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Abstract

Feeds and feeding constitute the major part of costs in intensive aquaculture. Any action to reduce feeding costs without negatively affecting fish production parameters and flesh quality would improve profitability of farming. Therefore, we studied the effects of feeding frequency on production parameters, stomach capacity and nutritional value of muscle in juvenile rainbow trout (Oncorhynchus mykiss) in an experiment with two stages. First, during the nine-week “starvation period” we fed rainbow trout (initial weight c. 40 g) with four different feeding protocols in an attempt to adapt the fish to a progressive decrease in the number of feeding days. During the second stage, a four-week “re-feeding period”, all fish were fed in excess on weekdays. Fish growth, feed intake, stomach size, and biomolecule content of muscle were monitored as response variables. During the starvation period, feed intake and growth decreased along with the number of feeding days. Compensatory growth during the refeeding was either only modest or absent. The fish in the starved groups were unable to significantly increase their stomach capacities. Starvation and re-feeding had only a slight effect on muscle fatty acid and amino acid composition. The used feeding protocols did not affect important production parameters (e.g. feed conversion or size variation). Our results suggest that despite differences in fish growth starvation and re-feeding hardly affect the nutritional value of fish. It is possible that several decades in captivity have made rainbow trout incapable to adjust their stomach size in respect to feeding frequency.

Keywords	Compensatory growth, stomach volume, salmonids, fatty acids, amino acids, feed conversion ratio
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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Dear Editor

> *We have gone carefully through the referees' comments and corrections and made the changes as suggested. However, we did not make every single suggestion of the reviewer 2 what s/he had made in the edited ms version, as we regarded some of those as opinions that can be taken into account.*

-Reviewer 1

- The manuscript was remarkably improved by the authors by including polynomial contrasts, but there are still some minor linguistic issues. I tried to edit them in the main text in track changes mode. My only suggestion is that the authors should benefit from the power of trend analysis in discussion section rather than relying on ANOVA results. Then, the manuscript is acceptable after a minor revision in my opinion.

> *We have made all suggested linguistic corrections as suggested by the referee. We have modified discussion by utilizing the results of the polynomial contrast analyses.*

-Reviewer 2

- Dear Editor,

The manuscript titled "Effects of progressive decrease of feeding frequency and re-feeding on production parameters, stomach capacity and muscle nutritional value in rainbow trout (Oncorhynchus mykiss)" is an original study of practical interest for fish farming.

According to the previous revision, the manuscript has been improved in many aspects.

As was previously commented, results obtained are not the expected but experimental design and methodology is consistent being results suitably discussed. Although the discussion may seem initially extensive, it is read fluently and is well supported by bibliographic references.

According to not expected results, maybe the authors should consider a possible trout adaptation to a slow progressive decrease of fasting days? Some studies with and immediate and absolute fasting during some weeks have manifested a later compensatory growth in a re-feeding period.

> *We have now added a sentence into discussion showing an example of this kind of late compensatory growth, L 373-376.*

By other hand, one of the main found in this study is that fatty acids and amino acids profile of flesh is not affected by fasting, being this aspect of interest for rainbow trout culture.

Anyway, authors should improve some minor aspects related to manuscript, to consider for publication.

According to sections of abstract and introduction, they are suitable and well supported. Some suggestions are given in Word document. Focus of the study should be more summarized and concrete.

> *We think that it is always good to present testable hypotheses in an article. However, we changed the wording of the second hypothesis to some extent (L 98-99).*

Referring to Material and methods some minor consideration have to be in count. Results are suitably described, but some data in text has to be contrasted with the tables of supplement 1 and 2.

> *We have made the corrections as suggested in the file provided.*

In tables and figures, some suggestions are given regarding to title and footnotes as well as description of experimental groups and fasting periods in head of tables.

> *Changes have been made as suggested*

In discussion of data, all comments support the (not expected) results, but some sentences that in my opinion can results too speculative.

> *We have deleted some parts of the text the reviewer regard too speculative. However, we do not think that the suggested future research (comparison of wild fish to hatchery fish) is speculation but an important question that rose based on our results (L 356-360).*

These general comments are detailed in the Word document in order to help the authors to improve this interesting document.

Highlights

- Rainbow trout were unable to adapt to progresive decrease of feeding frequency in 63 days.

- 28-day re-feeding period did not elicit compensatory growth.

- Starvation and re-feeding did not affect nutritional value of flesh.

Effects of progressive decrease of feeding frequency and re-feeding on production parameters, stomach capacity and muscle nutritional value in rainbow trout (*Oncorhynchus mykiss)*

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ABSTRACT

Feeds and feeding constitute the major part of costs in intensive aquaculture. Any action to reduce feeding costs without negatively affecting fish production parameters and flesh quality would improve profitability of farming. Therefore, we studied the effects of feeding frequency on production parameters, stomach capacity and nutritional value of muscle in juvenile rainbow trout (*Oncorhynchus mykiss*) in an experiment with two stages. First, during the nine-week "starvation period" we fed rainbow trout (initial weight c. 40 g) with four different feeding protocols in an attempt to adapt the fish to a progressive decrease in the number of feeding days. During the second stage, a four-week "re-feeding period", all fish were fed in excess on weekdays. Fish growth, feed intake, stomach size, and biomolecule content of muscle were monitored as response variables. During the starvation period, feed intake and growth decreased along with the number of feeding days. Compensatory growth during the re-feeding was either only modest or absent. The fish in the starved groups were unable to significantly increase their stomach capacities. Starvation and re-feeding had only a slight effect on muscle fatty acid and amino acid composition. The used feeding protocols did not affect important production parameters (e.g. feed conversion or size variation). Our results suggest that despite differences in fish growth starvation and re-feeding hardly affect the nutritional value of fish. It is possible that several decades in captivity have made rainbow trout incapable to adjust their stomach size in respect to feeding frequency.

Key words: compensatory growth, stomach volume, salmonids, fatty acids, amino acids, feed conversion ratio

1. Introduction

Feeds and feeding constitute the major part of costs in intensive aquaculture operations. Thus, any decrease in these costs through feed development or rationalization of feeding practices would improve the profitability of farming. In aquaculture research dealing with fish nutrition and husbandry, the focus is typically on growth and feed utilization, but also the composition of the flesh is important from the human nutrition point of view. One option for rationalization of feeding is optimization of feeding rhythmicity or frequency, and this has attracted a lot of attention from aquaculture researchers.

In studies dealing with feeding frequency or starvation and consequent re-feeding, the fish are typically fed with the same predetermined frequency throughout the experiment. However, if the time intervals between feedings are several days or weeks, it may take quite a long time for the fish to become acclimated to such sparse feeding regimens (Pirhonen and Forsman, 1998; Nikki et al., 2004; Blake et al., 2006). Acclimation in this case means an increase in the capacity to ingest feed, as the fish are known to adjust their stomach capacities according to the meal size (Ruohonen et al.,1997), and a decrease in feeding frequency leads to an increase in the amount of feed ingested per feeding and consequent increase in stomach volume (Känkänen and Pirhonen, 2009; Mattila et al., 2009).

After a period of starvation or feed restriction animals typically show a growth spurt when supplied food in excess. During re-alimentation animals can show maximal growth rates which are not otherwise observed (Metcalfe and Monaghan, 2001; Ali et al., 2003) as they attempt to regain the lost growth. This phenomenon is referred to as compensatory growth and it has been investigated widely also with fishes in order to be exploited in commercial farming to obtain improved feed efficiency without compromising weight gain or muscle nutritional quality. However, research results are variable in this respect, and compensation is often induced by hyperphagia rather than by improved feed efficiency (Ali et al., 2003; Fu et al., 2007; Huang, 2008; Känkänen and Pirhonen, 2009; Mattila et al., 2009). A decrease in feeding frequency or starvation can also decrease the relative liver size and visceral fat accumulation (Weatherley and Gill, 1981; Nikki et al., 2004; Känkänen and Pirhonen, 2009; Mattila et al., 2009; Güroy et al., 2011), but a clear feeding frequency related decrease can also be absent due to large individual variation (Nikki et al., 2004). Starvation can also alter physiological responses seen as changes in hematocrit or plasma ions (Einarsdóttir and Nilssen, 1996; Falahatkar, 2012; Caruso et al., 2011, 2012).

The dietary availability of long-chain ω-3 polyunsaturated fatty acids (PUFA) influences the growth and development of fish (Tocher, 2010; Glencross et al., 2014), but also the nutritional value of fish (Sargent et al., 1995; Jobling, 2003). Previous studies have shown that low dietary content of docosahexaenoic acid (DHA, 22:6ω3) results in lower growth rates compared to fish feed rich in this fatty acid (Murray et al., 2014; Taipale et al., 2018). However, fish physiology and metabolism can also influence fatty acids (FA) composition in fish tissues since fish have the ability to synthesize long chain ω-3 PUFA, DHA and eicosapentaenoic acid (EPA, 20:5ω3), from precursors such as α-linolenic acid (ALA, 18:3ω3; Tocher, 2003; Murray et al., 2014,2015). Nevertheless, the ability for conversion varies by fish species and age (Tocher, 2010). For example, adult and juvenile rainbow trout (*Oncorhynchus mykiss*) are reported to be able to synthesize EPA and DHA from high concentration of dietary ALA (Gregory and James, 2014), but the direct dietary source of DHA is crucial for larvae (Wirth et al., 1997; Taipale et al., 2018). On the other hand, it is known that, in addition to long chain PUFA, proteins and amino acids are required for growth and development of fish (Wilson and Halver, 1986; Rønnestad et al., 1999), and restricted availability of essential amino acids (EAA) causes growth retardation and fin rot (Ketola, 1982). A study with juvenile rainbow trout (Taipale et al., 2018) showed ability of this species to compensate for the low EAA but not low DHA content of diet.

To the best of our knowledge there are no earlier studies looking at the effects of a progressive decrease in feeding frequency in fishes. This study was planned to investigate these effects with a two-part approach. In the first part of the experiment (starvation period) we tested a hypothesis that rainbow trout would be able to increase feed intake by increasing stomach volume without significant influence on growth rate when the feeding frequency is decreased progressively. Our second hypothesis was that starved fish would be able to full growth compensation during the re-feeding period (second part of the experiment). In addition to responses in growth and feed conversion, we measured treatment effects on the liver, viscera, stomach capacity, body composition and muscle biochemistry.

2. Materials and methods

2.1. Animals and experimental conditions

The experiment was carried out on 0+ age all-female rainbow trout between March 27 and June 26, 2017. On March 7, the fish were transported from a commercial fish farm to the laboratory at the University of Jyväskylä where they were held in a stock tank (0.5 m^3) until 2 weeks prior to the start of the experiment. At this time, 240 fish (39.5 ± 7.8 g), were taken from the stock tank and placed in twelve 360 L flow-through stainless steel tanks (20 individuals per tank) supplied with flowing (1 L min^{-1}) well water. Each tank was aerated through an air stone. Dissolved oxygen concentration and pH were c. 9.0 mg L^{-1}, and 6.4 respectively. Experimental fish were exposed to a 12L: 12D photoperiod.

2.2. Experimental design

The experiment consisted of two periods. The first period (starvation period) lasted for 9 weeks and tested the possible adaptation to the decrease in feeding frequency. Four treatments were designated: control (fed every weekday, Monday to Friday); T1 (fed three days a week: Mon., Wed., Fri.), T2 (fed three days a week for the first two weeks, and then twice, Mon., Fri.) and T3 (fed three days a week for the first two weeks, then twice the following two weeks and thereafter once a week, Mon.). After these starvation periods, the fish were subjected to a 4-week feeding period, when all the fish were fed from Monday to Friday (Fig. 1). The number of days the experimental fish were fed during the first period were 45 (control), 27 (T1), 20 (T2) and 15 (T3). During the second period all the fish were fed for 20 days (Fig. 1). During the first 4 weeks of the starvation period (as well as during the 2-week acclimation), the fish were hand-fed with commercial trout pellets (Biomar Efico Enviro 920 Advance 3 mm; proximate composition according to manufacturer was fat 31.5%, protein 44.5%, fiber 1.6%, ash 5.5%, energy 25 MJ kg^{-1}) and thereafter fish were fed with bigger pellets (Royal Plus 3.5 mm, Raisio Ltd, Raisio, Finland; proximate composition according to manufacturer was fat 28%, protein 43%, fiber 1.1%, ash 6.5%, energy 24.4 MJ kg^{-1}). Fish were fed twice per day between 08.00–09.00 and 15.00–16.00 h. Fish were always fed *ad libitum* (as much as they were willing to eat), and eaten feed was recorded considering uneaten feed siphoned out from the tanks after feeding. Five individuals died during the experiment for unknown reasons (T1: three and T3: two individuals) and four fish (in one of the control group tanks) died because of a technical failure in the aeration system (day 56).

2.3. Sampling procedure

Every two weeks (on Mondays) fish were sampled. During weighing days, the fish were not fed in the morning. Fish were anesthetized using clove oil: ethanol mixture (1:10, clove oil concentration 40 mg L^{-1}) and measured for weight and length. Five animals were netted out and killed with a sharp blow on the head. From these fish blood samples were taken from the caudal vessel of five individuals per tank with a heparin-coated syringe at the end of both periods. Hematocrit was analyzed right away and the remaining blood was centrifuged (7000 rpm) to separate plasma (frozen at -20°C) for chloride assays (Sherwood Chloride Analyzer, Model 926S).

Digestive tract was removed and, when all visible visceral fat and liver had been separated and weighed, was frozen (-20°C) for later stomach weight and volume measurement. A piece of muscle (two fish per tank) was excised from under the dorsal fin, and separated into two microtubes, one for the analysis of lipids, fatty acids and amino acids (frozen at -80°C) and the other for the water content (dried at 75°C).

2.4. Growth performance indices

Specific growth rate was calculated as SGR (% day^{-1}) $= 100(\ln W_2 - \ln W_1)*t^{-1}$, where W_1 and W_2 were weights (g) at the start and end of the measuring period and t was the period in days. Condition factor (CF) was calculated as $100W*L^{-3}$, where L was the total length (cm).

Relative feed intake $feeding^{-1}$ was calculated as I_R = (total intake (g) * number of $feedings^{-1}$) * W^{-1}, where W was the average weight (g) of the fish. Feed conversion ratio was calculated as FCR = intake (g) * gain $(g)^{-1}$. Possible compensation (for weight gain and intake) in the treatment groups during the two periods was estimated by a compensation coefficient which was calculated as CC = $\Delta T * \Delta C^{-1}$, where ΔT was the average weight gain or intake (g) in the treatment group tanks divided by the number of feeding days and ΔC was the average weight gain and intake (g) in the control group tanks divided by the number of feeding days; thus, CC > 1.0 would indicate compensation. Hepatosomatic index was calculated as HIS (%) = 100 $W_L * W^{-1}$, where W_L was liver weight (g). The visceral-somatic index was calculated as VSI (%) = 100 $W_V * W^{-1}$, where W_V was visceral weight (g).

2.5. Stomach measurement

Stomach capacity (volume and weight) was measured at three sampling points: on days 28, 63, and 91. For the measurement of stomach volume, a string was tied around the pyloric sphincter and the esophagus was tied to a 50 cm (=50 mL) burette. Stomach volume was estimated as the volume of water required to dilate the stomach under a pressure head of 50 cm water (Jobling et al., 1977), i.e. the amount of water added to the burette (to keep it at 50 cm) was regarded as the volume (to 0.1 mL) of the stomach. After the volume measurement, the stomach was separated from the intestine and weighed (to 0.01 g).

2.6. Lipids and fatty acids assays

Approximately 1-5 mg of freeze dried muscle samples (two fish per tank, 3 tanks per treatment) were analyzed for lipids. This assay was also carried on feeds (Supplement 1 and 2). Total lipids were extracted with chloroform:methanol:water mixture (2:1:0.75). For the formation of fatty acid methyl esters (FAME), mild acidic methylation (1% sulphuric acid-methanol solution) was used and samples were incubated in a 90 °C water bath for 1.5 h. FAMEs were run by a coupled gas chromatography-mass spectrometry (GC-MS, Shimadzu

Ultra, Kyoto, Japan) using an Agilent® DB-23 column (30 m × 0.25 mm × 0.25 μm) as previously published temperature ramp (Taipale et al. 2016). The identification of FAMEs was based on retention times and their specific mass ions (Taipale et al. 2016). For quantification of FAMEs we used 566c fatty acid mixture (Nu chek Prep) and specific ions following the protocol of Taipale et al. (2016).

2.7. Proteins and amino acids assays

Freeze-dried muscle samples (two fish per tank, 3 tanks per treatment) and feeds (Supplement 1 and 2) were pulverized using a mortar and pestle, and 0.1-0.6 mg of homogeneously mixed sample was weighed in tin cups for the analysis of elemental nitrogen (Carlo Erba Flash EA 1112 elemental analyzer). Two replicates of the dried white muscle of pike *Esox lucius* L., as an internal working standard, were analyzed after every 10 samples in each sequence. Total protein content was analyzed by multiplying elemental nitrogen content with a coefficient of 6.25 (Mariotti et al., 2008).

For amino acid analysis we used 0.5–1 mg of diets or freeze dried muscle tissue of the same two fish per tank as for the FA analyses. Proteins were hydrolyzed with 1 mL of 6 M HCl at 110 °C for 20 h and the solvent evaporated to dryness overnight. Amino acids were run as their propyl chloroformates using EZ:faast kit for preparation (Phenomenex) and a GC-MS using ZB-AAA column (9.5 m x 0.25 μm x 0.25 mm) with a previously published protocol (Taipale et al. 2018). Amino acid identification was based on specific ions included by the EZ:faast library. For quantification, we used Sigma-Aldrich AA-18 standard mix of which we made four-point calibration curve (0.005 $\mu g\ \mu l^{-1}$; 0.05 $\mu g\ \mu l^{-1}$; 0.1 $\mu g\ \mu l^{-1}$; 0.2 $\mu g\ \mu l^{-1}$) which was derivatized using EZ:faast kit. Eight essential amino acids (valine, leucine, isoleucine, threonine, methionine, phenylalanine, lysine and histidine) were analyzed, but not arginine or tryptophan. Also, two conditionally essential amino acids (glycine and proline) and seven non-essential amino acids (alanine, serine, asparagine, glutamic acid, ornithine, glycine-proline and tyrosine) were quantified.

2.8. Statistical analyses

Statistical analyses were performed using Minitab 18 for Windows and SPSS 24.0. Possible differences in weight, length and condition factor (CF), SGR, feed intake, weight gain, FCR, were tested using one-way ANOVA and the tank average value as an observational unit (i.e. $n = 3$). A one-sample *t*-test was used to test the possible difference of average CC of the treatment groups from the control group value (1, expected value when no compensation). *Post-hoc* comparisons were tested by Tukey's test. $P = 0.05$ was taken as the level of significance. Polynomial contrasts (linear, quadratic and cubic) were used to detect possible significant trends in responses during the starvation and re-feeding periods by using the number of feeding days during the starvation period (45, 27, 20 and 15 days in the control, T1, T2 and T3, respectively) as model effects. In the case of significant ($P < 0.05$) trend(s), we report the most significant one of the three (linear, quadratic or cubic). Permutational multivariate analysis of variance (PERMANOVA, Anderson et al., 2008) was used to test significant differences in amino acids and fatty acids between treatments were significant. PERMANOVA was run with unrestricted permutation of raw data and type III sums of squares. All the multivariate analyses were operated on Bray-Curtis distances of untransformed data with the program PRIMER-E (v.7; Ivybridge, United Kingdom) and the PERMANOVA+ add-on.

3. Results

3.1. Intake and growth performance

At day 63 (end of the 9-week starvation period), fish weight and total length were significantly smaller in the treatments T1, T2 and T3 than in the control group, and condition factor (CF) was significantly smaller in T3 than in the controls, and the decreasing linear trend was significant (Table 1). At day 91 (end of the 4-week re-feeding period), fish in the T2 and T3 groups were still significantly smaller than the controls and there was still a significant decreasing linear trend in CF. The coefficient of variation of fish weight exhibited a significant increasing linear trend along with the decrease of feeding even if there were no significant differences between treatment averages (Table 1).

Weight gain (%) and SGR decreased along with the decrease of the feeding frequency, and all treatments were significantly different from each other at the end of the starvation period (Table 1). During the re-feeding period the trends were opposite: the less the fish had grown during the first period the more they tended to grow during the second period. However, weight gain (%) and SGR were significantly larger only in T3 than in the controls (Table 1).

In respect to feed intake during the starvation period, control fish ate significantly more than the fish in the other groups, and T3 fish consumed significantly less feed than the fish in T1 or T2, and a negative linear trend was significant (Table 2). During the re-feeding period, there were no significant differences in feed consumption between the treatments, and no trend was observed. On the other hand, relative feed intake (% of body weight) increased during both periods along with the increase in the length of starvation of the first period, and a positive linear trend was significant (Table 2). Feed conversion ratio (FCR) did not differ between treatments (T1: 0.99; T2: 0.98 and T3: 1.17) and control (1.10) during the starvation, re-feeding and total experimental periods. However, there was a significant trend in FCR during the starvation period (quadratic contrast): FCR decreased from the controls (1.16) to T2 (0.95) and then increased in T3 (1.39).

The possible compensation of the fish in the treatment groups (T1, T2, T3) was estimated by comparing intake and weight gain to those of the control fish during the days when the fish were fed. Compensation coefficient of feed intake (CC_{intake}) during the first period was in T2-group 1.17 ± 0.05 (Fig.2a) and of weight gain (CC_{gain}) 1.38 ± 0.05 (Fig. 2b), which were significantly higher than 1 but the significant difference disappeared during the second period. In the other two treatments (T1 and T3) CC did not differ from 1 either in terms of intake or weight gain (Fig. 2).

3.2. Stomach capacity

According to stomach capacity measurements in absolute terms (weight g, volume mL) there were no significant differences between the treatments or trends (Table 3). In relative terms stomach weight (% of body weight) was significantly smaller in the controls (0.77 ± 0.09) than in the group T3 (1.04 ± 0.05) at the end of the experiment while T1 and T2 did not differ from the other groups, and there was also a positive linear trend. Also in relative stomach volume, there was a linear positive trend at the end of the first period. The stomach weight to volume -ratio was significantly higher in the controls than in the groups T1 and T2 at the end of the first period, and there was a significant decreasing linear trend, but there were no significant differences or trends at the end of the experiment (Table 3).

3.3. Hematocrit and plasma chloride

At the end of the starvation period (day 63) hematocrit values varied between 44.5 and 50.5 and at the end of the experiment (day 91) between 40.6 and 44.5, not displaying statistical differences between feeding regimes. However, there was a significant decreasing trend in hematocrit along with the decrease in feeding frequency at the end of the first period. Plasma chloride varied between 121.3 and 126.3 mmol/L at the end of the first period and at the end of the experiment between 124.7 and 128.6 mmol/L without being significantly different between the treatments.

3.4. Liver and visceral indices and composition

The absolute liver size was significantly smaller in T3 fish than in the other groups at the end of the first period (day 63), and livers also in T1 and T2 groups were smaller than in the controls (Table 4). At the end of the re-feeding period, there were no differences in liver weight between the treatments. Relative liver size (HSI) or liver water content were not significantly different at any sampling points between the treatments, but in the group T3 HSI was significantly smaller on day 63 than on the other two sampling points (Table 4).

Visceral somatic (VSI) and visceral fat somatic (VFSI) indices did not differ significantly between the treatments but VFSI increased in all the treatments during the experiment (Table 4). There was an apparent decreasing linear trend in VFSI along with the decrease of feeding on days 63 (after starvation) and 91 (after re-feeding).

Muscle water content increased along with the decrease in feeding frequency at the end of the first (day 63) and second (day 91) periods (significant linear trends), and the control group had significantly lower water content than the fish in T3 on both sampling times (Table 5). Lipids tended to decrease along with the decrease in the feeding frequency, but the trend was significant only on day 63 and the treatment effects were significant only on day 28. Muscle protein decreased along with the feeding frequency at the end of the starvation period (day 63) (Table 5).

3.5. Fatty acid and amino acid profiles in muscle

Before the experiment ω-3 and ω-6 PUFA contributed 29.5±6.4% and 13.2±7.4% of all FA of rainbow trout muscle, respectively, and DHA was the major constituent of (22.1±5.0%) ω-3 PUFA (Supplement 2). Total FA content of fish muscle prior to the experiment was 41±14 µg mg^{-1} dry weight (DW) and remained at a similar level after the period 1 (treatment averages varied between 34 and 47 µg mg^{-1} DW^{-1}, Supplement 2), but increased slightly after the period 2 (varied between 47 and 83 µg mg^{-1} DW^{-1}). Two factor (treatment x period) PERMANOVA for the contents of ALA, LIN (linoleic acid; 18:2ω6), SDA (stearidonic acid; 18:4ω3), ARA (arachidonic acid; 20:4ω6) (Fig. 3c), EPA and DHA in fish muscle (Fig. 3d) revealed that treatments explained only 7.3% of variances (Pseudo-$F_{3, 42}$=1.32, p=0.275), whereas period explained 18.4% of variances (Pseudo-$F_{1, 42}$=10.06, p=0.001). Treatments did not differ from each other whereas periods 1 (day 63) and 2 (day 91) differed statistically from each other. PERMANOVA for fish muscle content of LIN and ARA separately for the periods 1 and 2 resulted in slightly higher explanation percentages for treatments, 24.6% and 18.2%, however, the difference between treatments was not statistically significant (PERMANOVA, $F_{(3, 23)}$ = 1.49-1.63, p=0.2-0.211). Correspondingly, treatments explained 15.4% and 23.3% of ALA, SDA, EPA and DHA for the periods 1 and 2, respectively, and the treatments did not differ (PERMANOVA, $F_{(3, 19)}$ = 0.91-2.02, p=0.13-0.46) in their ω-3 contents.

At the beginning of the experiment, essential amino acids (EAA, Supplement 2) formed 64.3±2.5% of all AA in fish muscle and remained similar in control and treatment groups after the first (64.3±2.5% of all AA) and second periods (64.1±3.2% of all AA). Total AA content of fish muscle was 482±73 µg AA mg^{-1} DW at the beginning of the experiment and did not differ between control and treatment groups after the first or second periods (Supplement 1). Leucine (21.7±1.7% of all AA) and lysine (15.4±1.4% of all AA) were the most abundant essential amino acids (Fig. 3a) and alanine (10.0±0.9% of all AA) was the most abundant non-essential amino acid (NEAA) (Fig. 3b) in fish muscle throughout the experiment. Two-factor PERMANOVA showed that treatments explained only 5.3% (Pseudo-$F_{3,44}$=0.94, p=0.485) and period 22.6% (Pseudo-$F_{1,44}$=12.095, p=0.002) of variance of NEAA content of fish muscle and whereas there were no differences in treatments but between periods. Correspondingly, treatments explained 9.2% (Pseudo-$F_{3,44}$=1.55, p=0.21) and periods 14.2% (Pseudo-$F_{1,44}$=7.16, p=0.011) of the variance of EAA content of fish muscle, and there were similarly no differences in treatments but between periods. When the periods 1 and 2 were separately tested, treatments explained only 12.1% and 15.9% of EAA (PERMANOVA, $F_{(3,19)}$ = 0.83-1.20, p=0.31-0.53) and 8.5% and 10.8% of NEAA (PERMANOVA, $F_{(3,19)}$ = 0.55-0.77, p=0.59-0.77) of variance of fish muscle. EAA and NEAA content of fish muscle did not differ among treatments.

4. Discussion

In this experiment, the growth response in the form of compensation to the decrease in feeding frequency was actually much less than expected. We increased the number of days of starvation during the course of the experiment as an attempt to acclimate the fish to the sparse feeding rhythm. Rainbow trout is a predatory fish and opportunistic feeder, and such qualities could be expected to allow growth of the stomach in terms of weight and/or volume to enable the fish to eat large amount at a single feeding bout. Enlargement of the stomach volume has been reported to occur in rainbow trout as a consequence of an increase in the food water content (fish fed with chopped herring, *Clupea harengus*, vs. fish fed with dry feed) (Ruohonen and Grove 1996) and also as a response to sparse feeding frequency (Nikki et al., 2004). In the current study feeding rhythms of the treatment groups elicited some changes in the stomach, and significant trends related to the severity of starvation were seen in those stomach variables which were relative of fish size (Table 3). However, the lack of stronger compensatory growth may be related to the duration of the first experimental period (63 days) and the fish may not have had sufficient time to become fully adjusted to the sparse feeding protocols. For example, brown trout (*Salmo trutta*) needed over two months before showing compensatory growth when fed only twice a week (Pirhonen and Forsman, 1998). On the other hand, pikeperch (*Sander lucioperca*) fed chopped fresh fish flesh clearly became adapted to the sparse (fed every fourth or every seventh day) feeding frequency in the latter half of the 58-day experiment (Mattila et al., 2009).

It is plausible that when the fish are fed with dry pellets (water content c. 10 %) there is a physiological limit to which they are capable to fill their stomachs, as the fish will need to moisturize the feed both by increasing drinking and by excreting gastric juices before digestion is possible (Ruohonen et al., 1997). Ruohonen et al., (1997) suggested that it is actually the water availability to moisturize ingested dry feed which constraints feed intake in rainbow trout rather than stomach capacity. Thus, the low feed water content may have restricted the fish to eat and grow more than what they did in the treatment groups. On the other hand, hatchery fish have been selected especially for fast growth, and it is known that the fastest growing fish are the ones with the highest feed intake (e.g. Nikki et al., 2004), and consequently probably with the largest stomachs. Some kind of upper level or plateau may have been reached in their stomach capacities, and the hatchery fish may not be able for further stomach volume increase in conditions when feed is not offered every day, thus limiting their ability for expected compensatory growth. Therefore, it would be interesting to compare feeding capacity of (semi-)wild fish to the domesticated rainbow trout.

Any decrease in feeding frequency during the first period affected fish growth (both in weight and length) negatively (Table 1) although some compensation in feed intake and weight gain were observed in the group T2 (Fig. 2). However, at their best the CC-values of the present experiment were only modest (1.38) when compared to the CC-values in pikeperch (about 1.9; Mattila et al., 2009) but quite close to those observed in rainbow trout (about 1.5; Taşbozan et al., 2016). It must be noted that if CC = 1 does not mean compensatory growth but it indicates only that feed deprived fish have been able to eat or grow as much as the controls during the feeding days. Therefore, the CC-values should be much higher than 1 in the feed deprived groups if a full growth compensation was anticipated. We expected to see compensatory growth especially during the second period in the treatment groups, but this did not occur (Fig. 2), and the fish from T2 and T3 remained significantly smaller than the controls despite the increasing trend in relative weight gain and SGR along with the decrease in feeding during the first period (Table 1). Quinton and Blake (1990) observed in rainbow trout clear growth compensation only on the third week of feeding after a three-week starvation period, which suggests that rainbow trout may need several weeks before growth rate starts to increase. Albeit fish from T1 were not significantly smaller than the controls, the difference in size at the end of the experiment was about 15%, which is negative from the fish farmer's point of view, especially when there was no difference in FCR.

We decided to feed the fish in the control group only during the weekdays based on the results obtained in our laboratory (Nikki et al., 2004) with individually grown rainbow trout of similar size than in the current study. Nikki et al. (2004) exposed the trout to starvation periods of fixed length (from 2 to 16 days) in order to keep them hyperphagic, in comparison to controls, during the feeding days. In that research, the trout grew largest when feed was withdrawn for 2 days, and the hyperphagic response lasted typically for 5 days. Also Taşbozan et al. (2016) found in group-reared rainbow trout that when fasted for two days per week the fish grew significantly larger than the controls. Based on these earlier observations we can assume that the control fish of the present experiment grew at least as well as they would have grown if they had been feed every day. The other advantage of fasting the fish during the weekends is that when the weighing of the fish is on Monday, the fish have more or less empty stomachs (Grove et al., 1978), which in turn aids in getting rather standardized body weight for all individuals in each treatment.

For the fish farmer, size homogeneity within a tank is important as it will decrease the need for size selection and the end product will be of similar market size. The variability in size is commonly expressed by the coefficient of variation, CV, and the increase of CV is typically related to the competition for food and increase of aggressiveness between individuals (Jobling, 1995). In brown trout (*Salmo trutta*) it was observed that a sparse feeding (twice per week) significantly decreased CV of intake, i.e. the fish on the restricted group ate very homogeneously when compared to the controls, but however, that did not affect CV of weight (Pirhonen and Forsman, 1998). In contrast, in the present experiment there appeared a significant trend for the CV to increase along with the increase of levels of starvation. The fish in our experiment were not restricted for food when they were fed but all fish were always fed to apparent satiation, and as such the increase in CV of weight can be interpreted to reflect inter-individual variability in feed intake or feed efficiency rather than competition for food or aggressive behavior.

Condition factor is a widely used morphometric index showing indirectly how lean or fat an individual animal of a given population is, even though it is only an approximate indirect index for actual body fat reserves in fishes (Rennie and Verdon, 2008; McPherson et al., 2011; Sutton et al., 2011). At the end of the starvation period, the significant decreasing trend in CF (Table 1) indicates that the increase of duration of starvation directly affect the ability of the fish to gain surplus energy from the feed. However, the condition factor in T3 and also in the other groups had increased significantly from the initial value (0.99) by the end of the starvation period. The T3-group fish were apparently slightly starving during the first period because during the re-feeding period it was the only group with a significant increase in condition factor (to 1.21), but however, the decreasing trend in CF was still significant (Table 1). Pirhonen and Forsman (1998) found in brown trout that the fish which were fed only twice a week exhibited a clear rise in condition factor after two months of rearing and that coincided with a clear rise in SGR.

One of the primary objectives of the potential use of compensatory growth in hatcheries would be to improve feed conversion ratio, and improvement in FCR has been indicated in some investigations (Gaylord and Gatlin, 2001; Oh et al., 2013; Xiao et al., 2013; Gao et al., 2015). However, this is not always the case and growth compensation has also been reached only by a hyperphagic response (Wang et al., 2000; Yengkokpama et al., 2013; Xiao et al., 2013). The present study did not find an indication of a significant change in FCR suggesting that the little what the fish were able to compensate for the feed deprivation was mostly achieved by hyperphagia. FCR was about 9 % less in the groups T1 and T2 than in the controls during the first period which would account for savings in the feed costs but on the other hand decrease in fish size at the end of the first period was over 20 % in the favor of controls outweighing the benefit from feed saving. The significantly higher FCR in the T3 group, also seen as a quadratic trend (Table 2), is another indication of too strong feed deprivation (Adakli and Taşbozan, 2015).

Liver water content, HSI and VSI were not significantly affected by the length of starvation. This indicates that the liver and viscera are not easily affected by starvation, which is in accordance with the findings in other fish species (Miglavs and Jobling, 1989; Rueda et al., 1998; Ali et al., 2016). There was a significant linear trend of VFSI to decrease along with the severity of starvation at the end of the first and second periods. However, VFSI increased in all treatments towards the end of the experiment showing that even in the least fed group (T3) the fish prefer to accumulate fat in the body cavity rather than converting this energy into growth.

Starvation under certain circumstances can be considered as a stress factor (Blom et al., 2000). In this study starvation and re-feeding periods did not affect significantly hematocrit and plasma chloride values albeit an increasing trend in hematocrit in the end of the first period was observed. The literature regarding the effect of starvation on hematocrit are conflicting. The increase of hematocrit in response to starvation has been reported in European eel (*Anguilla anguilla*) and beluga (*Huso huso*) (Johansson-Sjöbeck et al., 1975; Falahatkar, 2012) being a possible response to starvation stress. On the other hand, a decrease in hematocrit related to starvation has been reported in lake sturgeon (*Acipenser fulvescens*), channel catfish (*Ictalurus punctatus*) and binni (*Mesopotamichthys sharpeyi*) (Gillis and Ballantyne, 1996; Lim and Klesius, 2003; Najafi et al., 2015). No effect on the haematocrit was found in European sea bass (*Dicentrarchus labrax*), blackspot seabream (*Pagellus bogaraveo*), red porgy (*Pagrus pagrus*), olive flounder (*Paralichthys olivaceus)*, persian sturgeon (*A. persicus)* and grey mullet (*Mugil cephalus*) (Caruso et al., 2011, 2012; Kim et al., 2014; Yarmohammadi et al., 2015; Akbary and Jahanbakhshi, 2016). Also, a possible difference in plasma chloride between the treatments would have suggested hydromineral imbalance, due to stress (Waring et al., 1992; Einarsdóttir and Nilssen, 1996). Even if changes in plasma hydromineral balance can be regarded as a secondary stress response (Einarsdóttir and Nilssen, 1996; Barton, 2002) the absence of differences in plasma chloride can be interpreted as lack of stress in our treatment groups.

Muscle lipid and protein content decreased and water content increased during the starvation period along with the increase of days of starvation. A decrease in muscle lipids and an increase in water were expected due to starvation (Shearer, 1994). When starved fishes can also oxidize protein to produce energy directly or through gluconeogenesis (Walsh, 1998) which can explain the decreasing trend in muscle protein content at the end of the first period. On the other hand, muscle protein has also been observed to remain constant in fishes regardless of starvation (Shearer, 1994; Mattila et al., 2009).

DHA fatty acid and essential amino acids are crucial for optimal growth and development of juvenile fishes (Wilson and Halver, 1986; Rønnestad et al., 1999; Tocher, 2010). The decrease in growth in the treatment groups did not lower the contents of DHA and AA in fish muscle. Previous studies have shown that fishes highly retain DHA (Glencross et al., 2003, 2014; Murray et al., 2014) and EAA and use protein-sparing strategy under nutrient limitation (Cho and Kaushik, 1990). The fish in the starved groups most likely used dietary carbohydrates and lipids for energy and spared AA and DHA for cell growth and optimal performance. Conservation of AA and DHA show their essential roles in fish metabolism (Tidwell et al., 1992; Rønnestad et al., 1994; Abi-Ayad et al., 2000). In the present research, starvation did not influence the AA profile of muscle which is along with the previous findings of the ability of juvenile rainbow trout to compensate for the low availability of amino acids from their diet (Taipale et al., 2018). Altogether, our results showed that rainbow trout sustained DHA and AA at the same level over the starvation period and thus did not influence on nutritional value of fish.

5. Conclusion

The results obtained in this experiment failed to show that rainbow trout would be capable to adapt to sparse feeding frequency by consuming enough during the feeding days to keep growing with the pace of the control fish. Our first hypothesis about the stomach volume increase and consequent increase in feed intake was not supported. The second hypothesis that the increased stomach volume in previously feed restricted fish would allow full growth compensation was supported only partly: although the fish from the treatment 1 (fed three times per week during the period 1) were not significantly smaller than the controls in the end, they weighed about 15 % less than the controls. Taken together, we did not find evidence that progressive decrease in the feeding frequency would facilitate rainbow trout in getting acclimated to the sparse feeding by expressing sufficient compensatory growth but these tested feeding schedules severely decreased fish growth; however, none of these treatments significantly altered the nutritional value of fish.

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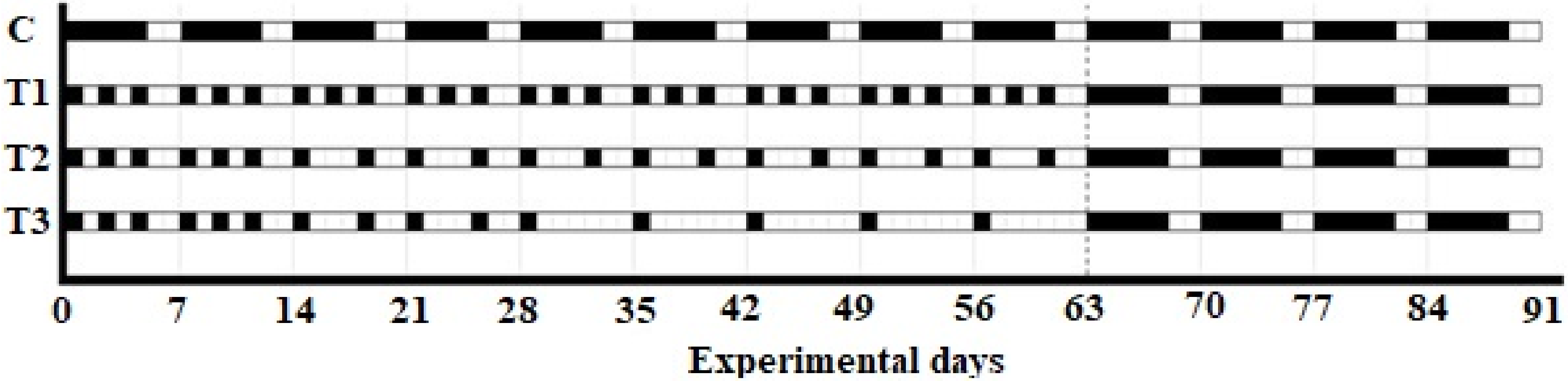
Figure 1. Feeding schedule in the experiment which first tested adaptation to starvation (period 1, 63 days) and the responses to re-feeding (period 2, 28 days) in juvenile rainbow trout (*Oncorhynchus mykiss*). C=Control (fed every weekday) and T1, T2 and T3 are treatments 1-3. Black bars indicate feeding days.

Figure 2. Compensation coefficients of feed intake (a) and weight gain (b) of rainbow trout (*Oncorhynchus mykiss*) fed according to different feeding regimes (treatment groups T1, T2, T3) for 91 days under starvation (period 1, 63 days) and re-feeding (period 2, 28 days). Each bar represents mean ± S.D, n = 3. CC>1 indicates compensation, and asterisk indicates a significant difference from 1.

Figure 3. Amino acid and fatty acid content (mean±sd; µg AA/FA mg^{-1} DW^{-1}) of rainbow trout (*Oncorhynchus mykiss*) muscle in the control group and different feeding treatments (T1, T2, T3) under starvation (period 1, 63 days) and re-feeding (period 2, 28 days). A) Essential amino acids (EAA: valine, leucine, isoleucine, threonine, lysine, phenylalanine, methionine), B) Non-essential amino acids (NEAA: alanine, glycine, serine, proline, aspartic acid and tyrosine), C) ω-6 polyunsaturated fatty acids (linoleic acid and arachidonic acid) and D) ω-3 polyunsaturated fatty acids (alfa-linolenic acid, stearidonic acid, eicosapentaenoic acid, docosahexaenoic acid).


C
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7
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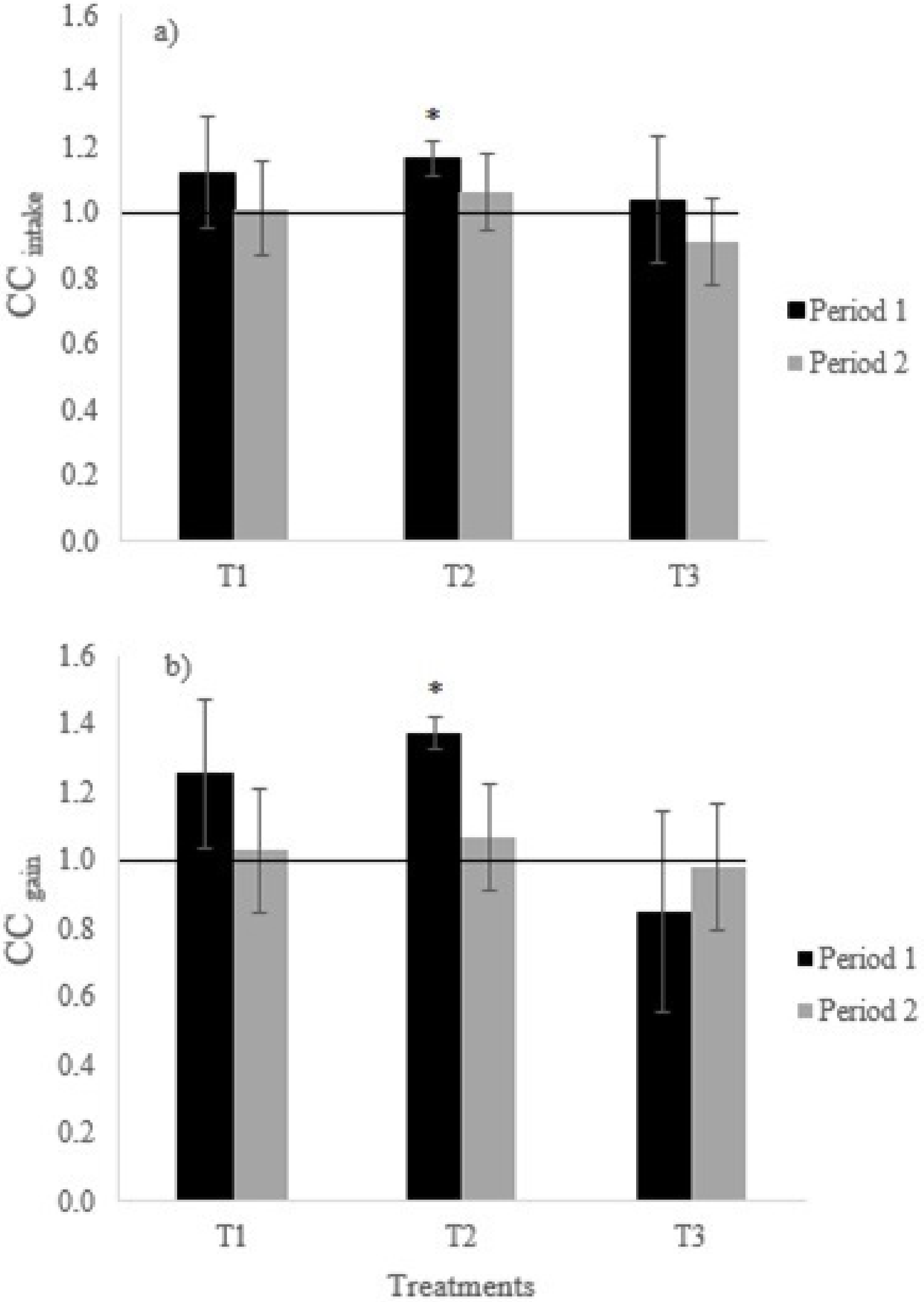
Experimental days


a)
CC intake
1.6
1.4
1.2
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T1
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T3
■ Period 1
■ Period 2
b)
CC gain
1.6
1.4
1.2
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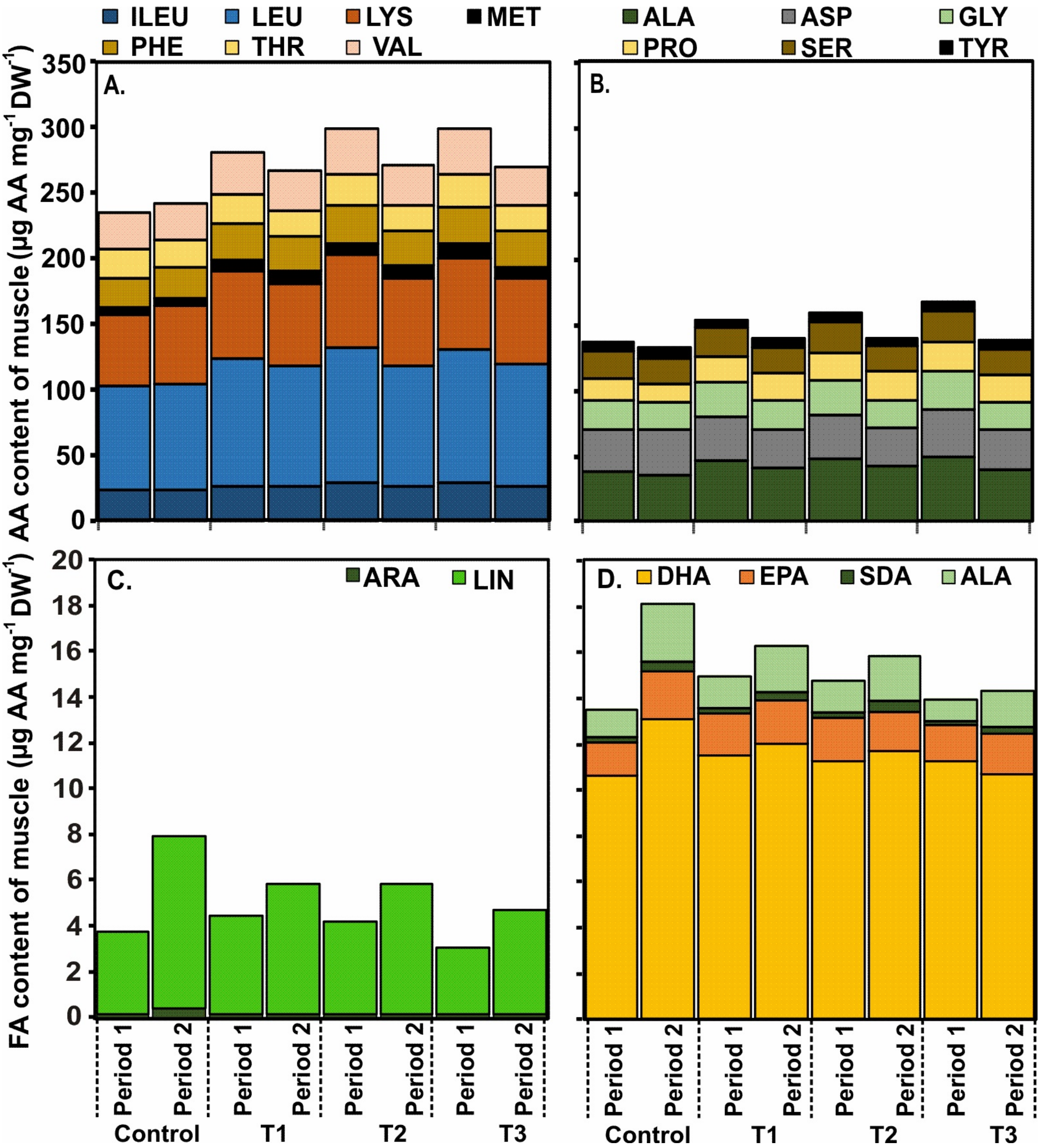
*
T1
T2
T3
Treatments
■ Period 1
■ Period 2

ILEU
LEU
LYS
MET
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GLY
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A.
B.
AA content of muscle (µg AA mg^{-1} DW^{-1})
0
50
100
150
200
250
300
350
C.
ARA
LIN
D.
DHA
EPA
SDA
ALA
FA content of muscle (µg AA mg^{-1} DW^{-1})
0
2
4
6
8
10
12
14
16
18
20
Period 1
Period 2
Control
T1
T2
T3

Table 1. Growth performance of rainbow trout (*O. mykiss*) during the experiment of different feeding regimes under starvation (days 0-63) and re-feeding (days 64-91) periods.

	Control	T1	T2	T3	P.C.
Weight (g)					
Day 0	40.97 ± 1.59^{A}	37.97 ± 1.27^{A}	39.60 ± 0.78^{A}	39.37 ± 2.70^{A}	N.S.
Day 63	173.47 ± 13.60^{cB}	134.17 ± 8.82^{bB}	114.03 ± 3.52^{bB}	81.17 ± 10.70^{aB}	L
Day 91	235.33 ± 27.84^{cC}	200.87 ± 12.66^{bcC}	174.13 ± 9.81^{abC}	134.70 ± 24.50^{aC}	L
Length (cm)					
Day 0	16.07 ± 0.21^{A}	15.77 ± 0.06^{A}	15.90 ± 0.20^{A}	15.97 ± 0.25^{A}	N.S.
Day 63	23.87 ± 0.40^{cB}	22.37 ± 0.49^{bB}	21.10 ± 0.30^{bB}	19.50 ± 0.70^{aB}	L
Day 91	26.43 ± 0.83^{cC}	25.23 ± 0.65^{bcC}	24.27 ± 0.40^{abC}	22.27 ± 1.15^{aC}	L
Condition Factor					
Day 0	0.99 ± 0.01^{A}	0.97 ± 0.04^{A}	0.99 ± 0.02^{A}	0.99 ± 0.03^{A}	N.S.
Day 63	1.27 ± 0.04^{bB}	1.20 ± 0.10^{abB}	1.21 ± 0.03^{abB}	1.09 ± 0.03^{aB}	L
Day 91	1.27 ± 0.03^{B}	1.25 ± 0.03^{B}	1.22 ± 0.01^{B}	1.21 ± 0.04^{C}	L
CV of fish weight					
Day 0	17.44 ± 1.87	20.43 ± 0.58	19.89 ± 3.65	20.87 ± 1.03	N.S.
Day 63	17.20 ± 2.37	23.77 ± 1.88	25.23 ± 4.19	26.71 ± 5.32	L
Day 91	16.96 ± 3.93	20.80 ± 1.25	24.02 ± 4.68	23.49 ± 4.25	L
Weight gain (%)					
Starvation period	323.02 ± 16.94^{dB}	253.25 ± 16.06^{cB}	187.98 ± 7.91^{bB}	105.86 ± 18.74^{aB}	L
Re-feeding period	35.54 ± 10.27^{aA}	49.79 ± 5.24^{abA}	52.68 ± 6.57^{abA}	65.26 ± 8.51^{bA}	L
Total	473.85 ± 55.78^{c}	429.22 ± 31.83^{bc}	339.53 ± 16.67^{ab}	241.26 ± 49.30^{a}	L
SGR					
Starvation period	2.25 ± 0.06^{dB}	1.97 ± 0.07^{cB}	1.65 ± 0.04^{b}	1.12 ± 0.14^{aA}	L
Re-feeding period	1.04 ± 0.26^{aA}	1.39 ± 0.12^{abA}	1.46 ± 0.15^{ab}	1.73 ± 0.18^{bB}	L
Total	1.88 ± 0.11^{c}	1.79 ± 0.07^{bc}	1.59 ± 0.04^{b}	1.31 ± 0.15^{a}	L

Values are mean ± SD, n = 3. Different superscript lower case letters indicate significant differences between treatments and upper case letters refer to significant differences between measurement periods within each treatment during the experiment ($p < 0.05$). P.C.: Polynomial contrast analysis; N.S.: not significant; L: linear model was the most significant one. See Fig. 1. for feeding protocols of the treatments. CV = coefficient of variation.

Table 2. Absolute intake, relative feed intake and feed conversion ratio in rainbow trout (*O. mykiss*) of different feeding regimes under starvation (days 0-63) and re-feeding (days 64-91) periods.

	Control	T1	T2	T3	P.C.
Absolute intake (g fish^{-1})					
Starvation period	117.43 ± 7.54^{cA}	78.70 ± 11.00^{b}	60.17 ± 2.63^{b}	39.17 ± 7.25^{aA}	L
Re-feeding period	62.32 ± 8.46^{B}	66.58 ± 4.54	63.79 ± 7.18	57.68 ± 5.38^{B}	N.S.
Total	183.76 ± 13.13^{c}	145.28 ± 10.96^{b}	123.97 ± 7.88^{ab}	96.86 ± 12.61^{a}	L
Relative feed intake (%)					
Starvation period	0.44 ± 0.00^{aA}	0.61 ± 0.06^{bA}	0.71 ± 0.02^{bcA}	0.80 ± 0.07^{cA}	L
Re-feeding period	0.19 ± 0.05^{aB}	0.24 ± 0.02^{abB}	0.29 ± 0.02^{bcB}	0.34 ± 0.02^{cB}	L
Total	0.31 ± 0.02^{a}	0.43 ± 0.04^{b}	0.50 ± 0.01^{bc}	0.57 ± 0.03^{c}	L
Feed conversion ratio					
Starvation period	1.16 ± 0.32	0.99 ± 0.04	0.95 ± 0.01	1.39 ± 0.20^{A}	Q
Re-feeding period	1.04 ± 0.12	1.00 ± 0.03	1.01 ± 0.04	0.94 ± 0.04^{B}	N.S.
Total	1.10 ± 0.21	0.99 ± 0.03	0.98 ± 0.02	1.17 ± 0.12	Q

Values are mean ± SD, n = 3. Different superscript lower case letters indicate significant differences between treatments and upper case letters refer to significant differences between measurements periods within each treatment during the experiment ($p < 0.05$). P.C.: Polynomial contrast analysis; N.S.: not significant; L: linear model was the most significant one; Q: quadratic model was the most significant one. See Fig. 1. for feeding protocols of the treatments. Relative feed intake = (total intake (g) * number of feedings^{-1}) * average fish weight^{-1}.

Table 3. Absolute and relative stomach weight, stomach volume and stomach weight to volume -ratio in rainbow trout (*O. mykiss*) of different feeding regimes under starvation (days 0-63) and re-feeding (days 64-91) periods.

Stomach variable	Control	T1	T2	T3	P.C.
Weight (g)					
Day 28	1.32 ± 0.14^{A}	1.28 ± 0.24	0.97 ± 0.18^{A}	1.17 ± 0.18	N.S.
Day 63	2.19 ± 0.31^{B}	2.01 ± 0.65	1.71 ± 0.33^{B}	1.53 ± 0.40	N.S.
Day 91	1.98 ± 0.43^{AB}	2.19 ± 0.22	1.89 ± 0.21^{B}	1.60 ± 0.39	N.S.
Weight (% of wet weight)					
Day 28	1.46 ± 0.29^{B}	1.75 ± 0.12^{B}	1.35 ± 0.07^{B}	1.68 ± 0.10^{B}	C
Day 63	1.23 ± 0.17^{AB}	1.40 ± 0.42^{AB}	1.41 ± 0.29^{B}	1.55 ± 0.34^{AB}	N.S.
Day 91	0.77 ± 0.09^{aA}	0.97 ± 0.16^{abA}	0.89 ± 0.05^{abA}	1.04 ± 0.05^{bA}	L
Volume (mL)					
Day 28	2.55 ± 0.86^{A}	2.61 ± 0.28^{A}	2.37 ± 0.54^{A}	2.01 ± 0.79^{A}	N.S.
Day 63	5.37 ± 0.65^{B}	6.44 ± 1.92^{B}	6.23 ± 0.99^{B}	4.40 ± 0.88^{AB}	N.S.
Day 91	7.21 ± 1.30^{B}	7.65 ± 0.13^{B}	6.03 ± 2.18^{B}	5.69 ± 2.07^{B}	N.S.
Volume (% of wet weight)					
Day 28	2.77 ± 0.78	3.66 ± 0.95	3.28 ± 0.49^{AB}	2.91 ± 1.04	N.S.
Day 63	3.01 ± 0.38	4.48 ± 1.19	5.10 ± 0.81^{B}	4.46 ± 0.75	L
Day 91	2.80 ± 0.15	3.35 ± 0.26	2.84 ± 0.97^{A}	3.72 ± 1.19	N.S.
Weight / Volume (g/mL)					
Day 28	0.56 ± 0.19^{B}	0.50 ± 0.12^{B}	0.42 ± 0.05	0.63 ± 0.21	N.S.
Day 63	0.41 ± 0.01^{bAB}	0.31 ± 0.03^{aAB}	0.27 ± 0.02^{a}	0.35 ± 0.05^{ab}	L
Day 91	0.27 ± 0.02^{A}	0.29 ± 0.03^{A}	0.35 ± 0.14	0.30 ± 0.09	N.S.

Values are mean ± SD, n = 3. Different superscript lower case letters indicate significant differences between treatments and upper case letters refer to significant differences between measurements periods within each treatment during the experiment ($p < 0.05$). P.C.: Polynomial contrast analysis; N.S.: not significant; L: linear model was the most significant one; C: cubic model was the most significant one. See Fig. 1. for feeding protocols of the treatments.

Table 4. Liver and visceral parameters in rainbow trout (*O. mykiss*) of different feeding regimes under starvation (days 0-63) and re-feeding (days 64-91) periods.

	Control	T1	T2	T3	P.C.
Liver weight (g)					
Day 28	0.97 ± 0.19	0.84 ± 0.26	0.99 ± 0.26	0.80 ± 0.09	N.S.
Day 63	1.82 ± 0.13^{c}	1.44 ± 0.11^{b}	1.25 ± 0.14^{b}	0.80 ± 0.05^{a}	L
Day 91	2.29 ± 0.37	2.29 ± 0.37	2.05 ± 0.18	1.69 ± 0.38	N.S.
Liver water content (%)					
Day 28	75.43 ± 0.54	74.05 ± 1.05	74.80 ± 0.66^{B}	74.80 ± 0.64	N.S.
Day 63	73.70 ± 1.90	73.82 ± 0.91	72.61 ± 0.44^{A}	74.14 ± 2.02	N.S.
Day 91	73.38 ± 0.48	73.84 ± 0.62	73.69 ± 0.44^{AB}	74.24 ± 0.51	N.S.
HSI (% of weight)					
Day 28	1.06 ± 0.14	1.12 ± 0.13	1.35 ± 0.19^{B}	1.15 ± 0.05^{B}	N.S.
Day 63	1.02 ± 0.03	1.01 ± 0.11	1.03 ± 0.15^{AB}	0.82 ± 0.08^{A}	N.S.
Day 91	0.89 ± 0.05	1.01 ± 0.21	0.97 ± 0.03^{A}	$1.10 \pm 0.03B^{B}$	N.S.
VSI (% of weight)					
Day 63	20.11 ± 0.62	21.89 ± 1.90	22.43 ± 1.89	20.39 ± 0.70	N.S.
Day 91	19.48 ± 1.33	17.34 ± 3.00	19.32 ± 1.44	20.50 ± 0.85	N.S.
VFSI (% of weight)					
Day 28	1.67 ± 0.69^{A}	1.39 ± 0.07^{A}	0.79 ± 0.41^{A}	1.20 ± 0.34^{A}	N.S.
Day 63	3.35 ± 0.72^{B}	3.21 ± 0.49^{B}	2.79 ± 0.61^{B}	2.06 ± 0.31^{AB}	L
Day 91	4.91 ± 0.15^{C}	4.23 ± 0.33^{C}	4.74 ± 0.40^{C}	3.45 ± 0.98^{B}	L

Values are mean ± SD, n = 3. Different superscript lower case letters indicate significant differences between treatments and upper case letters refer to significant differences between measurements periods within each treatment during the experiment ($p < 0.05$). HSI: Hepatosomatic index; VSI: Visceral somatic index; VFSI: Visceral fat somatic index. P.C.: Polynomial contrast analysis; N.S.: not significant; L: linear model was the most significant one. See Fig. 1. for feeding protocols of the treatments.

Table 5. Muscle composition of rainbow trout (*O. mykiss*) of different feeding regimes under starvation (days 0-63) and re-feeding (days 64-91) periods.

	Control	T1	T2	T3	P.C.
Muscle composition					
Water content (%)					
Day 28	74.66 ± 1.97	74.02 ± 1.30	75.53 ± 0.46	75.32 ± 0.61	N.S.
Day 63	75.58 ± 1.52^{a}	76.78 ± 0.77^{ab}	77.42 ± 0.46^{ab}	78.86 ± 0.17^{b}	L
Day 91	75.48 ± 0.08^{a}	75.77 ± 0.29^{ab}	75.95 ± 0.69^{ab}	76.98 ± 0.61^{b}	L
Lipids (%)					
Day 28	3.76 ± 0.52^{ab}	3.67 ± 0.58^{ab}	3.89 ± 0.76^{b}	2.43 ± 0.25^{a}	N.S.
Day 63	2.40 ± 1.33	1.90 ± 0.63	1.38 ± 0.46	0.86 ± 0.04	L
Day 91	2.05 ± 0.17	1.70 ± 0.17	1.65 ± 0.19	1.50 ± 0.81	N.S.
Protein (%)					
Day 28	19.43 ± 2.01	20.03 ± 1.45	18.61 ± 0.37	19.95 ± 0.65	N.S.
Day 63	20.72 ± 0.68^{b}	19.84 ± 0.54^{b}	19.56 ± 0.37^{ab}	18.34 ± 0.43^{a}	L
Day 91	20.56 ± 1.29	21.05 ± 0.39	21.19 ± 0.58	20.31 ± 0.31	N.S.

Values are mean ± SD, n = 3. Different superscript letters indicate significant differences between treatments ($p < 0.05$). P.C.: Polynomial contrast analysis; N.S.: not significant; L: linear model was the most significant one. See Fig. 1. for feeding protocols of the treatments.

Supplement 1. Amino acid content (μg AA mg^{-1} DW^{-1}) in two different feeds and muscle of rainbow trout (*O. mykiss*) of different feeding regimes at the end of the starvation (Day 63) and re-feeding (Day 91) periods.

	Feed 1	**Feed 2**	**Day 0**	**Control**		**T1**		**T2**		**T3**	
				Day 63	**Day 91**	**Day 63**	**Day 91**	**Day 63**	**Day 91**	**Day 63**	**Day 91**
Essential amino acids											
ALA	9.1 ± 0.6	9.1 ± 1.2	10 ± 1.0	10.5 ± 0.1	9.2 ± 1.2	10.7 ± 0.8	9.8 ± 0.6	10.4 ± 0.5	10.0 ± 0.6	10.3 ± 0.6	9.4 ± 1.0
ASN	0.1 ± 0.0	0.0 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
ASP	10.6 ± 1.1	9.9 ± 1.2	7.3 ± 0.7	9.1 ± 2.7	8.4 ± 2.4	7.5 ± 0.9	7.0 ± 1.0	7.2 ± 0.9	7.1 ± 0.9	7.9 ± 1.3	7.2 ± 1.2
GLU	4.3 ± 1.9	3.4 ± 3.0	0.9 ± 0.1	1.0 ± 0.2	4.0 ± 5.4	0.9 ± 0.3	1.3 ± 0.3	0.9 ± 0.2	1.4 ± 0.4	1.1 ± 0.5	3.1 ± 4.1
GPR	0.2 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
ORN	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SER	3.4 ± 0.6	2.3 ± 1.2	1.1 ± 0.8	1.9 ± 0.4	1.9 ± 0.9	1.4 ± 0.4	1.5 ± 0.9	1.5 ± 0.4	1.4 ± 0.5	1.6 ± 0.8	1.5 ± 0.7
TYR	2.5 ± 0.4	2.7 ± 0.8	4.5 ± 0.9	4.3 ± 0.3	3.9 ± 1.2	4.5 ± 0.5	5.1 ± 0.4	4.5 ± 0.6	5.0 ± 0.4	4.6 ± 0.3	4.9 ± 1.1
Σ EAA	**30 ± 4.6**	**28 ± 7.5**	**24 ± 3.6**	**27 ± 3.9**	**27 ± 11.2**	**25 ± 3.0**	**25 ± 0.4**	**25 ± 0.3**	**25 ± 0.4**	**26 ± 0.4**	**26 ± 1.0**
Non-ssential amino acids											
GLY	6.7 ± 0.3	6.1 ± 0.5	6.5 ± 1.0	5.9 ± 0.2	5.4 ± 0.5	6.1 ± 0.3	5.4 ± 0.5	5.8 ± 0.3	5.3 ± 0.4	6.0 ± 0.2	5.0 ± 0.1
HIS	0.2 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
ILEU	4.4 ± 0.0	5.6 ± 0.5	6.3 ± 0.8	5.9 ± 1.2	6.1 ± 0.4	6.1 ± 0.3	6.4 ± 0.3	6.2 ± 0.2	6.2 ± 0.2	6.3 ± 0.5	6.1 ± 0.4
LEU	20.5 ± 1.6	19.7 ± 0.6	23.1 ± 2.6	20.0 ± 3.6	20.7 ± 2.5	21.9 ± 0.9	22.4 ± 1.2	22.2 ± 0.8	22.3 ± 0.6	21.5 ± 0.8	22.1 ± 1.2
LYS	9.3 ± 1.0	11.4 ± 1.0	14.1 ± 1.2	15.1 ± 2.5	15.7 ± 2.3	15.4 ± 1.2	15.3 ± 1.7	15.5 ± 1.4	15.8 ± 0.6	14.6 ± 0.6	15.6 ± 0.9
MET	0.7 ± 0.1	0.7 ± 0.3	1.9 ± 0.4	1.4 ± 0.7	1.5 ± 0.4	1.8 ± 0.4	2.2 ± 0.5	1.9 ± 0.5	2.2 ± 0.3	2.2 ± 0.4	2.1 ± 0.6
PHE	6.9 ± 0.5	6.4 ± 0.1	6.9 ± 1.3	5.8 ± 1.1	6.0 ± 0.7	6.4 ± 0.4	6.4 ± 0.4	6.3 ± 0.5	6.3 ± 0.2	6.1 ± 0.3	6.5 ± 0.4
PRO	8.9 ± 0.4	10.1 ± 1.0	5.2 ± 0.5	5.6 ± 0.6	4.8 ± 0.5	5.0 ± 0.2	4.8 ± 0.2	4.9 ± 0.2	4.8 ± 0.2	5.0 ± 0.2	4.6 ± 0.4
THR	4.6 ± 0.2	5.1 ± 0.3	5.0 ± 1.2	5.8 ± 0.5	5.3 ± 0.4	5.0 ± 0.3	4.8 ± 0.7	5.1 ± 0.4	4.9 ± 0.3	5.2 ± 0.4	4.7 ± 0.4
VAL	7.5 ± 0.0	6.8 ± 0.7	7.1 ± 0.6	7.4 ± 0.9	7.1 ± 0.5	7.3 ± 0.3	7.3 ± 0.4	7.4 ± 0.3	7.2 ± 0.4	7.3 ± 0.5	6.9 ± 0.4

Σ NEAA	**70 ± 0.5**	**72 ± 0.6**	**76 ± 1.1**	**73 ± 1.4**	**73 ± 0.9**	**75 ± 0.5**	**75 ± 0.7**	**75 ± 0.5**	**75 ± 0.4**	**74 ± 0.4**	**74 ± 0.6**
Total AA content (µg AA mg^{-1} DW^{-1})	**244 ± 32**	**171 ± 30**	**482 ± 73**	**436 ± 55**	**419 ± 121**	**440 ± 56**	**413 ± 49**	**463 ± 56**	**418 ± 54**	**473 ± 69**	**425 ± 67**

Supplement 2. Fatty acid content (µg FA mg^{-1} DW^{-1}) in two different feeds and muscle of rainbow trout (*O. mykiss*) of different feeding regimes at the end of the starvation (Day 63) and re-feeding (Day 91) periods.

	Feed 1	**Feed 2**	**Day 0**	**Control**		**T1**		**T2**		**T3**	
				Day 63	**Day 91**	**Day 63**	**Day 91**	**Day 63**	**Day 91**	**Day 63**	**Day 91**
Saturated fatty acids											
c14:0	2.8 ± 0.0	1.8 ± 0.0	0.8 ± 0.3	1.1 ± 0.2	1.5 ± 0.2	1.1 ± 0.2	1.3 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	0.9 ± 0.2	1.2 ± 0.1
c15:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
c16:0	10.8 ± 0.2	9.4 ± 0.1	15.0 ± 1.6	14.9 ± 1.2	13.6 ± 0.6	13.5 ± 0.4	13.7 ± 0.8	13.6 ± 0.9	14.4 ± 1.3	14.4 ± 0.7	14.4 ± 1.4
c17:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
c18:0	3.9 ± 0.1	3.8 ± 0.0	5.1 ± 0.8	4.0 ± 0.5	3.5 ± 0.1	3.8 ± 0.3	3.4 ± 0.2	3.9 ± 0.1	3.7 ± 0.3	4.0 ± 0.2	3.9 ± 0.2
c20:0	0.4 ± 0.0	0.8 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
c22:0	0.2 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Σ SAFA	**18.5 ± 0.3**	**17.6 ± 0.2**	**21.5 ± 2.8**	**20.5 ± 2.0**	**19.4 ± 0.9**	**19.0 ± 0.9**	**19 ± 1.2**	**19.1 ± 1.1**	**20.0 ± 1.9**	**19.8 ± 1.1**	**20.1 ± 1.8**
Monounsaturated fatty acids											
16:1ω9	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
16:1ω7	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.3	1.5 ± 0.1	2.3 ± 0.3	1.4 ± 0.3	1.7 ± 0.3	1.1 ± 0.2	1.5 ± 0.2	1.0 ± 0.2	1.8 ± 0.3
18:1ω9c	35.8 ± 0.2	38.4 ± 0.4	24.3 ± 4.7	23.5 ± 2.2	32.0 ± 2.3	25.6 ± 2.9	28.4 ± 3.3	22.4 ± 4.2	26.1 ± 3.0	19.1 ± 1.8	26.3 ± 0.5
18:1ω7c	1.7 ± 0.2	1.6 ±0.2	2.3 ± 0.3	2.2 ± 0.1	2.2 ± 0.3	2.3 ± 0.1	2.4 ± 0.2	2.1 ± 0.2	2.3 ± 0.2	1.9 ± 0.1	2.3 ± 0.3
20:1ω9	0.3 ± 0.0	1.3 ± 0.0	1.6 ± 0.3	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	1.3 ± 0.2	1.2 ± 0.3	1.3 ± 0.1	1.2 ± 0.2	1.3 ± 0.2
20:1ω7	4.8 ± 0.2	1.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1ω9	1.3 ± 0.0	0.7 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
Σ MUFA	**43.9 ± 0.6**	**43.9 ± 0.8**	**30 ± 5.7**	**29 ± 2.6**	**38.6 ± 3.1**	**31.2 ± 3.6**	**34.4 ± 4.1**	**27.3 ± 5.0**	**31.8 ± 3.6**	**23.6 ± 2.4**	**32.2 ± 5.9**
ω-6 Polyunsaturated fatty acids											
18:2ω6c (LIN)	15.1 ± 0.0	15.5 ± 0.1	12.0 ± 2.8	9.7 ± 1.1	12.3 ± 0.9	10.8 ± 1.1	11.2 ± 1.2	9,9 ± 1.6	10.3 ± 1.3	8.5 ± 0.7	10.7 ± 1.6
18:3ω6	5.9 ± 0.1	6.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
20:2ω6	0.6 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.7 ± 0.1

20:3ω6	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.1
20:4ω6 (ARA)	0.3 ± 0.0	0.2 ± 0.0	0.9 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.3
22:3ω6	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:5ω6	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
Σ ω-6 PUFA	**21.9 ± 0.2**	**22.2 ± 0.1**	**14.4 ± 3.3**	**12.3 ± 1.4**	**14.6 ± 1.2**	**13.2 ± 1.4**	**13.4 ± 1.6**	**12.1 ± 1.9**	**12.6 ± 1.6**	**10.8 ± 0.9**	**13.1 ± 2.2**
ω-3 Polyunsaturated fatty acids											
18:3ω3 (ALA)	6.0 ± 0.1	6.8 ± 0.0	2.8 ± 0.6	3.2 ± 0.5	4.3 ± 0.3	3.6 ± 0.5	3.9 ± 0.3	3.5 ± 0.3	3.6 ± 0.5	2.9 ± 0.3	3.6 ± 0.6
18:4ω3 (SDA)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	1.0 ± 1.0	0.5 ± 0.1	0.8 ± 0.1
18:5ω3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3ω3	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:4ω3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:5ω3 (EPA)	3.8 ± 0.0	3.7 ± 0.0	3.7 ± 0.9	3.9 ± 0.4	3.0 ± 0.4	4.1 ± 0.8	3.6 ± 0.7	5.0 ± 0.9	3.6 ± 0.5	4.9 ± 0.4	4.1 ± 0.9
22:5ω3 (DPA)	1.4 ± 0.0	0.9 ± 0.0	1.6 ± 0.2	1.4 ± 0.2	1.4 ± 0.0	1.5 ± 0.2	1.4 ± 0.1	1.6 ± 0.3	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.2
22:6ω3 (DHA)	4.3 ± 0.0	4.9 ± 0.1	25.9 ± 7.8	28.8 ± 2.7	17.7 ± 2.9	26.5 ± 3.8	23.3 ± 4.0	30.5 ± 5.2	25.8 ± 3.5	35.5 ± 3.0	24.4 ± 5.2
Σ ω-3 PUFA	**15.7 ± 0.2**	**16.4 ± 0.1**	**34.1 ± 9.5**	**38.2 ± 4.0**	**27.5 ± 3.8**	**36.6 ± 5.5**	**33.2 ± 5.3**	**41.5 ± 6.9**	**35.7 ± 5.6**	**45.7 ± 3.8**	**34.7 ± 7.1**
Σ PUFA	**37.6 ± 0.3**	**38.6 ± 0.2**	**48.5 ± 12.8**	**50.5 ± 5.4**	**42 ± 5.0**	**49.8 ± 6.9**	**46.6 ± 6.9**	**53.6 ± 8.8**	**48.3 ± 7.2**	**56.5 ± 4.8**	**47.7 ± 9.3**
ω-3 : ω-6 -ratio	**0.7 ± 0.0**	**0.7 ± 0.0**	**2.4 ± 1.1**	**3.1 ± 0.4**	**1.9 ± 0.3**	**2.8 ± 0.5**	**2.5 ± 0.5**	**3.4 ± 0.8**	**2.8 ± 0.5**	**4.2 ± 0.6**	**2.6 ± 0.7**
Total FA content (µg FA mg^{-1} DW^{-1})	**120 ± 15**	**130 ± 16**	**41 ± 14**	**37 ± 4.7**	**83 ± 26**	**47 ± 13.4**	**55 ± 11**	**39 ± 14.2**	**47 ± 13**	**33.9 ± 4.2**	**47 ± 15.3**

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: