 minutes (n=12/treatment). Time points having at least one identical letter are not significantly different by the Tukey test (p<0.05).

For the mean turn angle parameter and during the first 5-minute interval, a significant effect of isogenic lines (24 fish analyzed for each line, F(1,30)=4.33, p<0.05) and a tendency for an interaction effect (line X stress) were found (F(1,30)=3.47, p=0.07; Figure 4). The mean turn angle was higher in R23T than in A22T group (p<0.05). A line effect was also found during the 10-15-minute interval (F(1,29)=5.94, p<0.05).

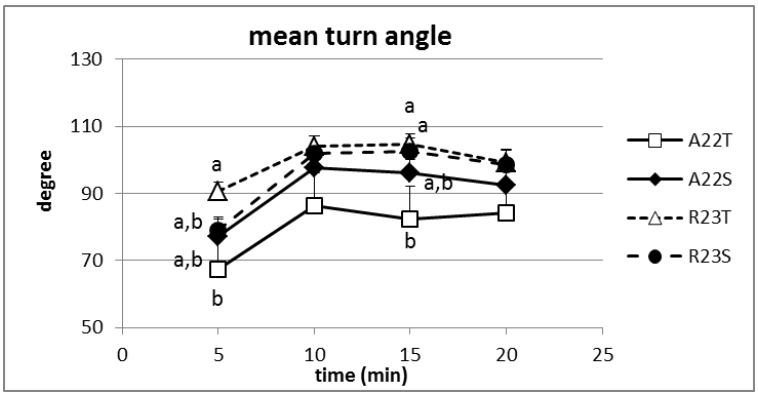


Figure 4. Mean (±SEM) turn angle (degree) of the trajectory of individuals from A22 and R23 isogenic trout lines, chronically stressed by hypoxia (S) or not (T), isolated in a novel tank for 20 minutes (n=12/treatment). Time points having at least one identical letter are not significantly different by the Tukey test (p<0.05).

Neuroendocrine responses to stress.

HPI axis responsiveness.

As shown in Deliverable D7.3, responsiveness of HPI axis to acute stress was shown to be an interesting parameter to assess chronic stress in rainbow trout. In the present study, we observed a significant increase ($p<0.05$) of plasma cortisol levels 1h and 3h after a 2 minutes confinement stress. Interestingly, the 2 lines responded differently with R23 line being highly responsive at 1h compared to A22 line (at 1h, cortisol levels are significantly different between the isogenic lines). It is also interesting to note that the shape of the cortisol response was quite different in the two lines, A22 showing a maximum cortisol level at 1h which stayed at the same level at 3h whereas a large peak was observed in R23 line at 1h which showed a significant reduction at 3h to reach cortisol level similar to those of the A22 line. Unexpectedly, at all time points (0h, 1h, 3h), we did not observe any effect of chronic exposure to poor water quality on plasma cortisol levels.

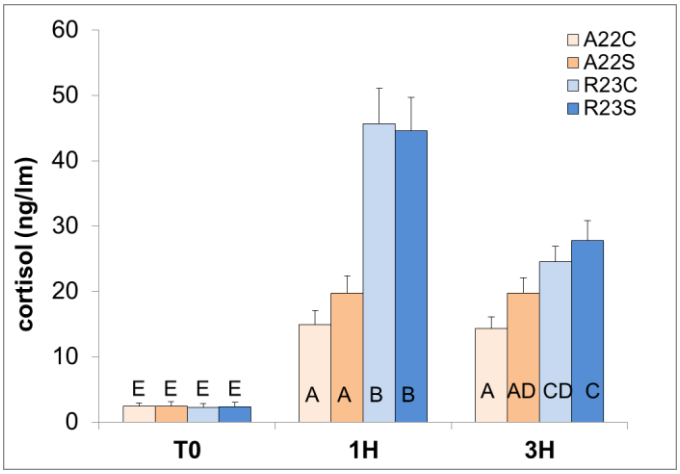


Figure 5b. Plasmatic cortisol. Values are mean ± SEM of 9 fishes. Mann-Whitney test was used for statistical analysis. Column with different letters were statistically different with at least p<0.05. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

Gene expression analysis

In order to have a refined phenotyping of HPI axis in the two isogenic lines chronically exposed or not to poor water quality, we have been measuring expression for several

candidate genes known to be the main actors pf the HPI axis in brain parts (telencephalon, preoptic area, hypothalamus) and interrenal. This included CRF, CRF-BP and corticosteroid receptors (in telencephalon, preoptic area and hypothalamus), MRC2 (ACTH receptor) , StAR and corticosteroid receptors (in interrenal). Exposure to environmental chronic stress induced a significant decrease ($p<0.05$) in A22 line of glucocorticoid receptors in interrenal (GR1 and GR2) whereas CRF-BP expression also decreased in telencephalon. In R23 line, the only significant change related to chronic stress exposure was observed in hypothalamus with a significant decrease in GR1 expression.

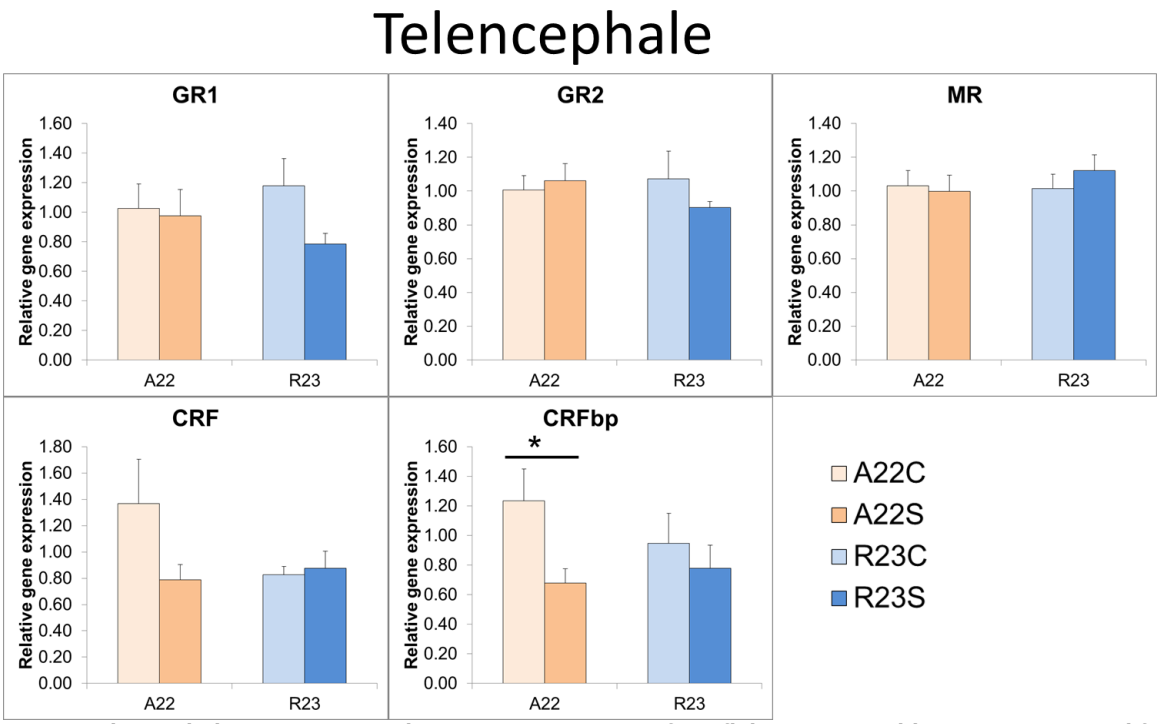


Figure 6. Telencephale responses. Values are mean ± SEM of 6-9 fishes. Mann-Whitney test was used for statistical analysis. *: p<0.05.

Pre Optic Area

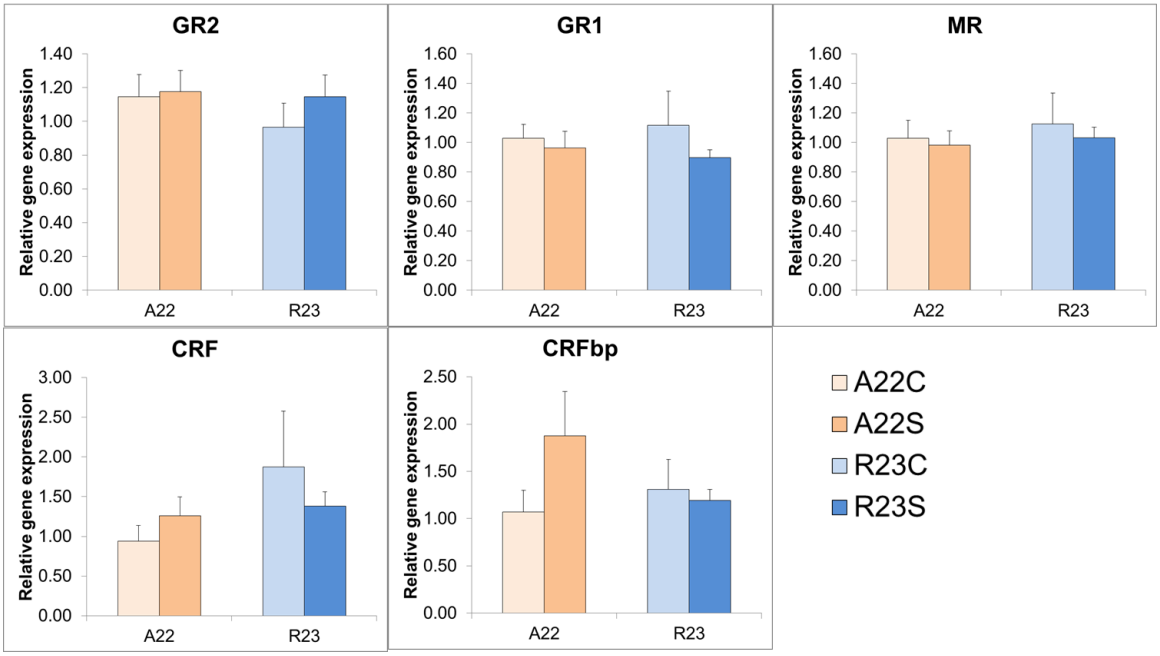


Figure 7 Pre Optic Area responses. Values are mean ± SEM of 7-9 fishes. Mann-Whitney test was used for statistical analysis.

Hypothalamus

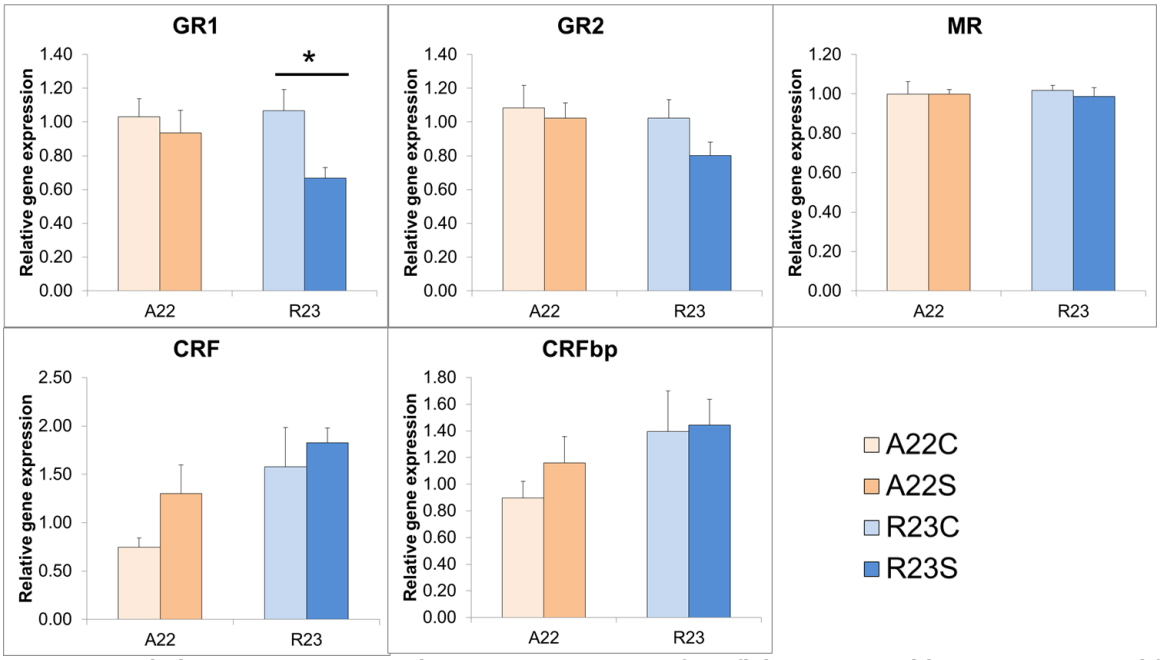


Figure 8. Hypothalamus responses. Values are mean ± SEM of 5-9 fishes. Mann-Whitney test was used for statistical analysis. *: p<0.05.

Interrenal

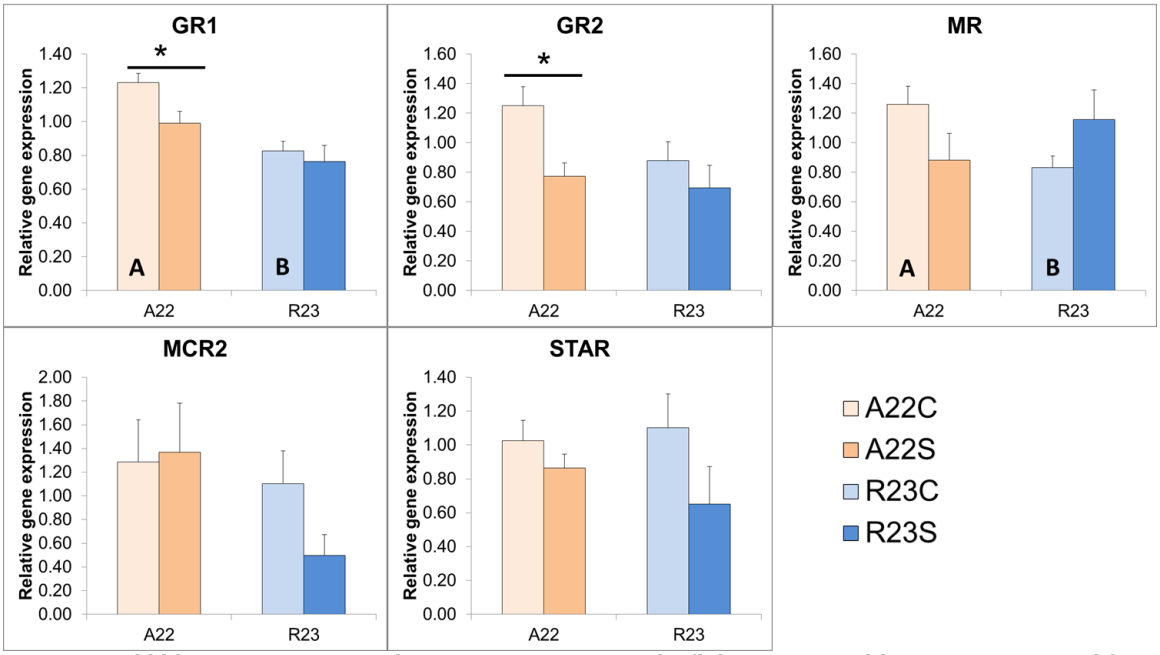


Figure 9. Head kidney responses. Values are mean ± SEM of 6 fishes. Mann-Whitney test was used for statistical analysis. *: p<0.05.

Gill functions

Plasmatic parameters

Chronic exposure to poor water quality did not modify chloride and urea concentration in plasma after 4 weeks of experiment whatever isogenic line of rainbow trout. In contrast, osmotic pressure and calcium concentration were significantly reduced after 4 weeks in bad water quality in plasma of fishes from line R23. No significant difference was measured between the two isogenic lines (figure 10).

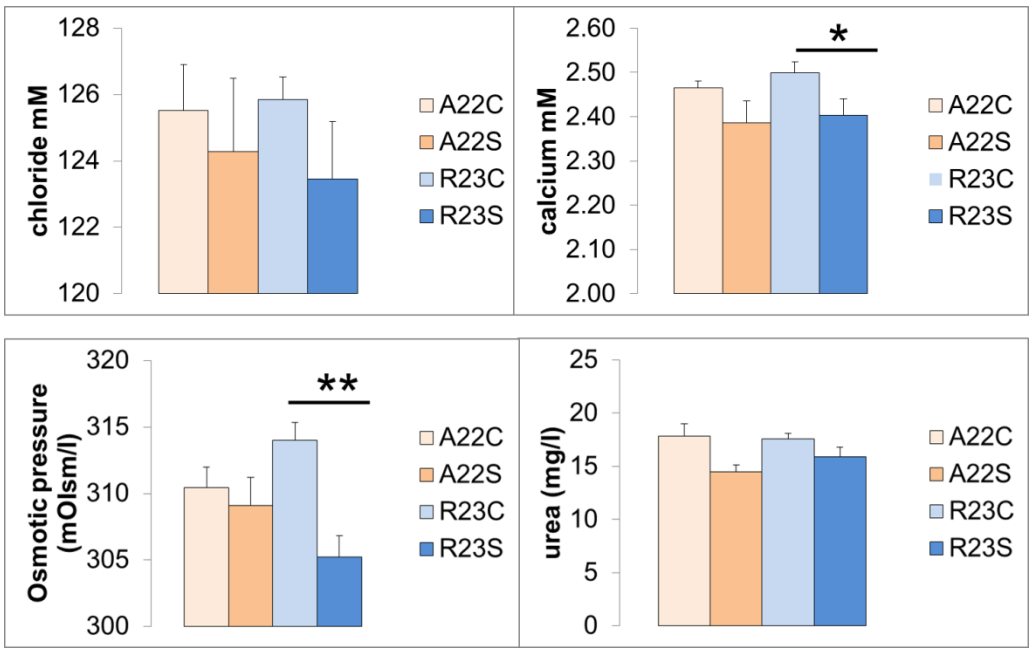


Figure 10: Plasma ion, osmotic pressure and urea concentration 4 weeks after chronic stress. Values are mean ± SEM of 9 fishes. Mann-Whitney test was used for statistical analysis. *p<0.05, **p<0.01. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

Gill gene expression

In a previous study, exposure of rainbow trout for 3 weeks to a bad water quality modified the expression in gills of several genes in relation with remodeling (apoptosis, proliferation), defense against pathogen or oxidative stress, muscle contraction and metabolism, cytoskeleton organization (see deliverable 7.3). The expression of several of these genes was measured in two isogenic lines after 4 weeks in bad water quality.

The expression of hypoxia inducible factor (HIF-1α), one important gene induced during low oxygen level, presented a higher expression in gill of stressed trout A22 and R23 (figure 11).

Gene expression of an anti-apoptotic protein (bcl2) was not significantly different between control and stressed fishes whatever isogenic line (figure 11).

Transcripts of Lactate dehydrogenase A, an enzyme involved during anaerobic metabolism, were down regulated in R23 line as in our previous study (figure 11).

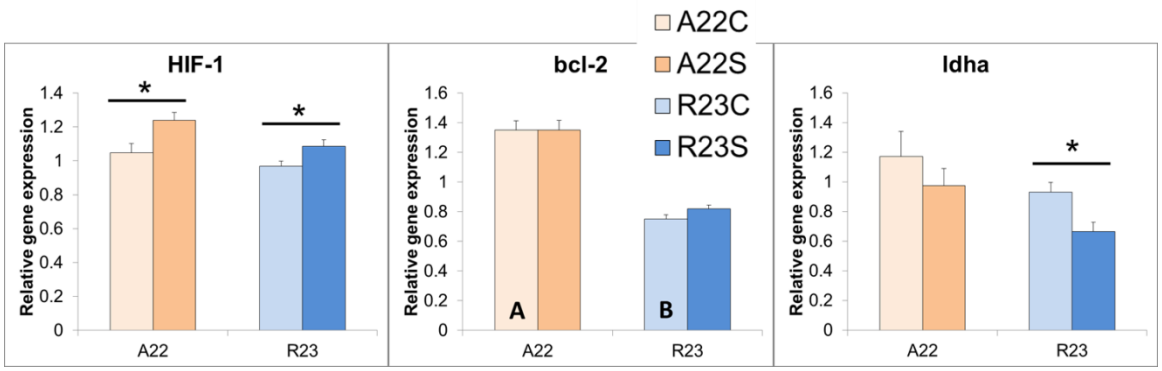


Figure 11. Some candidate genes in relation with hypoxia, apoptosis and anaerobic metabolism in gills after chronic stress. Values are mean ± SEM of 9 fishes. Mann-Whitney was used for statistical analysis, * p<0.05, letters in columns indicate significant difference between A22 and R23 fishes in control condition. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

Several transcripts of ammonia and urea transporters, implicated in nitrogen waste excretion by gill, were also measured in fish gill. Rhbg and Rhcg2 mRNA were down regulated in fish gill of A22 line exposed to bad water quality. In contrast, gene expression of urea transport (Ut) was significantly increased in R23 line after chronic stress (figure 12).

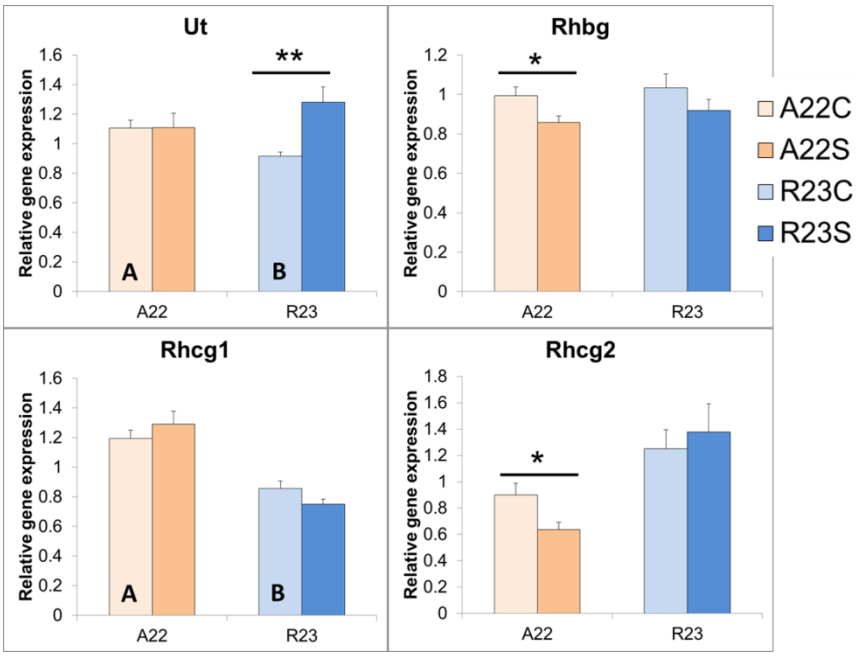


Figure 12. Some candidate genes in relation with apoptosis, hypoxia and nitrogen transporters in gills after chronic stress. Values are mean ± SEM of 9 fishes. Mann-Whitney was used for statistical analysis, * p<0.05, letters in columns indicate significant difference between A22 and R23 fishes in control condition. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

Several genes in relation to defense were also measured in two isogenic lines after 4 weeks in bad water exposure.

- First, defense against ROS (reactive Oxygen Species) was analyzed by measuring gene expression of 4 enzymes having antioxidant properties: catalase (cat), glutathione peroxidase (gpx1), superoxide dismutase in the cytoplasm (sod1) or in mitochondria (sod2). Most of genes had a similar expression in gills between control and stressed fish whatever isogenic line. Surprisingly, one enzyme presented a down regulation after 4 weeks in bad water in A22 and R23 lines: the superoxide dismutase in cytoplasm: sod1 (figure 13).

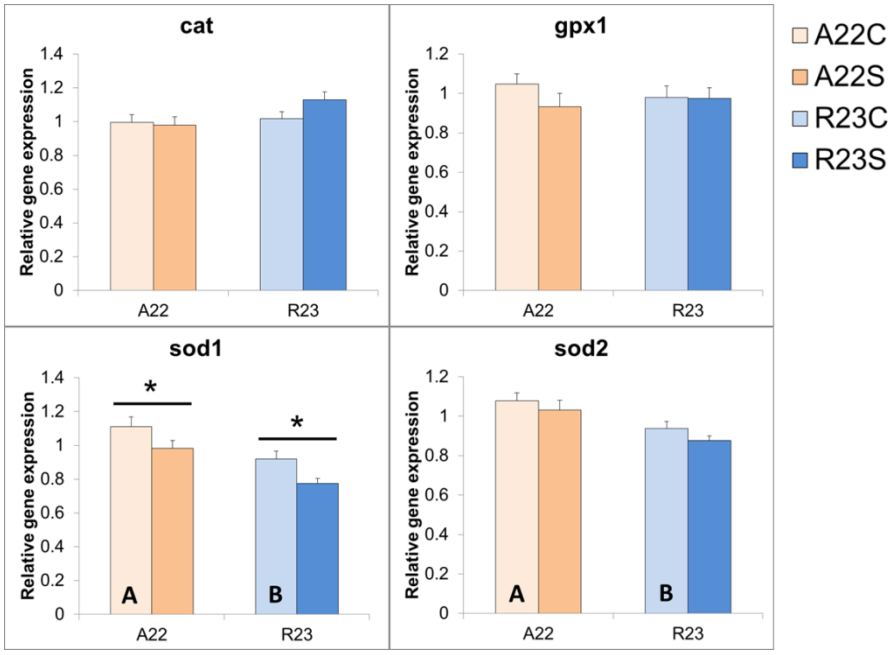


Figure 13. Some candidate genes in relation with oxidative stress in gills after chronic stress. Values are mean ± SEM of 9 fishes. Mann-Whitney was used for statistical analysis, * p<0.05, letters in columns indicate significant difference between A22 and R23 fishes in control condition. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

- Second, potential defense against pathogen was identified by measuring transcript of two genes involved in innate immune response and more particularly in inflammatory reaction: inducible nitric oxide synthase (iNOS) and complement C3. In a previous microarray analysis, these genes presented an important increase of their expression after 3 weeks in bad water condition. In this study, higher expression of iNOS and C3 were also observed after chronic stress in bad water in A22 and R23 isogenic lines (figure 14).

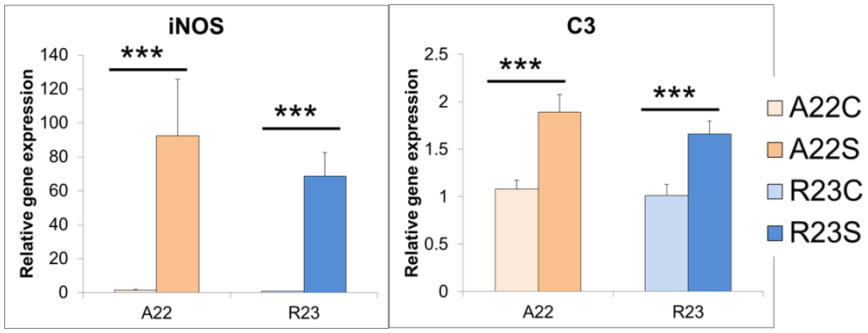


Figure 14. Some candidate genes in relation with immune system in gills after chronic stress. Values are mean ± SEM of 9 fishes. Mann-Whitney was used for statistical analysis, * p<0.05, letters in columns indicate significant difference between A22 and R23 fishes in control condition. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

Exposure to chronic stress modifying calcium concentration and osmotic pressure in plasma of fishes from R23 line, mRNA of some ion transporters was measured in gill. No significant difference for epithelium calcium channel (ECaC) and isoform α1a of Na/K-ATPase pump (Nak1a) between control and stressed fishes. However, a down regulation of gene expression of isoform α1b of Na/K-ATPase pump (Nak1b) was measured in A22 and R23 after exposure to bad water quality (figure 15).

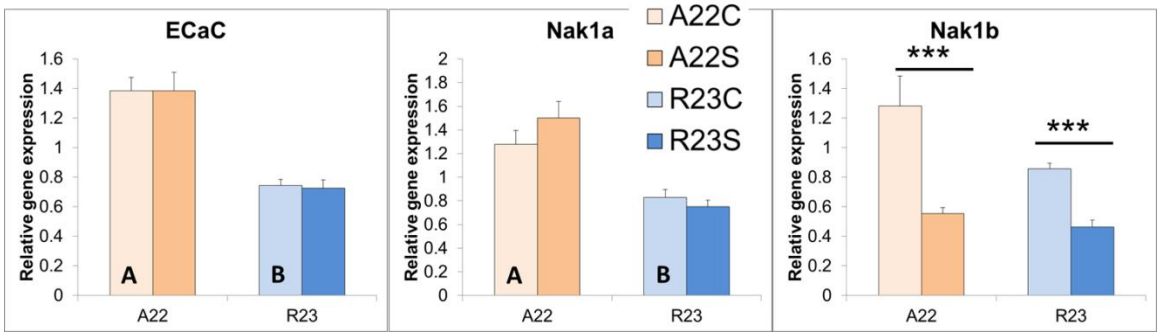


Figure 15. Some candidate genes in relation with ion transporters in gills after chronic stress. Values are mean ± SEM of 9 fishes. Mann-Whitney was used for statistical analysis, ** p<0.01, *** p<0.001, letters in columns indicate significant difference between A22 and R23 fishes in control condition. A22C: A22Control, A22S: A22Stressed, R23C: R23Control, R23S: R23Stressed

Independently of the effect of chronic stress, several genes presented a higher expression in A22 versus R23 in control condition: bcl2, ut, rhcg1, sod1, sod2, ECaC, Nak1a.

Discussion

In the present study, a chronic stress of bad water exposure was performed with two trout isogenic lines and those conditions were very similar to the stress protocol developed in deliverable D7.3. However for technical and practical problems, it was not possible to reproduce exactly the same environmental stress protocol. Experiments were performed at a different period in the year, consequently temperature was different: it increased from 10.5 to 12.5 °C for the first experiment and decreased from 14 to 10°C for the second experiment. Moreover, water renewal was also different. In control condition, it was 3 and 6 renewal per hour for the first (D7.3) and second experiment (present study) respectively. In stressed conditions, water renewal was similar in both experiments, at ~1 renewal/h. Consequently, we observed a larger differences in water quality between control and chronic stress groups in the present study although the absolute values measured for oxygen and ammonia level were quite close in the stress group of both experiments. Overall, we have been able to create an environmental chronic stress situation in both experiments and for all fish lines, exposure to poor water quality led to a significant decrease in growth.

Behavioral responses to chronic hypoxia exposure

In accordance with some authors (Destrez et al., 2013), we consider that increased fearfulness observed in animals may be the consequence of a previous chronic stress. In the present study, we have observed hypoxia-exposed and control animals from two isogenic lines, the fear responses were analyzed. We have shown a stress effect between control and chronic stress groups for the time spent in thigmotaxis with chronic stressed fish remaining more frequently against the walls of the tank. This avoidance of the exposed area indicates an increased fearfulness in this group, which can be detected in isogenic lines. In experiment 1 (deliverable D7.3) carried out on non-isogenic lines, the lack of behavioural differences in the emotional reactivity test may have been related to the treatment used (too mild hypoxia) not inducing a strong enough negative affective state to elicit behavioural changes as suggested above.

The mean turn angle observed during the emotional reactivity test was higher in R23 than in A22 line, indicating a more erratic swimming pattern in R23. Behavioural phenotypic differences have already been observed between these two isogenic lines, with a higher locomotor behaviour in R23 fish (Sadoul et al, in preparation). In accordance with this previous study, our present data showed also a line effect in plasma cortisol levels, with a HPI axis more reactive in R23 than in A22 trout after an acute stress.

In any case, the behavioral tests carried out on isogenic lines in the present study confirm what we have observed also in deliverable D7.3 using standard trout line, i.e. behavioral responses can be indicative of a chronic stress status related to exposure to poor quality water.

HPI axis responses

The present study was aiming to confirm the previous results on the response of HPI axis to chronic poor water quality exposure. The first experiment (sub-task 7.1.2, deliverable D7.3) was carried out in standard trout line. The present work was developed using two isogenic trout lines showing divergent responses to stress in previous studies (Guerden et al., 2013; Sadoul et al., in preparation; Sadoul et al., 2015). Our objectives were to confirm and precise the multi-parameters approach developped in the first experiment using fish having the same

genome and expressing quite different stress response phenotypes. Our results only partially agree with previous data obtained in experiment 1 (task 7.1.2):

- ***Results which are in agreement between the experiments 1 (subtask 7.1.2) and 2 (present study):***
  In both studies, plasma cortisol levels in fish chronically exposed to poor water quality are not significantly different from control: This is observed in the present study with the two isogenic lines as it was also reported with standard fish line in sub-task 7.1.2 (deliverable D7.3). This absence of chronic environmental stress on cortisol does not agree with previous studies on chronic stress and cortisol (Piato et al., 2011; Tudorache et al., 2013) but these experiments were carried out on another fish species and with a different chronic stress protocol. Conversely, our results agree with Pickering and Stewart (1984) despite the fact that they used chronic confinement stress with normal water. Overall, these results and our present data indicate that plasma cortisol level cannot be taken as a good indicator of chronic stress status, at least in rainbow trout.

- ***Results which are in partial disagreement between experiment 1 and experiment 2:***
  Whereas HPI axis responsiveness to acute stress (2 minutes confinement) was stimulated after 3 weeks exposure to poor water quality in experiment 1, this effect of chronic environmental stress was not observed in the present study in both isogenic lines. . In any case, this suggest us, that HPI axis responsiveness to acute stress as assessed by measurement of plasma cortisol levels cannot be considered as a unique criteria for assessment of chronic stress status in rainbow trout. This why we have developed analysis of molecular markers of the HPI axis.

- ***Results which are not agreeing between experiment 1 and experiment 2***: When analyzing expression of candidates genes which are main regulator of the HPI axis in the present study, we did not find the same results as observed in experiment 1 (not the same genes in the same organs). More surprising is that we observed in experiment 2 a <u>decrease</u> of expression for a few genes in ithe hypothalamus and interrenal whereas in experiment 1, we observed an <u>increase</u> in expression for a few genes in the interrenal. Finally, when we compare expression of these candidate genes between the 2 isogenic lines, we did not find any clear significant differences, whereas these 2 lines express a clear difference in HPI axis responsiveness. This suggests us that maybe we have not been analyzing the right candidate genes which control HPI axis responsiveness in our experiment. Further studies would be necessary for finding genes of which expression would be more relevant of HPI axis responsiveness.

<u>Gill functions</u>

Phenotyping of two trout isogenic lines with physiological and transcriptomic tools, already used in the project (sub-task 7.1.2, deliverable 7.3), allowed to compare two experiments of fish exposed chronically to bad water quality and to determine the importance of genetic in stress response.

- <u>Results in accordance between the two experiments whatever fish line</u>

Gene expression in gills of hypoxia inducible factor (HIF-1α), inducible nitric oxide synthase (iNOS) and complement C3 was upregulated after chronic stress. Interestingly, these transcripts/proteins are known to increase after hypoxia but also after exposure to pathogens (Soitamo et al 2001; Douxfils et al. 2012, Elks et al. 2013; Palazon et

al 2014). Furthermore, HIF-1α protein was also upregulated in other situations (presence of metal (Heerden et al. 2004), low temperature (Rissanen et al 2006)). In contrast, a chronic exposure of zebrafish to hypoxia induced a down regulation of few immune genes in gills (van der Meer et al. 2005). However in this last study, hypoxia was the only stressor. In our condition, closer to what occurs in freshwater aquaculture systems, hypoxia was also associated to higher CO2 and NH4 levels. Furthermore, low water renewal can be also responsible of higher concentration of pathogens such as bacteria in water. Consequently these transcripts differentially expressed in our two experiments (Deliverable 7.3 and this study) are very interesting genes to follow in chronic situation implicating hypoxia and/or pathogen exposure and possibly others stressors. In the first experiment (D7.3), using a microarray approach, others immune genes have been up-regulated in chronic stress situation. Further analysis will be necessary to determine which kind of immune pathway was activated: innate and/or adapted?

- Results in accordance with previous study but only for one fish line

In the present study, fish line A22 showed a significant difference between control and stressed fish; after 4 weeks in bad water quality; for some ammonia transporters: rhbg and rhcg2. In the previous study (see D7.3), the same transcripts of ammonia transporters (rhbg and rhcg2) appeared down regulated between 2 and 21 days in bad water quality, however without a significant difference between control and stressed fishes. This reduction can be induced by the need to limit ammonia entry via gills. **Fish line A22 seems more sensitive to external ammonia concentration than R23** and trout used in the first experiment (see D7.3). However for this last group, ammonia stress was not so important: chronic stress was 3 weeks (D7.3) against 4 weeks for fish line and the difference of ammonia concentration between control and stressed tank was inferior due to a higher difference in water renewal (for D7.3 in control tanks: 3 renewal/h, in stressed tank ~0.8-1 renewal/h, for fish line study in control tanks: 6 renewal/h, in stressed tank ~0.8-1.4 renewal/h).

Gene expression of lactate dehydrogenase A (glycolytic enzyme involved during anaerobia) was down regulated after 4 weeks in bad water condition in fish of R23 line. Similarly, this down regulation was also observed in our previous experiment (see D7.3). In contrast, transcripts of ldha were up regulated after hypoxia in *Gillichthys mirabilis*. However, it was only 6 hours of hypoxia (no other stress), on liver tissue and with a euryoxic species (Gracey et al. 2001). In our conditions, stressors were multiple for 4 weeks; oxygen level was not constant, fluctuating between 3 and 6mg/l, and trout is a hypoxia-intolerant species. This decrease of lactate dehydrogenase A can be an adaptive response to our chronic stress, however further analysis of our microarray experiment (see D7.3) and more qPCR will be necessary to understand metabolic ways modified after chronic stress. Anyway, **in contrast to fish of A22 line, those of R23 line can modulate their responses to chronic stress by reducing some pathways of glycolysis**.

- Results in disagreement with previous study but only for one fish line

At the plasma level, calcium concentration and osmotic pressure were significantly reduced after 4 weeks in bad water condition for fish of R23 line only. **These results suggest that R23 line has more difficulty to maintain their calcium homeostasis and osmotic pressure after chronic stress**. At mRNA level, expression of epithelial calcium channel (ECaC) was not significantly different between control and stressed fish. However, the expression being higher for A22 versus R23, this can explain the difference measured after chronic stress. To look for the impact on osmotic pressure, expression of gene expression of two isoforms of Na/K-ATPase pump was measured. The activity of this ionic pump, involved in osmoregulation, was significantly reduced in Amazonian cichlid after 20hours in severe

hypoxia (Richards et al 2007). Expression of Nak1a was not modified after chronic stress but it was higher for A22 vs R23line, this can explain the difference in osmotic pressure response after chronic stress. Interestingly, in the present study, gene expression of Nak1b was significantly reduced after chronic stress whatever fish line. In previous study (see D7.3), Nak1b being absent from microarray slide it was not identified as involved after chronic stress. It will be interesting to look for this gene by qPCR in the first study.

- Results in disagreement with previous study whatever fish line

Gene expression responses of antioxidant and antiapoptotic proteins to chonic exposure to bad water quality were different between our previous experiment (sub-task 7.1.2, D7.3) and this study with fish lines. Consequently, we decided that these pathways should not deserve more investigation in fish exposed to such chronic stress situation. In the first experiment glutathione peroxidase 1 and superoxide dismutase 2 were up and down regulated respectively. With fish lines, superoxide dismutase 1 was down regulated. In the two experiments, catalase mRNA was not modified. Response to an antiapoptotic gene was also different between the two experiments: down regulation after chronic stress in the previous study and no different expression after stress with fish lines.

Conclusion:

The main objective of this experiment was to assess new phenotyping methods developed in D7.3 for characterizing chronic stress in rainbow trout and to see whether these markers would give consistent results using two isogenic lines. Overall, these approaches allowed us i) to confirm the importance to have a multi-parameters approach for studying such biological situation and the relevance of parameters associated with gill functions and behavior ii) to highlight the limits of using HPI axis responsiveness and molecular markers of HPI axis for characterizing chronic stress.

In that context, the main conclusions of the present study are:

- A multi-parameter approach is necessary to have a relevant assessment of chronic stress status in rainbow trout exposed to poor water quality.
- Behavioral test can be used for detecting environmental chronic stress status within a multi-parameters approach.
- Gills are also an interesting tissue for detecting gene expression changes in chronically stressed rainbow trout exposed to poor water quality.
- HPI axis responsiveness may not be always relevant for assessment of chronic stress.
- Isogenic trout lines R23 and A22 express significantly different phenotypes: i) their HPI axis responsiveness to acute confinement stress are different, R23 behaving as a ‘high’ responsive fish line compared to A22 ii) R23 has more difficulty to maintain their calcium homeostasis and osmotic pressure after chronic stress iii) Fish line A22 seems more sensible to external ammonia concentration than R23 iv) R23 line can modulate its responses to chronic stress by reducing some pathway of glycolysis.

General conclusion.

The experiments conducted in the present study were 3 cases studies aiming to phenotype complex traits related to growth performance or to health and welfare. New phenotyping methods have been used in these studies which have been developed on trout isogenic lines, where all fish within a line are genetically uniform: This gave us highly standardized

experimental conditions by either removing genetic variation (experiments with one line) or partitioning it only among lines (experiments with two or more lines) giving ideal conditions to identify genotype by environment interaction. The main informations we can take out of these cases studies are:

i) Despite the fact that we have not been able in all experiments to compare different isogenic lines, this biological material appeared to be well suited for analyzing complex situations such as effects of feed abundance during first-feeding, investigation of the "oxystatic" theory or characterization of chronic effects of poor water quality.

ii) The first two cases studies were related to phenotyping of growth performance under nutritional stress (feed restriction during first-feeding, impact of dietary CAD) and used a large sub-set of biochemical, physiological and endocrine parameters in order to determine phenotypic effects of such nutritional stressors. In addition to classical parameters related to growth performances, we have been using metabolic chamber units which lead us to measure nitrogen and energy partitioning associated with metabolic parameters at organ levels. Overall, the present results of these two case studies provide in-depth phenotypic characterization of growth performance and information on the metabolic pathways/process which have been impacted by nutritional stress.

iii) The third case study is interesting in the fact that experiments were carried out on two isogenic trout lines which clearly show different phenotypic characteristics in control conditions (different acute stress responsiveness, different Ca++homeostasis after chronic stress, different sensitivity to external ammonia). Exposure of these two lines to chronic stress conditions during several weeks highlight interesting points:

- Despite these phenotypic differences related to genome structure, chronic environmental stressful challenge (poor water quality) has similar effects on expression of several genes related to response or hypoxia (HIF-1α, iNOS, complement C3). These results were not only obtained in the above experiment with the 2 isogenic lines but also in another similar experiment carried out in April 2014 on a standard trout line (cf. D7.3 report). This clearly suggest that gills are an important target tissue for detecting chronic environmental stress (i.e. related to water quality) and that further studies should be developed to establish sets of non-lethal biomarkers using gill biopsies.

- Our general approach for characterizing chronic stress was to develop a multi-parameters analysis based on the measurement of various biological functions, i.e. behaviour, osmoregulation metabolism, stress endocrinology. Data obtained in the present experiment and also a previous chronic stress experiment (cf. D7.3 report) do confirm the importance : However, as indicated in figure 16, when comparing these 2 sets of experiments carried out at two different periods and with different trout lines (D7.3 and D7.5), parameters related to HPI axis responsiveness or related to behavior did not agree: in April 2014 (D7.3), chronic exposure to poor water quality significantly increased HPI axis responsiveness (i.e. cortisol levels), an observation we did not get in December 2014 when using two isogenic lines. Conversely, exposure to low water quality modified fearfulness in two isogenic trout lines (present report) whereas similar treatment applied last April on a standard trout line did not modify behavioral parameters in emotional reactivity test. However, when looking at the literature, these two sets of parameters (cortisol analysis and behavioral measurement) do respond strongly when considering other kind of chronic stressors,

(i.e. repeated acute stressors, dominance, confinement, pollutants… Grassie et al., 2013; Berneire et al., 2008; Madoro et al., 2015). In that context, we suggest that the nature and the duration of the chronic stressor is important and may have variable effects depending with biomarkers of chronic stress one is measuring. In that context, we consider that multi-parameters approach for characterizing chronic stress in a fish experiment is more than necessary.

	Behavioral parameters	**gill parameters**	**corticotrope axis parameters**
Standard trout line chronic exposure to poor water (April 2014)	Emotional reactivity: **no change**	1) Genes related to hypoxia: **increase** 2) antioxidant and antiapoptotic genes: **no changes**	plasma cortisol after acute stress: **increase**
isogenic lines trout lines exposed to poor water quality (December 2014)	fearfulness test: **increase**	1) Genes related to hypoxia: **increase** 2) antioxidant and antiapoptotic genes: **no changes**	plasma cortisol after acute stress: **no change**

Figure 16: Summary table presenting responses of biological parameters (behavior, gill and corticotrope axis) in rainbow trout exposed to chronic poor water quality during two experiments (April 2014 – cf? D7.3 report- and December 2014 – present report).

References

Auperin, B., Baroiller, J.F., Ricordel, M.J., Fostier, A., Prunet, P., 1997. Effect of confinement stress on circulating levels of growth hormone and two prolactins in freshwater-adapted tilapia (Oreochromis niloticus). Gen Comp Endocrinol 108, 35-44.

Bernier N.J., Alderman S.L., Bristow E.N. 2008. Head or tails? Stressor-specific expression of corticotropin-releasing fatcor and urotensin I in the preoptic area and caudal neurosecretory system of rainbow trout. J. Endocrinol. 196, 637-648.

Destrez, A., Deiss, V., Leterrier, C., Boivin, X., Boissy, A., 2013. Long-term exposure to unpredictable and uncontrollable aversive events alters fearfulness in sheep. Animal 7, 476-484.

Douxfils, J., Deprez, M., Mandiki, S.N.M., Milla, S., Henrotte, E., Mathieu, C., Silvestre, F., Vandecan, M., Rougeot, C., Mélard, C., Dieu, M., Raes, M., Kestemont, P., 2012. Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication – Clues to physiological acclimation and humoral immune modulations. Fish & shellfish immunology 33, 1112-1122.

Elks, P.M., Brizee, S., van der Vaart, M., Walmsley, S.R., van Eeden, F.J., Renshaw, S.A., Meijer, A.H., 2013. Hypoxia inducible factor signaling modulates susceptibility to mycobacterial infection via a nitric oxide dependent mechanism. PLoS Pathog 9, e1003789.
Geurden, I., Borchert, P., Balasubramanian, M.N., Schrama, J.W., Dupont-Nivet, M., Quillet, E., Kaushik, S.J., Panserat, S., Medale, F., 2013. The Positive Impact of the Early-Feeding of a Plant-Based Diet on Its Future Acceptance and Utilisation in Rainbow Trout. PloS one 8.
Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. Proceedings of the National Academy of Sciences of the United States of America 98, 1993-1998.
Grassie C., Braithwaite V.A., Nilsson J., Nilsen T.O., Teien H.C., Handeland S.O., Stefansson S.O., Tronci V., Gorissen M., Flik G., Ebbesson L.O.E. 2013. Aluminium exposure impacts brain plasticity and behavior in Atlantic salmon. J. Exp. Biol. 216, 3148-3155.
Lucas, J., Perrichon, P., Nouhaud, M., Audras, A., Leguen, I., Lefrancois, C., 2014. Aerobic metabolism and cardiac activity in the descendants of zebrafish exposed to pyrolytic polycyclic aromatic hydrocarbons. Environmental science and pollution research international 21, 13888-13897.
Madaro A., Olsen R.E., Kristiansen T.S., Ebbesson L.O.E., Nilsen T.O., Flik G., Gorissen M. 2015. Stress in Atlantic salmon: response to unpredictable chronic stress. J. Exp. Biol. (in press).
Olsen, R.E., Sundell, K., Hansen, T., Hemre, G.I., Myklebust, R., Mayhew, T.M., Ringo, E., 2002. Acute stress alters the intestinal lining of Atlantic salmon, Salmo salar L.: An electron microscopical study. Fish Physiology and Biochemistry 26, 211-221.
Palazon, A., Goldrath, A.W., Nizet, V., Johnson, R.S., 2014. HIF transcription factors, inflammation, and immunity. Immunity 41, 518-528.
Piato, A.L., Capiotti, K.M., Tamborski, A.R., Oses, J.P., Barcellos, L.J.G., Bogo, M.R., Lara, D.R., Vianna, M.R., Bonan, C.D., 2011. Unpredictable chronic stress model in zebrafish (Danio rerio): Behavioral and physiological responses. Progress in neuro-psychopharmacology & biological psychiatry 35, 561-567.
Pickering, A.D., Stewart, A. (1984). Acclimation of the interrenal tissue of the brown trout, Salmo trutta L., to chronic crowding stress. J. Fish. Biol. 24: 731-740.
Richards, J.G., Wang, Y.S., Brauner, C.J., Gonzalez, R.J., Patrick, M.L., Schulte, P.M., Choppari-Gomes, A.R., Almeida-Val, V.M., Val, A.L., 2007. Metabolic and ionoregulatory responses of the Amazonian cichlid, Astronotus ocellatus, to severe hypoxia. Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology 177, 361-374.
Rissanen, E., Tranberg, H.K., Sollid, J., Nilsson, G.E., Nikinmaa, M., 2006. Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (Carassius carassius). The Journal of experimental biology 209, 994-1003.
Rouger, Y., Aubin, J., Breton, B., Fauconneau, B., Fostier, A., Le Bail, P.Y., Loir, M., Prunet, P., Maisse, G., 1998. Réponse au stress induit par le transport chez la truite arc-en-ciel (Oncorhynchus mykiss). Bulletin Français de la Pêche et de la Pisciculture, 511-519.
Sadoul B, Foucard A, Valotaire C, Prunet P, Dupont-Nivet M, Quillet E, Médale F, Colson V. Behavioural and endocrinal effects of a plant-based diet in two isogenic rainbow trout lines (in preparation).
Soitamo, A.J., Rabergh, C.M., Gassmann, M., Sistonen, L., Nikinmaa, M., 2001. Characterization of a hypoxia-inducible factor (HIF-1alpha) from rainbow trout. Accumulation

of protein occurs at normal venous oxygen tension. The Journal of biological chemistry 276, 19699-19705.
Tudorache, C., Schaaf, M.J., Slabbekoorn, H., 2013. Covariation between behaviour and physiology indicators of coping style in zebrafish (Danio rerio). The Journal of endocrinology 219, 251-258.
van der Meer, D.L., van den Thillart, G.E., Witte, F., de Bakker, M.A., Besser, J., Richardson, M.K., Spaink, H.P., Leito, J.T., Bagowski, C.P., 2005. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. American journal of physiology. Regulatory, integrative and comparative physiology 289, R1512-1519.
van Heerden, D., Vosloo, A., Nikinmaa, M., 2004. Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor-1alpha (HIF-1alpha) levels in rainbow trout (Oncorhynchus mykiss). Aquatic toxicology 69, 271-280.