The effect of plant-based diet and suboptimal environmental conditions on digestive function and diet-induced enteropathy in rainbow trout (*Oncorhynchus mykiss*)

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Plant-based diet at suboptimal environment

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Abstract

This experiment investigated intestinal enteropathy and digestive function of rainbow trout challenged with soybean meal-based diet (SBM) at optimal or suboptimal environments created by normal or reduced water flow, respectively. Oxygen level remained above 7 mg L^{-1} for optimal environment and between 4 to 5 mg L^{-1} for suboptimal environment. Triplicate groups of fish (mean body weight 74.1 g) were fed fishmeal-based diet (FM) or SBM at optimal environment in period 1 (28 days). In period 2 (42 days), fish were subjected to a change from FM to SBM or remained on the same diet as used in period 1. The fish were also exposed to change from optimal to suboptimal environment or remained under optimal conditions. The fish subjected to change from FM to SBM, regardless of their environment, showed similar degree of enteropathy from day 14. Lipid and starch digestibility was lower in SBM-fed fish at suboptimal environment compared to fish fed the same diet at optimal environment. Crude protein digestibility, however, was highest in SBM-fed fish at suboptimal environment throughout period 2. In conclusion, in SBM-fed rainbow trout, exposure to suboptimal environment did not change the degree of enteropathy, however, lipid and starch digestibility were further reduced.

Introduction

The use of plant ingredients in salmonid feeds to improve sustainability of aquaculture, may lead to challenges including impaired digestive function, reduced growth, and increased risk of developing gastro-intestinal disorders such as soybean meal-induced enteritis (SBMIE). The negative effects of plant ingredients are attributed to the presence of non-starch polysaccharides (NSP) and anti-nutritional factors (ANF). SBM has been used as a model to study the effect of plant ingredients on gut health and function of salmonids (Krogdahl *et al.,* 2003; Romarheim *et al.,* 2008; Urán *et al.,* 2008; Mosberian-Tanha *et al.,* 2016). The inclusion of SBM has shown to adversely affect the apparent digestibility coefficients (ADC) of nutrients and energy (Opstvedt *et al.,* 2003; Romarheim *et al.,* 2006). Furthermore, it has been shown that SBM can reduce activity of digestive enzymes in the distal intestine (DI) of Atlantic salmon (*Salmo salar*) (Krogdahl *et al.,* 2003; Chikwati *et al.,* 2013). The reduced activity of digestive function may partly be due to the morphological changes caused by SBMIE. Although DI is not the main site for macronutrient absorption, some important components such as taurine and bile acids have been shown to be re-absorbed in the DI (Nordrum *et al.,* 2000) with possible implications for the absorption of lipid in the proximal parts of the intestine. Morphological changes associated with SBMIE may disturb the capacity of digestion and re-absorption of nutritionally important substances in the DI and thus contribute to the lower ADC of nutrients. ADC of lipid in particular has shown to be reduced in Atlantic salmon fed SBM (Krogdahl *et al.,* 2003; Romarheim *et al.,* 2006). Changes in digestive function appears to be a more sensitive parameter than changes in the gut morphology as observed in Atlantic cod (*Gadus morhua*), where feeding SBM reduced lipid digestibility (Førde-Skjærvik *et al.,* 2006) in the absence of SBMIE (Refstie *et al.,* 2006).

Aquaculture is also facing challenges from the environment. Sub-optimal environmental conditions are partly caused by seasonal changes in water temperature and consequently dissolved oxygen (DO) (Oppedal *et al.,* 2011) or on a long-term basis by global warming leading to alterations in water quality parameters such as increased temperature and CO_2 level (Lough & Hobday 2011). However, the adverse conditions may also be induced by some production procedures such as reduced water flow/exchange rate in intensive fish farming (Ellis *et al.*, 2002). Water DO level is one of the important environmental factors affected by change in temperature or water flow rate. Low water DO level may induce environmental hypoxia with physiological consequences in fish (Wu 2002). Adverse effect of low water DO on feed intake and growth has been reported in Nile tilapia (*Oreochromis niloticus*) (Tran-Duy *et al.,* 2012) and rainbow trout (Glencross 2009). Exposure of the fish to low DO level resulted in impaired intestinal barrier function and also induced morphological changes in the distal intestine in Atlantic salmon (Sundh *et al.,* 2010). Reduced water flow rate is not only associated with stress or low water DO but also increased accumulation of fish excretions such as ammonia in the ambient water (Ellis *et al.,* 2002). High ambient ammonia concentration has been reported to reduce feed intake and increase mortality in juvenile lake trout (*Salvelinus namayeush*) (Beamish & Tandler 1990) and under chronic exposure it also causes gill damage and hyperplasia (Meade 1985). In contrary, in another experiment, chronic exposure to sublethal levels of ammonia did not change feed intake in Atlantic salmon kept at 12 ºC (Kolarevic *et al.*, 2013).

It is not known how the combination of a suboptimal environment (such as hypoxia) and a plant-based diet (such as SBM-based diet) may affect digestive function and intestinal health in rainbow trout. In an experiment, changes in the intestinal morphology induced by dietary plant

ingredients was found to be aggravated in Nile tilapia (*Oreochromis niloticus*) kept at hypoxia (Tran-Ngoc *et al.,* 2016). In the current experiment, it is hypothesised that the effect of a dietary challenge on gut morphology and digestive function may be aggravated when rainbow trout is exposed to a challenging environment. This experiment was, therefore, conducted to evaluate if exposure to hypoxia (induced by lowering the water flow rate) will aggravate the effect of a SBM-based diet as a dietary challenge on digestive function and intestinal morphology of rainbow trout.

Materials and methods

The experiment was performed in accordance with the Dutch law on the use of experimental animals and approved by the ethical committee of Wageningen University for animal experiments (DEC: 2014006.a).

Fish and rearing conditions

Six hundred juvenile rainbow trout with mean initial body weight (± SE) of 74.1 ± 0.3g were randomly allocated into 12 tanks (50 fish per tank) supplied with freshwater at the start of the experiment. The tanks were all connected to a recirculation system which allowed on-line measurement of actual and cumulative water flow per tanks, oxygen concentration, temperature, pH and conductivity. The details of measurement units and water sampling is described elsewhere (Saravanan *et al.,* 2012).

Two isoenergetic and isonitrogenous diets were formulated; one fishmeal-based control (FM) and one containing 400 g kg^{-1} soybean meal (SBM) as experimental diet. Cellulose was added to the diets as a filler. Yttrium oxide (Y_2O_3) was added to the diets as inert marker for digestibility calculations (Austreng *et al.,* 2000). The formulation and composition of the diets

are shown in Table 1. The ingredients were ground in a hammer mill (Condux LHM20/16, Hanau, Germany) fitted with a 1-mm sieve. The diets were produced by Research Diet Service (Wijk bij Duurstede, The Netherlands) by using a twin-screw extruder (Clextral, Firminy, France) equipped with a 3 mm die. The pellets were then dried in a tray-drier at 70 ˚C for 3 hours and cooled to ambient temperature. Restrictive feeding was used to ensure that the fish in all treatment groups consume the same amounts of feed, thus, the same amount of SBM as a dietary challenge. The intention was to exclude the effect of feeding level on the degree of SBMIE and ADC values. The feeding rate was 1.5% of mean biomass of 12 tanks during period 1 and was reduced to 1.25% at the start of period 2. Each diet was assigned randomly to triplicate tanks (200 L capacity) according to the treatments and fed to the fish manually twice daily throughout the experiment at 9:00 and 16:00 hours for maximum 1 hour. The water flow rate was set at 7.5 L min^{-1} for all tanks during period 1. Photoperiod was maintained at 12 L: 12 D, water temperature at 14.0±0.5˚C and pH between 7.0 and 7.5 throughout the experiment.

Experimental design

The experiment consisted of four treatments and divided into two periods; Period 1; was adaptation period of 28 days to diets and all fish were kept under optimal conditions by setting the water flow rate at 7.5 L min^{-1} and Period 2; an experimental period of 42 days where fish were subjected to either a dietary challenge and/or exposed to suboptimal environment by reducing the water flow rate from 7.5 L min^{-1} to 2.25 L min^{-1}. Water DO level is the key limiting factor when the water flow rate is reduced, however, this treatment also leads to accumulation of metabolites or fish excretions such as ammonia. To simplify nomenclature, low water flow

rate is termed hypoxia (HY) and optimal water flow rate is termed normoxia (NO). Thus, the four treatments tested in this experiment are as follows:

Treatment 1: Period 1, FM at normoxia → Period 2, FM at hypoxia (FMNO → FMHY)

Treatment 2: Period 1, FM at normoxia → Period 2, SBM at hypoxia (FMNO → SBMHY)

Treatment 3: Period 1, FM at normoxia → Period 2, SBM at normoxia (FMNO → SBMNO)

Treatment 4: Period 1, SBM at normoxia → Period 2, SBM at hypoxia (SBMNO → SBMHY)

Treatment 1 was designed to evaluate if exposure to hypoxia alone would affect digestive function and impair intestinal health. Treatments 2 and 3 were designed to evaluate if change from FM to SBM is more detrimental to digestive function and SBMIE, as an indicator of diet-induced enteropathy, at hypoxia compared to normoxia. Treatment 4 was designed to evaluate if under steady state dietary challenge any change in the environment from normoxia to hypoxia will aggravate digestive function and SBMIE.

Normoxia resulted in a mean water DO level of above 8 mg L^{-1} in the outlet (>78% saturation). If necessary, pure oxygen was injected into the inlet to maintain the intended DO level. Hypoxia resulted in a mean water DO level of below 6 mg L^{-1} in the outlet (< 55% saturation). The minimum DO level in the outlet, however, was maintained above 3.8 mg L^{-1} to avoid extreme reduction in feed intake and increased mortality. For this purpose pure oxygen was injected into the inlet water. The mean of DO level (mean ±SD) in the inlet was 10.3±0.3 mg L^{-1}. Water parameters including daily oxygen concentration and pH and also during week five of period 2, total ammonium nitrogen (TAN), nitrite and nitrate were measured for each tank by the method described elsewhere (Saravanan *et al.,* 2012).

Sampling procedure

Faeces collection was performed daily throughout the last two weeks of the period 1 and pooled to determine digestibility of nutrients in this period. The faeces collection continued throughout period 2 at four sampling time points, days 0-7, 8-14, 15-21 and 22-42 (faeces samples collected daily and were pooled within these periods). Each tank was connected to one settling tank as previously described (Saravanan *et al.*, 2012). A faecal collection bottle (250 ml) was attached to the bottom of the settling tank while placed in a thermostatic box connected to a cooling system to avoid the bacterial degradation of nutrients in the faeces. The faeces collected within weeks from each tank was pooled in the same tray and stored at -20˚C in an aluminium box until further analysis. The settling tank was also used to check and count the uneaten pellets in the respective respiration tank at every feeding for accurate calculation of feed intake. For this purpose another set of 250 ml-bottles were attached to the settling tanks during feeding.

DI tissue samples from 3 fish were taken per tank on days 0, 7, 14, 21 and 42 of period 2. The tissue samples were fixed in neutral buffered formalin (4% formaldehyde) and embedded in paraffin before staining by hematoxylin and eosin (H&E). Blinded evaluation and scoring of the following five morphological parameters was performed on each tissue:

1) Subepithelial infiltration of leukocytes: increased accumulation of leukocytes in the subepithelial area down to stratum compactum.
2) Supranuclear vacuolisation (SNV) of epithelial cells: reduced vacuolisation of the epithelial cells.
3) Atrophy of intestinal folds.

4) Vacuolar degeneration of the epithelial cells: increased vacuolar degeneration in the base of the intestinal folds.
5) The presence, if any, of granulomatous change and the degree of this change: increased accumulation of fibroblasts, macrophages and presence of giant cells in the subepithelial area.

A score was given to each parameter which ranged from 0 to 3. Increase in the score of each parameter indicates a more severe morphological changes. The overall histopathology score for each fish was calculated by taking the average score of the morphological parameters to express the degree of change in that individual.

Analytical procedure

Feed and oven-dried faeces samples were ground in a blender before further analysis. Dry matter was determined by drying the samples for 4 hours at 103 ˚C until a constant weight was obtained. Crude protein was determined by the Kjeldahl method based on N content × 6.25 (ISO 5983/NEN 3145). Feed and faecal samples were hydrolysed by 3N HCl before crude fat analysis as described in Saravanan *et al.,* (2012). Crude fat content was measured following petroleum-ether extraction (Soxhlet method). Gross energy content was determined using a bomb calorimeter (IKA-C7000, IKA-Aanalysentechnik, Weitersheim, Germany). Gross ash was determined after combustion of dried samples in a muffle furnace at 550 ˚C (ISO 5984/NEN 3323). Yttrium was measured by inductively coupled plasma mass spectrometry (ICP-MS) after acid digestion of feeds and faeces. Starch content was determined enzymatically as glucose, liberated by α-amylase and amyloglucosidase hydrolysis (AOAC Method 996.11).

Calculations and statistics

Apparent digestibility coefficients (ADC, %) were calculated as:

$$ADC_X = (1-Y_{diet}/Y_{faeces} \times X_{faeces}/X_{diet}) \times 100$$

where X represents dry matter, crude protein, crude lipid, starch or energy, Y_{diet} and Y_{faeces} represent the yttrium concentrations in the diet and faeces, respectively, and X_{diet} and X_{faeces} are the concentrations of X in the diet and faeces respectively.

Feed conversion ratio was calculated as:

$$FCR = \text{Feed intake (g, DM)} \times \text{fish weight gain (g)}^{-1}$$

Daily feed intake is expressed per kg current body weight (BW_n): daily feed intake (g DM) divided by BW_n.

BW_n was calculated as: $BW_n = BW_{n-1} + (\text{daily feed intake, g DM} \times FCR^{-1})$.

Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA). All data were tested for normality and homogeneity by Kolmogorov-Smirnov and Bartlett tests. Data from ADC of dry matter in period 1 and overall histopathological score violated the normal distribution assumption after log10-transformation; and thus these data were subjected to non-parametric Kruskal-Wallis test followed by multiple pairwise comparisons (Dwass-Steel-Critchlow-Fligner) if the test was significant. ADC of crude protein, lipid, starch, ash and energy were subjected to one-way analysis of variance (ANOVA) in GLM procedure to test the effect of diet in period 1. The effect of treatment and sampling time on ADC of dry matter, crude protein and ash in period 2 was analysed using a two-way ANOVA in GLM procedure. ADC of lipid and starch at the end of period 2 (day 22-42) were subjected to a one-way ANOVA. Least square means comparison was used to determine which groups differed significantly from

each other. Regression analysis was performed to determine the variables that correlated with feed intake at the end of period 2. Differences were declared statistically significant if $P < 0.05$.

Results

Water quality parameters

The water pH level remained stable (ranged from 7.0 to 7.5) throughout the experiment (period 1 and 2) for all treatment groups and hypoxia did not change the pH level ($P>0.05$). The fluctuations in pH was too small to have had a significant effect on toxicity of TAN. The water DO level (expressed as mg L^{-1}) was above 7.0 mg L^{-1} during period 1 in all tanks (Fig. 1). At the start of period 2, water DO level was reduced to below 5 mg L^{-1} immediately after reduction of water flow rate in the tanks assigned to hypoxia and remained between 4 to 5 mg L^{-1} during this period (Fig. 2). Peaks were observed, however, on the oxygen curve corresponding to the DI tissue sampling days. The mean water concentration of TAN during week five of period 2 was significantly higher at hypoxia compared to that observed at normoxia (P=0.002) (Fig. 3). During the same week, water level of nitrite and nitrate at hypoxia were 0.008±0.0003 and 0.22±0.015 mg N L^{-1} (mean ± SE, n=9 tanks) respectively. At normoxia the concentrations were 0.007 ±0.0009 and 0.18±0.016 mg N L^{-1} (mean ± SE, n=3 tanks). The difference in concentration of nitrite and nitrate was insignificant among treatments.

Feed intake and growth

Feed intake (g kg^{-1} body weight) of FM-and SBM-fed fish remained stable throughout period 1 (Fig. 1). The mean feed intake (g $fish^{-1}$ day^{-1}) over period 1 was not changed significantly in

response to diet (P>0.05) (Table 3). In period 1, there was no significant difference in weight gain (g $fish^{-1}$ day^{-1}) of fish fed FM and SBM diet (1.70 vs. 1.66).

Feed intake of all treatment groups was not significantly changed during period 2, however, it was reduced in fish fed FM and SBM diets and kept at hypoxia during the last two weeks of period 2 (Fig. 2). Feed intake in fish subjected to change from FM to SBM at normoxia (FMNO → SBMNO) in period 2 remained unchanged for the whole period. The mean daily feed intake (g $fish^{-1}$ day^{-1}) over period 2 was significantly higher in the fish fed SBM at normoxia (FMNO → SBMNO) than that in other treatment groups (i.e. fish kept at hypoxia) (P=0.014).

Regression analysis revealed that feed intake showed reduction with increasing TAN concentration (R^2= 0.45, P=0.02) (Fig. 4). However, no significant relation was found between changes in feed intake and water DO level (R^2=0.25, P=0.1), pH (R^2=0.15, P=0.21), ADC of crude protein (R^2=0.08, P=0.36), dry matter (R^2=0.15, P=0.20), lipid (R^2=0.01, P=0.72) and starch (R^2=0.03, P=0.58) at the end of period 2.

Histopathological evaluation

The changes in histopathological scores over time are shown in Fig. 5. These changes were confined to the distal intestine and characterised by reduced apical SNV, reduced height of simple and complex intestinal folds (partial atrophy), and increased number of leukocytes (e.g. lymphocytes, granulocytes and eosinophilic granular cells) in the subepithelial area, the degree of vacuolar degeneration in the base of the folds and the degree of granulomatous change, if present. Exact mean histopathological scores for all treatment groups are given in Table S1.

Exposure to hypoxia did not exert adverse effect on morphological changes in fish fed FM throughout the experiment (steady state diet), but exposed to hypoxia during period 2 (FMNO

→ FMHY) (P>0.05). Fish fed the SBM diet during period 1, however, developed SBMIE in the DI. The degree of SBMIE remained unchanged in this treatment group over time during period 2 where the fish was exposed to hypoxia (SBMNO → SBMHY) (P>0.05). The two groups of fish subjected to change from FM to SBM, regardless of their environment (i.e. FMNO → SBMHY and FMNO → SBMNO) showed similarly increased histopathological score over time in period 2. By day 14, they reached the same degree of SBMIE as in fish fed SBM throughout the experiment but exposed to suboptimal condition (SBMNO → SBMHY) (Fig. 5). Thus, the degree of SBMIE was stable and similar from day 14 onwards among fish challenged with SBM during period 2, regardless of their environmental conditions.

Digestibility

There was no significant effect of diets on the ADC of starch in period 1 (Table 2), however, ADC of lipid was reduced in fish fed SBM compared to the fish fed FM (P=0.0001). The effect of treatments on ADC of dry matter, crude protein, ash and energy during period 1 are shown in Table 2. ADC of crude protein, ash and energy was higher in fish fed SBM (P <0.05) compared with those fed the FM diets, while the ADC of dry matter tended to increase in these fish (P=0.08).

During period 2, there was no significant difference in any of the ADC values of the fish subjected to change from FM to SBM diet and exposed to hypoxia (FMNO → SBMHY) and of the fish subjected to hypoxia and fed SBM diet throughout the experiment (SBMNO → SBMHY). The fish subjected simultaneously to changes in diet and environment (FMNO → SBMHY) and the fish fed SBM continuously (steady state), but subjected to hypoxia in period 2 (SBMNO → SBMHY) showed the lowest ADC of lipid and starch at the end of period 2 (Fig. 6). ADC of lipid and starch were highest in the group fed FM throughout the experiment, but

exposed to hypoxia (FMNO → FMHY) (P=0.001). ADC values of lipid and starch were higher in the fish subjected to dietary change from FM to SBM and kept at normoxia (FMNO → SBMNO) than in the fish fed SBM and exposed to hypoxia during period 2 (FMNO → SBMHY and SBMNO → SBMHY) (P=0.002). In the fish fed FM throughout the experiment but exposed to hypoxia in period 2 (FMNO → FMHY), the ADC of dry matter reached its highest value by day 42. ADC of dry matter, was, however, gradually reduced from day 7 to 21 in the fish subjected to changes in both diet and environment (FMNO → SBMHY). Similar trend was also observed in the fish challenged by SBM but kept at normoxia (FMNO → SBMNO). There were, however, no differences in ADC of dry matter among any groups challenged by SBM regardless of the type of the environment by day 42. ADC of crude protein and ash in all treatment groups remained unchanged throughout period 2. ADC of crude protein was, however, highest in groups fed SBM at hypoxia (FMNO → SBMHY and SBMNO → SBMHY) at all time points and lowest in fish fed FM (steady state), but subjected to change to hypoxia (FMNO → FMHY). At hypoxia, changing from FM to SBM increased the ADC of ash significantly at day 7 compared to steady state FM feeding (FMNO → FMHY). The difference in ADC of ash was insignificant among treatments by day 42. ADC of energy was found to be highest in the fish challenged by SBM and kept at normoxia (FMNO → SBMNO) (P=0.01), however, no significant difference was observed among other treatments (P >0.05). The interaction between treatments and sampling time was not significant (P >0.05) (Table 4).

Discussion

This study was performed to investigate if exposure to suboptimal environment (i.e. hypoxia) aggravates the effect of SBM on digestive function and intestinal enteropathy in rainbow trout

over time. We evaluated the gastrointestinal status by monitoring digestive function and progression of SBMIE in rainbow trout in response to the challenges over time.

It is known that oxygen is less available to aquatic than air-breathing animals and the uptake of oxygen from water is more challenging (Kramer 1987). Thus, it is likely that reduction of DO level in this study was a challenging factor. We observed that the fish activity (locomotion) was lower in the hypoxia tanks. This is in accordance with previous observations of Nile tilapia kept at different degrees of hypoxia (Tran-Duy *et al.*, 2012). Reduced activity of the fish could be a response to reduced DO level as a mechanism of adaptation (Kramer 1987). Reduction in feed intake is another response which is reported to occur under hypoxic conditions (Tran-Duy *et al.*, 2012) as feed intake is an oxygen demanding process. In this study, however, the feed intake during the four weeks after exposure to hypoxia remained unchanged in all treatment groups, indicating that low DO level did not affect feed intake. Fish were fed restrictively which may explain why the low DO level did not adversely affect feed intake. Glencross (2009) reported that feed intake under hypoxia did not differ from normoxia when fish were fed restrictively for 28 days. The reduction in feed intake during the last two weeks of period 2, however, could be a response to accumulation of ammonia due to the reduced water flow rate. Previous publications have reported adverse effect of elevated environmental ammonia level on feed intake in rainbow trout (Ortega *et al.*, 2005) and European sea bass (*Dicentrarchus labrax*) (Dosdat *et al.*, 2003) and juvenile lake trout (*Salvelinus namayeush*) (Beamish & Tandler 1990). The highest TAN concentration in this study was well below the levels tested in those experiments, however, the slight but significant accumulation of ammonia may have been a challenging factor to the fish already affected by reduced DO level at hypoxia. Thus, it is possible that the combination of increased TAN and reduced water DO level caused reduction

in feed intake in this experiment. Kolarevic *et al.,* (2013) also showed that exposure to sublethal levels of TAN at normoxic condition did not change feed intake significantly in Atlantic salmon.

The development of SBMIE in rainbow trout fed the SBM diet during period 1 was expected and coincided with previous findings (Baeverfjord & Krogdahl 1996; Romarheim *et al.,* 2008). Exposure to hypoxia in this experiment did not aggravate SBMIE in fish fed SBM. The lack of interactive effect between SBM and hypoxia in period 2 could be due to the high inclusion level of SBM (400 g kg^{-1}) used in the present experiment leading to histopathology score of 2 or higher in all fish from day 14. Thus it was difficult to evaluate the impact of additional environmental challenge induced by reduced water flow on intestinal health. Furthermore, feeding FM at hypoxia did not result in any signs of inflammation in the DI of rainbow trout. Sundh *et al.,* (2010) reported atrophy of intestinal folds in Atlantic salmon kept at hypoxia and temperature of 16 ºC (corresponding to 50% saturation). It is possible that rainbow trout is more resistant to the adverse change in the environmental conditions such as hypoxia than Atlantic salmon. SBM diet, however, induced significant morphological changes after 7 days of period 2 in fish subjected to SBM independent of the environment, which is in agreement with the study in Atlantic salmon (Urán *et al.,* 2009). At day 14 and onward, all SBM-fed fish had similar histopathology score regardless of their environment, implying that there was no effect of feed intake, steady state SBM consumption and suboptimal conditions (reduced water flow rate) on this parameter, even at longer time of exposure.

The reduction in ADC of lipid in fish fed SBM compared to the fish fed FM in period 1 confirms previous reports (Refstie *et al.,* 1998; Romarheim *et al.,* 2006; Øverland *et al.,* 2009). This trend was also observed 42 days after the change from the FM to SBM diet at normoxia and hypoxia.

The ADC of starch in this study was close to the values previously reported in rainbow trout (Krogdahl *et al.*, 2004; Romarheim *et al.,* 2006). Earlier publications have shown that starch can be highly digestible for carnivorous fish after hydrothermal treatment of the feed resulting in starch gelatinisation (Bergot & Breque 1983; Panserat 2009). Furthermore, lower intake of dietary starch under restrictive feeding has also reported to improve ADC of starch (Bergot & Breque 1983). The fact that ADC of starch did not differ significantly between SBM and FM during period 1 is in accordance with some earlier studies (Romarheim *et al.*, 2006; Romarheim *et al.,* 2012). The further reduction in ADC of lipid and starch in two groups of fish kept at hypoxia and fed SBM (steady state and subject to change from FM to SBM), suggests that there is an adverse additive effect of dietary challenge and suboptimal environment in the present study on digestive function of the fish. The degree of SBMIE did not differ between hypoxia- and normoxia-treated fish. This indicates that the changes in ADC of lipid and starch is independent of SBMIE. A possible explanation is that reduced activity of the fish at hypoxia, may have led to slower gastrointestinal peristaltic movement than that at normoxia, which consequently increased the interaction time of lipids and starch with ANFs including NSPs in SBM diet. This in turn aggravated the adverse effect of ANFs on ADC of these nutrients. There are different types of ANFs in SBM, the function of which are not yet fully understood (Francis *et al.,* 2001). Some fraction of ANFs may interact with components essential for lipid digestion and reduce the ADC of lipid. An example is saponins which have been suggested to reduce lipase activity, leading to reduced ADC of lipid (Han *et al.*, 2000). NSPs may also reduce digestibility of different nutrients such as starch by increasing the viscosity of the digesta (Leenhouwers *et al.,* 2006) or reducing brush border enzymes activity and bile acid concentration (Kraugerud *et al.*, 2007). Another explanation is that at hypoxia, reduced water

DO level contributed to further reduction in ADC of lipid due to higher oxygen demand of dietary lipids for oxidation. On the other hand, storage of dietary starch energy in the form of body fat is also more oxygen demanding than deposit of dietary fat (Reeds *et al.,* 1982). The suboptimal environment may also have increased the interactions between carbohydrates and lipids in the GIT, resulting in amylose-lipid complexes, which has shown to increase resistance of amylose to α-amylase (Holm *et al.,* 1983). Overall this result also indicates that digestive function is more sensitive than the DI enteropathy in rainbow trout exposed to a dietary challenge under suboptimal conditions.

The lower ADC of crude protein in fish fed the FM diet during period 1 compared to the fish fed the SBM diet contradicts previous results (Øverland *et al.,* 2009). Cellulose inclusion level was relatively high in the FM diet, but Hansen & Storebakken (2007) showed that cellulose does not affect ADC of protein, lipid and starch. Reduced ADC of FM compared to SBM may be due to the faeces collection method used in this experiment. In this experiment faeces was collected in bottles mounted to the settling tanks and remained in the bottle for 23 hours which may result in leaching of nutrients. Leaching has been discussed previously as a problem associated with the use of columns for faeces collection (Storebakken *et al.,* 1998; Vandenberg & De La Noüe 2001). The same method of faeces collection was used in this experiment for all treatment groups, however, leaching rate of nitrogen may differ for different diets. Physical and chemical properties of the faecal matter from SBM diet is different from that of FM diet. For example SBM diet has shown to contain less dry matter due to diarrhea (Refstie *et al.,* 2000; Refstie *et al.,* 2005). The properties of faecal matter from SBM diet may have resulted in a higher rate of nitrogen leaching than for that for FM diet. This proposed effect of faeces collection method, however, was not reflected in ADC of starch and lipid. The observed stability in ADC of crude

393 protein during the first four weeks of period 2 may be explained by the stable feed intake during
394 this period. However, reduction of feed intake during the last two weeks of period 2 did not
395 affect ADC of crude protein in fish kept at hypoxia regardless of the diet. The finding is in
396 accordance with a previous report of no change in ADC of crude protein in European sea bass
397 with chronic exposure to high water TAN level (Dosdat *et al.*, 2003).

398 The higher ADC of dry matter and energy in fish fed the SBM diet in the present experiment
399 may be a result of the high inclusion level of cellulose in the FM diet. The results are in
400 agreement with Glencross *et al.,* (2012) whom also showed reduced ADC of dry matter and
401 energy with higher percentage of cellulose in diet. However, the results show no significant
402 difference in ADC of energy after 42 days of feeding in period 2 among the fish fed FM and
403 SBM (steady state and subject to change from FM to SBM) at hypoxia. The reason for this
404 observation may be the overall result of lower ADC of lipid and starch in fish subjected to SBM
405 at hypoxia and reduced ADC of dry matter and crude protein in the fish fed FM at the same
406 environment.

407

408 **Conclusions**

409 To conclude, the suboptimal environment used in this experiment did not induce or aggravate
410 the changes associated with SBMIE or adversely affect the ADC of nutrients in rainbow trout.
411 However, fish subjected to the dietary challenge at suboptimal environment showed further
412 reduction in digestibility of starch and lipid without change in the degree of SBMIE when
413 compared to the fish exposed to dietary challenge alone. These results indicate that there was

414 an interaction between feeding plant-based diets and exposure to suboptimal environmental
415 condition on digestive function of rainbow trout.

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Figure legends

Fig. 1. Daily feed intake (g kg^{-1} body weight) of rainbow trout (*Oncorhynchus mykiss*) (means ± SE) fed fish meal (FM) or soybean meal (SBM) and kept at normoxia (high water flow rate) in period 1. Each data point on diet curves, is the mean of three tanks for one day. The DO level is the mean of all tanks (*n*=12).

Fig. 2. Feed intake (g kg^{-1} body weight) of rainbow trout (*Oncorhynchus mykiss*) (means ± SE) subjected to change in diet and/or environment (hypoxia) in period 2. (A) Treatment groups subjected to challenging environment (hypoxia). One treatment remained on the fish meal (FM) diet supplied in period 1 (steady state dietary condition) (FMNO → FMHY). One treatment group was subjected to change from FM diet to soybean meal (SBM) diet (FMNO→ SBMHY) and another treatment remained on SBM diet (steady state dietary challenge) (SBMNO→SBMHY). (B) Treatment group kept at normoxia. Fish in this group was subjected to change from FM diet supplied in period 1 to SBM diet (FMNO→SBMNO) in period 2. Each data point is the diet mean of three tanks for one day. The DO line in (A) is the mean of all low flow tanks in period 2 (*n*=9) and in (B) is the mean of high flow tanks (*n*=3).

Fig. 3. Total ammonia nitrogen (TAN) level in each treatment group during week five of period 2. Ambient TAN level increased (*P*=0.002) in the three treatments exposed to hypoxia regardless of their dietary regimen. Values are mean (*n*=3) ± SE.

Fig. 4. The regression of water total ammonia nitrogen (TAN) level against feed intake (g kg^{-1} body weight) during week five of period 2.

Fig. 5. Morphological changes in the distal intestine of rainbow trout (*Oncorhynchus mykiss*) (*n*=9 fish treatment^{-1}) over time. Scores are based on average of the five parameters used in evaluation of SBMIE; sub-epithelium infiltration of leukocytes, supranuclear vacuolisation of apical epithelial cells, atrophy of intestinal folds and the degree of basal-fold vacuolar degeneration and granuloma. Fish was challenged with soybean meal and/or hypoxia during period 2. NO, normoxia; HY, hypoxia. Values at day 0 are histopathological scores at the end of period 1.

Fig. 6. Apparent digestibility of starch and lipid of rainbow trout (*Oncorhynchus mykiss*) subjected to change in diet and/or hypoxia at the end of period 2. Values are means (*n*=3) ± SE.

Table 1 Diet formulation and chemical composition of experimental diets fed to rainbow trout (*Oncorhynchus mykiss*)

	FM	SBM
Ingredients (g kg^{-1})		
Fish meal [a]	540.0	250.0
Soybean meal [b]	-	400.0
Wheat flour [c]	170.0	140.0
Rapeseed oil	100.0	120.9
Fish oil [d]	40.0	40.0
Cellulose	143.4	30.0
Monocalciumphosphate [e]	-	10.0
DL-methionine [f]	-	2.5
Yttrium oxide [g]	0.1	0.1
Vitamin/mineral premix [h]	6.5	6.5
Proximate analysis		
Dry matter (g kg^{-1})	949	957
Crude protein (g kg^{-1})	430	427
Crude lipid (g kg^{-1})	206	220
Non-starch polysaccharides (g kg^{-1}) [i]	155	164
Starch (g kg^{-1})	130	113
Ash (g kg^{-1})	79	76
Gross energy (MJ kg^{-1})	23.0	23.2

FM, fishmeal; SBM, soybean meal

[a] TripleNine Fish Protein, Esbjerg, Denmark.

[b] Cargill, Amsterdam, The Netherlands.

[c] Meneba, Weert, The Netherlands.

[d] Coppens International, Helmond, The Netherlands.

[e] Tessenderlo Chemie, Rotterdam, The Netherlands.

[f] Evonik Industries AG, Hanau, Germany.

[g] Sigma–Aldrich, USA.

[h] Vitamin/mineral premix provided (kg^{-1} diet): α- tocopherol acetate, 100 IU; sodium menadione bisulphate, 10 mg; retinyl acetate, 3000 IU; cholecalciferol, 2400 IU; thiamin, 10 mg; riboflavin, 10 mg; pyridoxine, 10 mg; nicotinic acid, 20 mg; folic acid, 2 mg; ascorbyl phosphate,100 mg; inositol, 400 mg; biotin, 0.2 mg; pantothenic acid, 40 mg; cyanocobalamin, 0.015 mg; choline chloride, 2000 mg; anti-oxidant BHT (E300-321), 100 mg; calcium propionate, 1000 mg; Fe (as $FeSO_4.7H_2O$), 50 mg; Zn (as $ZnSO_4.7H_2O$), 30 mg; Co (as $CoSO_4.7H_2O$), 0·1 mg; Cu (as $CuSO_4.5H_2O$), 10 mg; Se (as Na_2SeO_3), 0.5 mg; Mn (as $MnSO_4.4H_2O$), 20 mg; Mg (as $MgSO_4.7H_2O$), 500 mg; Cr (as $CrCl_3.6H_2O$), 1 mg; I (as $CaIO_3.6H_2O$), 2 mg.

[i] Calculated non-starch polysaccharides=1000- (crude protein+ crude lipid+ starch+ ash).

Table 2 Apparent digestibility coefficients (ADC, %) of nutrients and energy of rainbow trout (*Oncorhynchus mykiss*) subjected to change in diet and/or environment [1]

Treatments	FMNO → FMHY	FMNO→ SBMHY	FMNO→ SBMNO	SBMNO→ SBMHY	Pooled SEM
Dry matter					
Period 1[2,3]	72.3	72.1	72.0	79.2	2.5
Period 2					
Day 7	$74.6^{B,c}$	$82.6^{A,a}$	$81.7^{AB,a}$	$80.3^{B,b}$	2.3
Day 14	$75.5^{B,c}$	$81.7^{B,a}$	$81.1^{AB,ab}$	$79.7^{B,b}$	0.8
Day 21	$75.9^{B,b}$	$80.6^{C,a}$	$80.7^{B,a}$	$80.7^{AB,a}$	0.7
Day 42	$78.0^{A,b}$	$81.9^{AB,a}$	$82.8^{A,a}$	$82.1^{A,a}$	0.5
Crude protein					
Period 1[2]	92.9^{b}	92.7^{b}	92.2^{b}	94.3^{a}	0.6
Period 2					
Day 7	94.3^{b}	96.2^{a}	95.0^{b}	95.9^{a}	0.4
Day 14	94.4^{b}	95.9^{a}	94.8^{ab}	95.7^{a}	0.5
Day 21	94.4^{c}	95.7^{a}	94.6^{bc}	95.5^{ab}	0.5
Day 42	94.3^{b}	95.8^{a}	95.1^{ab}	95.5^{a}	0.4
Ash					
Period 1[2]	51.2^{b}	50.9^{b}	50.5^{b}	57.8^{a}	0.9
Period 2					
Day 7	$52.1^{B,b}$	$57.8^{B,a}$	56.6^{a}	59.0^{a}	1.1
Day 14	55.8^{AB}	58.1^{B}	57.3	59.1	1.5
Day 21	56.1^{AB}	57.9^{B}	57.4	58.8	1.5
Day 42	57.9^{A}	60.4^{A}	58.6	60.2	1.3
Gross energy [4]					
Period 1[2]	80.5^{b}	80.0^{b}	80.2^{b}	83.4^{a}	0.7
Period 2					
Day 42	84.2^{b}	84.3^{b}	86.2^{a}	84.7^{ab}	0.6
Starch					
Period 1[2]	89.4	89.6	90.0	89.2	1.3
Lipid					

Period 1[2]	93.7[a]	93.5[a]	94.8[a]	89.0[b]	1.2

[1] Values represent the means (n=3) with pooled SEM. Means in a row with different lower case letters indicate significant difference among treatments in period 1 (one-way ANOVA, $P < 0.05$) and in period 2 (two-way ANOVA, $P < 0.05$). Means in each column with different capital letters indicate significant difference over time during period 2 within a treatment (two-way ANOVA, $P < 0.05$). One-way ANOVA was used for data from ADC of gross energy at the end of period 2. ADC of starch and lipid for the end of period 2 (day 22-42) are presented in Fig. 6 (one-way ANOVA).

[2] Fish were fed either fish meal (FM) or soybean meal (SBM) for 4 weeks during period 1.

[3] A Kruskal-Wallis one-way ANOVA was used for ADC of dry matter in period 1.

[4] Mean of gross energy digestibility coefficient includes the effect of cellulose inclusion as an inert ingredient.