Stable isotope profiling for large-scale evaluation of feed efficiency in Atlantic salmon *(Salmo salar)*

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Running title: Individual feed efficiency in aquatic species

Abstract

In growing animals, individual variation in feed efficiency may arise from individual differences in growth rate and protein metabolism. Over a period of time, these factors will affect the ratio between “new” vs. “old” protein, which can be quantified using isotope profiling. The aim of the current study was to investigate the relationship between relative weight gain and atom percentage excess ^{15}N in muscle, liver and mid-intestine. A 50-day experiment was conducted with a total of 375 fish initially fed a standard diet, subsequently replaced by one out of five experimental diets, enriched with ^{15}N. In general, fast-growing fish are expected to have a better feed efficiency, and the results show that this is captured by isotope profiling in liver and muscle tissues. Furthermore, individual variation in isotope content, i.e. relative fraction of “new” protein, among fish with comparable growth rates was observed, most expressed around ~50% isotope saturation, indicating differences in protein degradation and replacement not attributed to growth. The results suggest that isotope profiles can be used as individual indicator traits for feed efficiency and that inclusion levels of stable isotopes of 1-2% gave the most reliable results.

Keywords: Atlantic salmon, growth, maintenance requirements, protein metabolism, protein turnover, selective breeding

1. Introduction

Advanced selective breeding programs for Atlantic salmon *(Salmo salar, Salmonidae)* have been carried out since the early 1970's (Gjedrem, Gjøen, & Gjerde, 1991), with growth as the pivotal trait. After five generations of selection, Thodesen, Grisdale-Helland, Helland, and Gjerde (1999) compared selected salmon to wild salmon, reporting a 25% improvement in feed efficiency, defined as growth per unit of feed. Recording of individual feed efficiency involves recording of the individual feed intake as well as individual weight gain. While the latter is relatively easy to obtain, individual feed intake is not easily attainable in large-scale aquaculture systems. Research have shown that feed intake cannot fully explain individual variation in growth, due to different individual growth responses when consuming the same amount of feed (Carter, Houlihan, Buchanan, & Mitchell, 1993b; Houlihan, Carter, & McCarthy, 1995). Although genetic variation in feed efficiency obviously exists, the underlying mechanisms are unknown, but can be assumed partly due to individual variation in protein metabolism.

Traditionally, individual feed efficiency in fish has been improved through selection for increased growth rate, assuming a favourable genetic correlation to feed efficiency (Thodesen, Gjerde, Grisdale-Helland, & Storebakken, 2001). A fast-growing animal will likely be more feed efficient by using a relatively larger fraction of the feed for growth and less for maintenance of existing body tissue. On a family level, Thodesen et al. (2001) estimated a favourable correlation of 0.8 between feed efficiency and growth rate. They stated an apparently decreasing response in feed efficiency with increasing growth rates, suggesting that direct selection for improved feed efficiency would be beneficial. Hence, it is timely to address other indicator traits related to feed efficiency as a tool to improve selective breeding for feed efficiency in aquatic species.

Diets with distinct stable isotope ratios, for example $^{15}N/^{14}N$ and $^{13}C/^{12}C$, can be used to trace metabolism of nutrients (Houlihan et al., 1995). Feeding diets with enriched levels of specific isotopes will result in an isotopic change in the organism (Le Vay & Gamboa-Delgado, 2011). Changes in the isotopic composition of tissues after a change in diet occur through two processes (Jardine, McGeachy, Paton, Savoie & Cunjak, 2003; Fellerhoff, 2002). Firstly, the process involves the metabolic breakdown of tissues that were synthesized during feeding on the

previous diet, and their subsequent replacement with tissues synthesized on a new diet. Secondly, the growth of new tissue that occurs after a diet switch will reflect the isotopic composition of the current diet, and will contribute to the overall reflection of that diet in the isotopic composition of the fish (Figure 1). Growth rate is a result of the net *difference* between absorption of new nutrients from feed and loss of nutrient components in faeces, while efficiency depends on the *ratio* between them. Hence, growth rate may not explain all variation in efficiency (i.e., a slow- or moderately growing individual may still be efficient if the degradation losses are sufficiently low). By combining growth rate and change in isotope profile, the efficiency can be more accurately assessed than by using relative increase in growth alone. The change in isotope ratio can be used to determine the rate of protein metabolism in various tissues, as a result of metabolic activity, nutritional state, partitioning of nutrients, physical activity and tissue growth (Bloomfield, Elsdon, Walther, Gier, & Gillanders, 2011).

The current experiment is part of a study aiming at investigating the use of stable isotope in feed to assess feed efficiency in Atlantic salmon. The current study was a pilot with five inclusion levels of ^{15}N in feed and sampling fish at seven time points with the aim to study the relationship between relative weight gain and atom percentage excess (APE) ^{15}N in muscle, liver and mid-intestine. From this, one objective was to draw inference as to how phenotyping could be carried out and yet another to discuss the potential of the indicator traits in selective breeding for improved feed efficiency in aquaculture species.

2. Materials and methods

2.1 Fish, housing and health

The experiment was carried out at the fish laboratory at the Norwegian University of Life Sciences (NMBU), Aas, Norway, following the laws and regulations for experiments on live animals in EU (Directive 2010/637EU) and Norway (FOR-2015-06-18-761). The experiment was approved by the Norwegian Food Safety Authority (FOTS ID 9484). A total of 510 Atlantic salmon with an average initial body weight of 21 g were randomly distributed into 15 tanks (34 fish per tank). Prior to start of the 50-day experimental period, all fish were pit-tagged with a 2 x

12 mm unique glass tag (RFID Solutions, Hafrsfjord, Norway), and initial length and weight were recorded. The tanks, each with a 270 l capacity, were supplied with recirculated freshwater. Water flow rate was 7-8 l min^{-1}, and the fish were kept under 12 h/12 h light-dark regime, with temperature in range of 15-16 ℃. Dissolved oxygen was measured daily and kept above 8 mg l^{-1} in the outlet water (Handy Delta, OxyGuard® AS, Farum, Denmark). There were no mortality or sign of disease during the experimental period.

2.2 Dietary treatments and feeding

The dietary treatments consisted of four ^{15}N-marked diets with different inclusion levels of ^{15}N. Diet SP1 and SP2 contained 1% and 2% ^{15}N marked *Spirulina* whole cells (Larodan, Sweden), while diet L0.1 and L0.2 had 0.1% and 0.2% ^{15}N marked L-Lysine (Sigma, Norway). A control diet was formulated with no added (0%) ^{15}N. All diets were formulated to meet requirements for Atlantic salmon for protein (NRC, 2011), and their composition are given in Table 1. The feed ingredients were mixed at the feed laboratory at NMBU, Ås, Norway. All dry ingredients, except gelatine, were mixed in a Forberg mixer (vacuum coater) (Forberg AS, Hegdal, Larvik, Norway). Gelatine was dissolved in cold water and then heated to 55 ℃ in a microwave oven. Fish oil, dissolved gelatine, water and the dietary ingredients were mixed with a Moretti Foreni kneading machine (Spiry 25, Mondolfo, Italy). This resulted in a firm dough that was cold pelleted using an Italgi pasta extruder (P35 A, Carasco, Italy) equipped with a 2.5 mm die. Feed was cooled to room temperature and dried at 45-60 ℃ to about 95% dry matter, in a batch dryer with a DANIA 9 kW fan (Inelco A7S, Fjerntslev, Denmark). The feed was stored at 4 ℃ until feeding. The diets were fed to triplicate groups of fish for a period of one hour, twice a day (08:00 and 17:00) by automatic belt feeders. The feeding level equalled 1.5% of body weight the first day and then 10% in excess, based on the level of uneaten feed. Uneaten feed was collected from the water outlet, and feed intake was calculated as described by Helland, Grisdale-Helland, and Nerland (1996).

2.3 Chemical analysis

The diets were ground, and analyses were performed in duplicates. Diets were analysed for dry matter by drying to constant weight at 104 ℃, ash by combustion at 550 ℃, crude protein by Kjeldahl nitrogen x 6.25 according to Commission regulation (EC) No 152/2009 and starch as described in McCleary, Solah, and Gibson (1994). Lipid was analysed after extraction with petroleum ether and acetone (70/30) on an Accelerated Solvent Extractor (ASE 200) (Dionex Corp, Sunnyvale, CA, USA). Gross energy was determined with PARR 1281 Adiabatic Bomb calorimeter (Parr Instruments, Moline, IL, USA) according to ISO 9831. Amino acids except tryptophan were analysed according to Commission regulation (EC) No 152/2009 on a Biochrom 30 Amino Acid Analyser (Biochrom Ltd., Cambridge, UK). For tryptophan, a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) equipped with a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan) was used, and the analysis was carried out according to Commission regulation (EC) No 152/2009. The chemical composition of the diets is given in Table 2.

2.4 Sampling

Four fish from each tank were sampled at different time points (2, 4, 8, 16, 32 and 50 days). The remaining fish (n = 150) were kept for another experiment. Fish were anesthetized with metacaine (MS-222^{TM}; 1 g l^{-1} water) and killed with a sharp blow to the head prior to dissection. Slaughter weight and length were recorded for all fish, and tissue samples (muscle, liver and mid-intestine) were collected in cryotubes, snap-frozen in liquid nitrogen and kept in a freezer at -80 ℃ until freeze-drying, grinding and stable isotope analysis. Prior to the experiment, tissue samples from 15 fish were collected to determine the initial isotopic atom percentage (IA %). Tissue sampling was standardized; muscle was sampled in front of the dorsal fin (1 x 1 cm cube), the whole liver was sampled and mid-intestine from the end of pyloric ceca to distal intestine.

2.5 Stable isotope analysis

Tissue samples were freeze-dried and homogenized with two stainless steel beads 5 mm (Qiagen, Retsch GmbH, Haan, Germany) in a TissueLyser (Qiagen, Retsch GmbH, Haan, Germany) for two minutes at 20 Hertz. Diets and ingredients were ground in an Ultra Centrifugal Mill ZM 100

(Retsch GmbH, Haan, Germany) to a homogeneous powder. Molecules containing ^{14}N and ^{15}N differ in mass, and the ratio of these isotopes can be detected with an element analysis isotope ratio mass spectrometry (EA-IRMS). First, the sample was subjected to element analysis by being dropped into a heated reactor which contains an oxidant such as copper or chromium oxide. Samples were combusted at 1000 ℃ to produce N_2, NOx, H_2O, O_2 and CO_2. The abundance of the isotopes in the sample was then determined by mass spectrometry (Iso-analytical, 2018). Samples of approximately 1 mg were weighed into small tin capsules (8 x 5 mm, Elemental Microanalysis, Devon, UK). Samples were analysed for N-isotope composition using a Nu Horizon isotope-ratio mass spectrometer (IRMS) (Nu Instruments, Wrexham, UK) coupled to an Eurovector element analyser (EA) 3028 (Eurovector S.p.A, Redavalle, Italy) at the Institute for Energy Technology (Kjeller, Norway). Analysed content of ^{15}N in the diets is given in Table 2.

Isotopic signatures were reported as $\delta^{15}N$ values, and converted to atom percentage excess (APE) as follows (Fry, 2006):

$$\text{APE } ^{15}\text{N} = \left(\left(\frac{(\delta^{15}N_{Sample} + 1000)}{\left(\delta^{15}N_{Sample} + 1000 + \left(\frac{1000}{\delta^{15}N_{Standard}}\right)\right)} \right) 100 \right) - IA\ \%$$

where $\delta^{15}N_{Sample}$ and $\delta^{15}N_{Standard}$ are the proportion of ^{15}N in the ratios $^{15}N/^{14}N$ in the sample and in the reference standard (atmospheric N_2; $\delta^{15}N_{Standard}$ = 0.003663 IAEA (International Atomic Energy Agency) 305) and *IA %* is the initial atom percentage in $N_{standard}$. The APE ^{15}N after feeding with enriched feed will be proportional to the fraction of newly deposited amino acids in the tissue, resulting from both tissue growth and replacement of previously deposited protein, denoted as protein metabolism. Atom percentage excess ^{15}N is the total atom percentage ^{15}N in the sample adjusted for the IA %. When enrichment of ^{15}N was low (~0.5% ^{15}N) calibration of ^{15}N was performed against the international standards IAEA 305B and IAEA N-1, while when enrichment of ^{15}N was high (2% ^{15}N), calibration standards IAEA 311 and IAEA N-1 were used. Three samples of the internal reference material (IFE Trout) was analysed in the beginning, middle and end of a sequence (75-78 samples per sequence) and for every sixth tissue sample. The average $\delta^{15}N$ in IFE Trout, was 11.60‰ with a standard deviation of 0.20. The

corresponding $\delta^{15}N$ values for samples analysed according to IAEA 305B and IAEA 311 were 375.3 ± 0.96‰ and 4693 ± 4.49‰, respectively. The content of ^{15}N before feeding with enriched feed was expected very low. For 15 fish the IA % was on average 0.370 with a standard deviation of 0.0001, respectively.

2.6 Calculations and statistics

Feed conversion ratio (FCR) was calculated on a tank level as follows:

$$FCR = FI\ (FW\text{-}IW)^{-1}$$

where FI is feed intake (g dry matter) and FW and IW are final and initial weights (g) of fish, respectively. An average FCR over three tanks given the same diet was calculated. Further, relative weight gain for an individual, i.e., growth relative to final body weight, was calculated as follows:

$$relative\ weight\ gain = ((FW\text{-}IW)\ FW^{-1})\ 100)$$

An effect of diet on averages of recorded variables was tested by use of the following univariate model:

$$\bar{y}_{ij} = \mu + diet_i + \varepsilon_{ij}$$

where $\bar{y}_{ij}$ is average feed intake, initial and final weights, weight gain, relative weight gain and FCR in tank j (j = 1…15), μ is the overall mean, diet is the fixed effect of i^{th} diet (i = 1…5) and ε_{ij} is a random residual.

Simple linear regression as well as Pearson correlations between relative weight gain and APE ^{15}N in muscle, liver and mid-intestine were calculated per diet and sampling time points. All statistical analyses above were carried out by use of SAS® software, V.9.4 (SAS Inst. Inc., Cary, NC).

3. Results

All diets contained the same level of *Spirulina* and L-Lysine, but with different inclusion levels of their ^{15}N marked counterparts (Table 1). Chemical analysis of the diets (Table 2) showed that there were some minor differences in total amino acid content between diets, being somewhat lower for SP2 and L0.2. The enrichment percentage of ^{15}N was slightly under the formulated values in all diets, but all diets were isotopically distinct.

All diets were consumed as expected. Overall average and standard deviation for start and final weights per fish were 21 ± 2.5 g and 36.2 ± 14.1 g, respectively, with a relative weight gain over the 50 days of 35.6 ± 19.0%. Corresponding average feed intake over the 15 tanks throughout the 50-day period was 489.4 ± 42.4 g, and average FCR was 0.68 ± 0.013. The model showed no significant ($P < 0.05$) effect of diet on any of the traits tested, as expected due to the same chemical composition. Descriptive statistics of the different traits by diet are therefore given in Table 3.

Table 4 shows the correlations between APE ^{15}N and relative weight gain in muscle, liver or mid-intestine over time in diets with added ^{15}N. Correlations were generally positive (one exception), ranging 0.12-0.98, -0.09-0.94 and 0.03-0.90 in muscle, liver and mid-intestine, respectively (Table 4). The relationship between relative weight gain and APE ^{15}N in the muscle for all diets over time is shown in Figures 2A-E. For diets enriched with ^{15}N, there was a strong positive relationship between relative weight gain and APE ^{15}N in the muscle (Figures 2B-E), which is expected due to the fact that protein growth is necessarily based on deposition of newly consumed and thus enriched protein. As expected, a stable ^{15}N concentration over time was observed for fish given the control diet (Figure 2A). The same tendency was also evident for liver and mid-intestine (data not shown). The main differences between muscle, liver and mid-intestine were that liver and mid-intestine were nearly in equilibrium with the diets around day 50, due to faster metabolic rate in these tissues.

Among inclusion levels, the SP2 diet (2% inclusion level of ^{15}N) was the only diet estimated with significant correlations ($P < 0.05$) between APE ^{15}N and relative weight gain both in muscle

and liver at all time points (r = 0.82-0.97 and r = 0.59-0.94, respectively). These, correlations were, slightly lower and less stable in liver than in muscle tissue. For mid-intestine, many of the correlations between APE ^{15}N and relative weight gain were not significant.

Figure 3 shows that the level of isotope in muscle tissue for fish fed the SP2 diet over time asymptotes towards an equilibrium atom percentage. Moreover, Figure 3 indicates that the individual variation in isotope level was low in the beginning of the 50 day experimental period and increased in accordance with the individual relative weight gain before it approaches equilibrium. Similar shape of curves was, in fact, obtained for the other diets (data not shown). This implies that for all the diets, the individual variation in APE ^{15}N is likely highest when the tissue is, on average, 50% saturated with the isotope in the feed, leaving room for individual variation around the value. For the SP2 diet this occurred for an APE ^{15}N in body tissue being 1% (Figure 3).

4. Discussion

The basic idea of the current project was to develop a method for use of isotope enriched feed to assess individual feed efficiency in fish, without recording their individual feed intake. By switching from normal to isotope-enriched feed, the isotope profile of the fish will change accordingly. The change in the nitrogen isotope profile can be explained by protein metabolism, i.e., growth dilution in addition to replacement of existing body tissue (losses). Here, Atlantic salmon in the freshwater phase were fed diets with different inclusion levels, and the corresponding changes in relative weight gain and APE ^{15}N of tissues were monitored over time. Generally, the largest positive relationship between the two variables was found in muscle with APE ^{15}N approaching an asymptote over time for all diets. This implies that diets with variable inclusion of isotopes, either by enrichment or natural variation in isotope content, may be used for assessing feed efficiency. It is recommended to test until ~50% saturation with the isotope level in the feed.

The finding that the body content of the feed-enriched isotope increases with body growth is hardly surprising. Figures 2B-E show that fish of similar relative weight gain, but different

growth rates (i.e., reaching the same relative weight gain at different time points), had clearly different isotope contents, with the fastest-growing fish having the lowest content of enriched isotope. This cannot be attributed to growth dilution (as relative weight gain is similar), implying that fast growth results in reduced replacement of existing body tissue, and better FCR, likely due to less maintenance costs from a shorter growth period. The experiment indicates that individual differences in FCR are likely to be captured by individual differences in isotope profiles. Furthermore, within each time point, Figure 2, there is individual variation in protein metabolism between fish of similar relative weight gain. This can partly be explained by measurement errors, but potentially by variation in protein degradation between fish at the same relative weight gain. Variation in levels of APE ^{15}N (i.e., variable replacement of body nitrogen) may be explained by variation in maintenance requirements (per unit body protein and day), and relates to individual differences in protein efficiency. Likewise, Figure 2 shows individual variation in relative weight gain for fish at the same APE ^{15}N, again pointing to potential differences in maintenance requirements. It remains to explore whether some of this variance has a genetic component.

Stable isotope analysis is a well-established method to obtain measurements of protein metabolism in fish. Protein efficiency, where amount of ingested protein is accreted as growth in muscle has been shown to vary between groups of individuals, where efficient fish obtain a higher growth rate for a reduced protein degradation (Carter et al., 1993b; McCarthy, Houlihan, & Carter, 1994; Morgan, McCarthy, & Metcalfe, 2000). These studies have all been based on the flooding dose method (Garlick, McNurlan, & Preedy, 1980), injecting radioactive [^{3}H] phenylalanine in the caudal vein, with subsequent tissue measurements over a shorter period of time (1-6 hours). The isotope profiling in this study, however, is based on adding stable isotopes to feed and considers cumulative individual measures over longer period of time, herein up to three weeks dependent on the growth rate of the fish. Another advantage of labelling the feed, is the ability to trace the nutrient deposition and loss without disturbing the fish. Labelling the feed allows to measure the change in isotope profile for a large number of individuals, making the indicator trait more relevant in a selective breeding context. As for the flooding dose method, the footprint of isotopes is measured in tissues, herein muscle, liver and mid-intestine. In fish, muscle alone accounts for as much as 40-60% of the total body weight and is the main protein

accretion site (Verri, Terova, Dabrowski, & Saroglia, 2011), directly reflecting the whole-body growth (Peragon, Barroso, Garcıa-Salguero, de la Higuera, & Lupianez, 1999). Moreover, it has been proposed that protein metabolism in white muscle of fish could be used as a biochemical index for protein accretion and growth (Fauconneau, Gray, & Houlihant, 1995), which is confirmed in this study. In addition, significant correlations between APE ^{15}N and relative weight gain in liver and mid-intestine existed for all diets (Table 4). Liver as a highly metabolic tissue with high protein metabolism, as well as epithelial renewal in the mid-intestine can explain the correlation between APE ^{15}N and relative weigh gain in these tissues. Correlations between mid-intestine APE ^{15}N and relative weight gain throughout the experiment were unstable, and many were non-significant (Table 4), likely because the tissue was difficult to rinse properly. In summary, our results show that isotope profile changes in muscle, liver and mid-intestine can be effectively traced by feed labelling. The clear association between individual relative weight gain and individual APE ^{15}N in different tissues over time demonstrates that isotope profiling from labelling the feed accurately assess deposition of new protein especially in muscle, but also in the liver.

The EA-IRMS technique is an accepted method for analysis of $^{15}N/^{14}N$ ratios, the analysis can be used for samples with low ^{15}N-concentrations (Grassineau, 2006) and has very good precision (< 0.1% relative standard deviation) (Matthews & Hayes, 1978). Thus, enrichments of ^{15}N with an APE of 0.004 can be detected (Matthews & Hayes, 1978). In this experiment the SP2 diet with an enrichment as high as 1.974 APE ^{15}N gave the most stable correlations throughout the 50-day sampling period (Table 4). This stability was considered important because the purpose of this experiment was to investigate the potential of using the change in isotope profile in a relation to relative weight gain with a consequence for individual feed efficiency. In addition, an increasing isotopic variance was observed (Figure 3) throughout the experiment, which can be explained by individual differences in growth and tissue replacement. However, the relationship between average level and time was non-linear, and the level asymptotes when the body tissues approach equilibrium with ^{15}N in the diet. At equilibrium, the isotope profile is fully dictated by the diet, and no variation among fish is expected (as observed prior to the feed trial). In contrast, the variation between fish will be highest when, on average, ~50% saturation is reached (i.e., saturation varies among fish). Due to high growth rates, fish reached ~50% saturation at day 25

for the SP2 diet. Relative growth rates vary considerably among species and life-stages within species, and the experiment should be adapted to each specific case.

Growth can be explained by increased protein synthesis, reduction in protein degradation and high accretion rate of proteins to skeletal muscle (Carter, Houlihan, Brechin, & McCarthy, 1993a; McCarthy et al., 1994; Morgan et al., 2000). The strong relationship between muscle protein metabolism and relative weight gain can be explained by the fact that 80% of the synthesized proteins in white muscle are retained as net protein accretion (Houlihan & Laurent, 1987). McCarthy et al. (1994) compared groups (n = 6) of protein "efficient" and "inefficient" rainbow trout (*Oncorhynchus mykiss*) where faster growing and more efficient fish showed reduced degradation rates, and differences in growth correlated to protein metabolism differences. Differences in protein degradation rates were important determinants of their reported variation of growth efficiencies. Our individual differences corresponded well with the results of McCarthy et al. (1994): fast-growing fish showed lower protein degradation in muscle, when compared at the same relative weight gain, as explained above. Correspondingly, Hawkins, Day, Rusin, and Worrall (1989) have reported genotype-dependent differences in protein metabolism in mussels *(Mytilus edulis)*. Results in the current study indicate that efficient fish are characterized by a high ratio between relative weight gain and change in isotope profile, meaning high gain for low cost, by individual variation in feed efficiency.

According to literature (e.g., Thodesen et al., 2001), a favourable correlation is expected between feed efficiency and growth rate. Given that isotope profiling can be used to assess feed efficiency on an individual level, this should be observed as a lower content of APE ^{15}N in faster-growing (more efficient) fish, which should be evident from the isotopic profiles. The findings in this study support this. The results indicate that individual isotope profiles can be used as an indicator trait of individual feed efficiency in fish.

5. Conclusions

Results show that the stable isotope ^{15}N added to feed and accumulated over time in muscle and liver has the potential to be a promising biomarker for revealing insight into individual feed

efficiency of fish. Results show that fast growth results in reduced replacement of existing body tissue and improved FCR, interpreted as reduced degradation rates, i.e., reduced maintenance requirement. Efficient fish are characterized by a high ratio between relative weight gain and change in isotope profile, meaning high gain for low cost or improved feed efficiency. Adding stable isotopes to feed can be done for a considerable number of fish and has therefore a potential in a breeding context. It is recommended to feed with stable isotopes to reach 50% saturation.

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Competing interests

The authors declare that they have no competing interest.

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Table 1

Formulated composition of experimental diets.

	Dietary treatments				
Formulation, g kg^{-1}	Control	SP1	SP2	L0.1	L0.2
Fish meal[†]	460	460	460	460	460
Gelatinized potato starch[‡]	130	130	130	130	130
Wheat gluten[§]	129.6	129.6	129.6	129.6	129.6
Spirulina[¶]	20.0	10.0	0.0	20.0	20.0
Spirulina ^{15}N[††]	0.0	10.0	20.0	0.0	0.0
L-Lysine[‡‡]	2.0	2.0	2.0	1.0	0.0
L-Lysine ^{15}N[§§]	0.0	0.0	0.0	1.0	2.0
Fish oil[¶¶]	170.0	170.0	170.0	170.0	170.0
Gelatine[†††]	80.0	80.0	80.0	80.0	80.0
Premix fish[‡‡‡]	6.3	6.3	6.3	6.3	6.3
Monocalcium phosphate [§§§]	2.0	2.0	2.0	2.0	2.0
Y_2O_3[¶¶¶]	0.1	0.1	0.1	0.1	0.1

[†]Norse LT 16-001, Norsildmel, Egersund Sildoljefabrikk AS, Egersund, Norway.

[‡]Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden.

[§]Vital Wheat Gluten, Amilina, Panevezys, Lithuania.

[¶]CIL-ULM-8453 Spirulina Whole cells (unlabelled), Cambridge Isotope Laboratories, Larodan, Solna, Sweden.

[††]CIL-NLM-8401 Spirulina Whole cells (U-^{15}N, 98%+), Cambridge Isotope Laboratories, Larodan, Solna, Sweden.

[‡‡]L5751 L-Lysine dihydrochloride (98% unlabelled), Sigma-Aldrich, St. Louis, USA.

[§§]CIL-NLM-143 L-Lysine*2HCl (alfa-^{15}N, 95-99%), Cambridge Isotope Laboratories, Larodan, Solna, Sweden.

[¶¶]NorSalmOil, Norsildmel, Bergen, Norway.

[†††]Rousselot® 250 PS, Rousselot SAS, Courbevoie, France.

[‡‡‡]Farmix, Trouw Nutrition, LA Putten, The Netherlands. Per kg feed; Retinol 2500.0 IU, Cholecalciferol 32400.0 IU, α-tocopherol SD 0.2 IU, Menadione 40.000 mg, Thiamine 15.0 mg, Riboflavin 25.0 mg, d-Ca-Pantothenate 40.002 mg, Niacin 150.003 mg, Biotin 3000.0 mg, Cyanocobalamin 20.0 mg, Folic acid 5.0 mg, Pyridoxine 15.0 mg, Ascorbate polyphosphate 0.098 g, Cu: CuSulfate 5H2O 11.998 mg, Zn: ZnSulfate 89.992 mg, Mn: Mn(II)Sulfate 34.993 mg, I: K-Iodine 1.999 mg, Se: Na-Selenite 0.200 mg, Cd Max. 0.0003 mg, Pd Max. 0.028 mg, Ca 0.915 g, K 1.380 g, Na 0.001 g, Cl 1.252 g.

[§§§]Bolifor®MCP-F.KPP Oy, Animal Nutrition, Helsingborg, Sweden.

[¶¶¶]Yttrium oxide (Y_2O_3), Metal Rare Earth Limited, Shenzhen, Guangdong, China.

Table 2

Analysed chemical composition of experimental diets.

	Dietary treatments[†]				
Chemical composition	Control	SP1	SP2	L0.1	L0.2
Analysed content, kg^{-1}					
Dry matter, g	910.6	910.6	911.2	922.0	924.3
Ash, g	76.4	75.8	76.5	76.2	76.6
Crude protein, g	503.6	507.2	503.3	506.6	509.8
Starch, g	121.0	119.0	121.0	124.0	125.0
Lipid, g	170.4	170.6	174.7	183.4	182.1
Gross energy, MJ	22.3	22.2	22.2	22.5	22.6
Analysed content, %					
^{15}N, measured (%)[‡]	0.003	0.964	1.974	0.081	0.151
Essential amino acids, g kg^{-1}					
Arginine	32.3	32.2	30.4	32.4	31.4
Histidine	9.7	9.5	8.8	9.6	9.0
Isoleucine	20.3	19.9	18.3	19.9	18.5
Leucine	35.0	34.6	32.2	34.7	33.2
Lysine	31.3	30.5	28.4	30.8	29.3
Methionine	11.2	10.9	10.2	11.1	10.4
Phenylalanine	20.8	20.6	19.3	20.6	19.4
Threonine	19.1	18.9	17.8	19.2	18.2
Valine	23.2	22.8	21.3	22.9	21.5
Tryptophan	4.7	4.6	4.2	4.6	4.6
Non-essential amino acids, g kg^{-1}					
Alanine	28.7	28.7	27.3	28.9	28.0
Aspartic acid	40.3	39.7	37.4	40.2	37.3
Glycine	36.3	36.8	35.6	36.6	35.8
Glutamic acid	95.3	95.2	88.6	93.6	89.3
Cysteine	5.5	5.4	5.1	5.3	5.4
Tyrosine	11.7	11.7	10.7	11.8	10.9
Proline	38.1	38.4	36.5	37.4	35.5
Serine	22.3	22.3	21.3	22.5	21.9
Sum amino acids	485.7	482.9	453.2	482.2	459.7

[†]All analyses was a mean of duplicates except atom percentage excess ^{15}N being an average of triplicates.

[‡]Atom percentage excess ^{15}N.

Table 3

Means and standard deviations of diets for initial and final weights as well as for weight gain, relative weight gain, feed intake and feed conversion ratio, over the 50 day test period.

	Dietary treatments				
	Control	SP1	SP2	L0.1	L0.2
Initial weight, g	711.1 ± 10.3	717.2 ± 6.0	713.0 ± 18.6	699.5 ± 3.4	709.6 ± 14.4
Final weight, g	1478.9 ± 57.6	1446.6 ± 49.5	1364.1 ± 28.2	1456.1 ± 66.0	1412.8 ± 20.5
Weight gain, g	767.8 ± 64.6	729.4 ± 54.6	651.1 ± 41.9	756.6 ± 64.1	703.1 ± 30.0
Relative weight gain, %	36.3 ± 2.4	35.7 ± 1.3	33.9 ± 1.84	35.7 ± 1.48	36.5 ± 1.9
Feed intake, g	520.7 ± 41.6	498.8 ± 41.1	437.9 ± 9.4	516.7 ± 39.2	473.0 ± 20.2
Feed conversion ratio	0.68 ± 0.003	0.68 ± 0.008	0.67 ± 0.03	0.68 ± 0.007	0.67 ± 0.005

Table 4

Pearson correlation coefficients (r) between atom percentage excess (APE) ^{15}N in muscle (M), liver (L) and mid-intestine (MI) and relative weight gain over time (n = 12, at 2, 4, 8, 16, 32 and 50 days) in four ^{15}N enriched diets. Significant correlations ($P < 0.05$) are indicated with asterisks (*).

	Days																	
	2			4			8			16			32			50		
	Trait																	
Diet	M	L	MI	M	L	MI	M	L	MI	M	L	MI	M	L	MI	M	L	MI
SP1	0.87*	0.66*	0.56	0.31	0.76*	0.65*	0.51	0.60*	0.49	0.92*	0.72*	0.75*	0.83*	0.62*	0.78*	0.98*	0.90*	0.86*
SP2	0.89*	0.88*	0.22	0.82*	0.72*	0.63*	0.97*	0.94*	0.90*	0.92*	0.91*	0.70*	0.85*	0.59*	0.25	0.95*	0.83*	0.54
L0.1	0.77*	0.39	0.41	0.81*	0.48	0.68*	0.53	0.51	0.24	0.72*	0.16	0.47	0.67*	0.78*	0.90*	0.75*	-0.09	0.43
L0.2	0.55	0.73*	0.59*	0.12	0.23	0.03	0.82*	0.49	0.78*	0.25	0.40	0.70*	0.46	0.68*	0.83*	0.92*	0.34	0.36

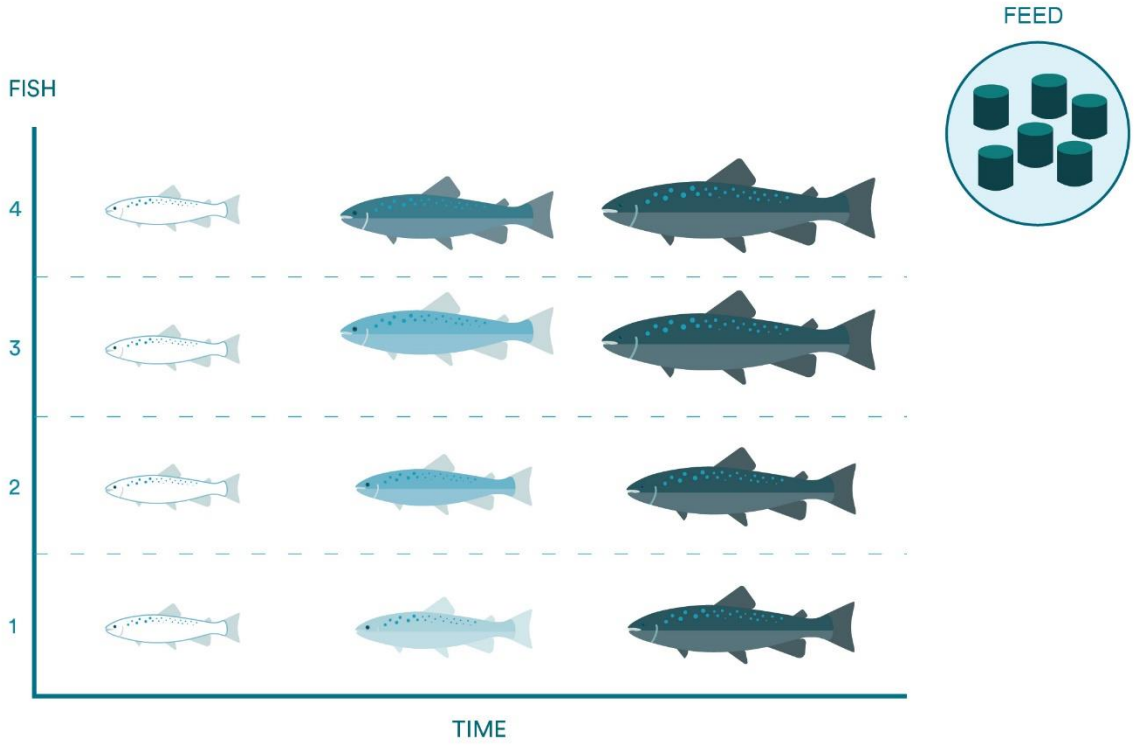

Figure 1. A schematic drawing illustrating the overall reflection of the regulation of growth over time, synthesized on a new diet, with different colored fish resembling the metabolic breakdown of tissues that were synthesized during feeding on the previous diet, and their subsequent replacement and growth of tissues synthesized on a new diet.

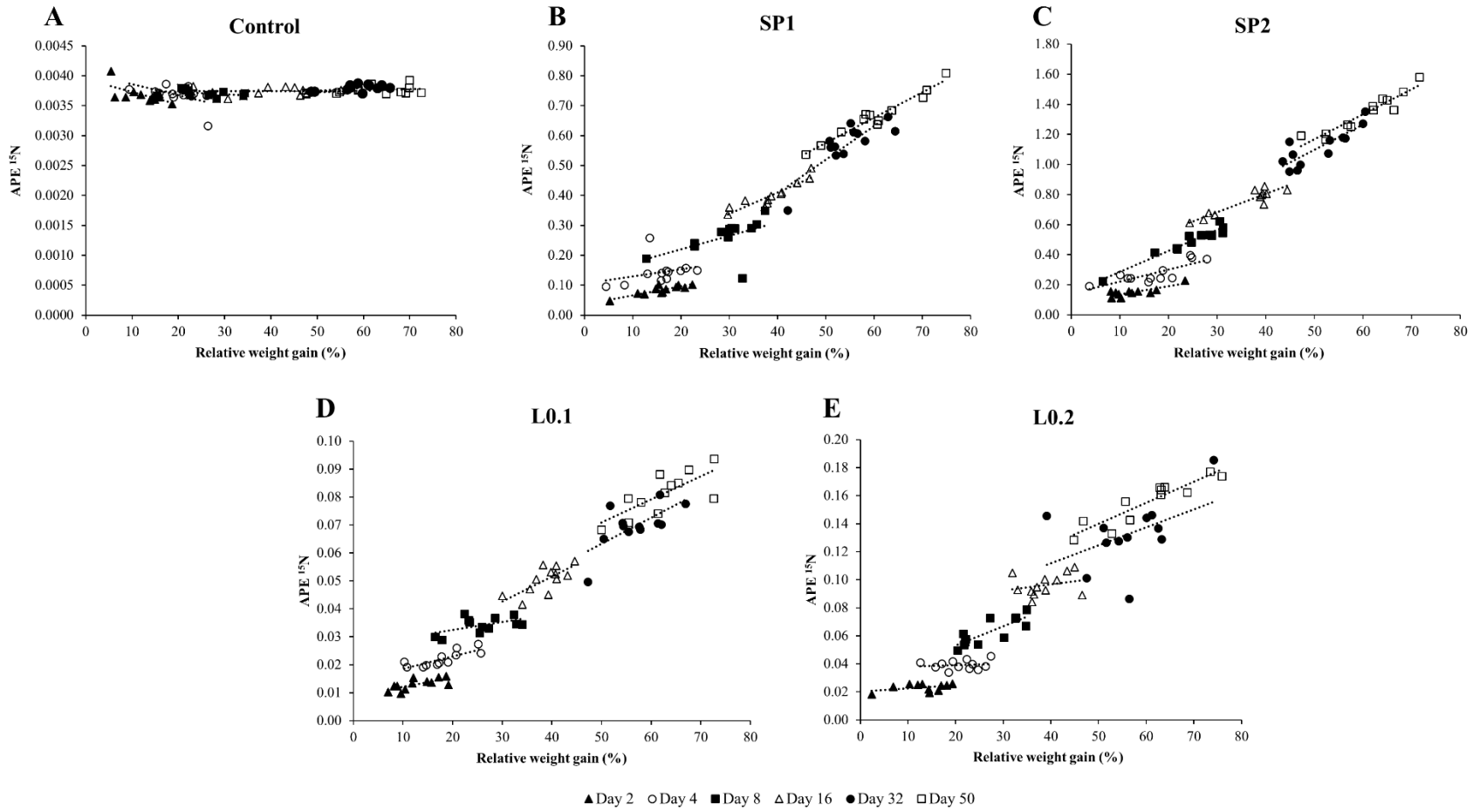

Figure 2. Regression lines for the relationship between atom percentage excess (APE) ^{15}N in muscle and individual relative weight gain over time (n = 12 at 2, 4, 8, 16, 32 and 50 days), for the (A) control (0% inclusion of ^{15}N), (B) SP1 (1% inclusion of ^{15}N from Spirulina), (C) SP2 (2% inclusion of ^{15}N from Spirulina), (D) L0.1 (0.1% inclusion of ^{15}N from L-Lysine) and (E) L0.2 (0.2% inclusion of ^{15}N from L-Lysine) diets.

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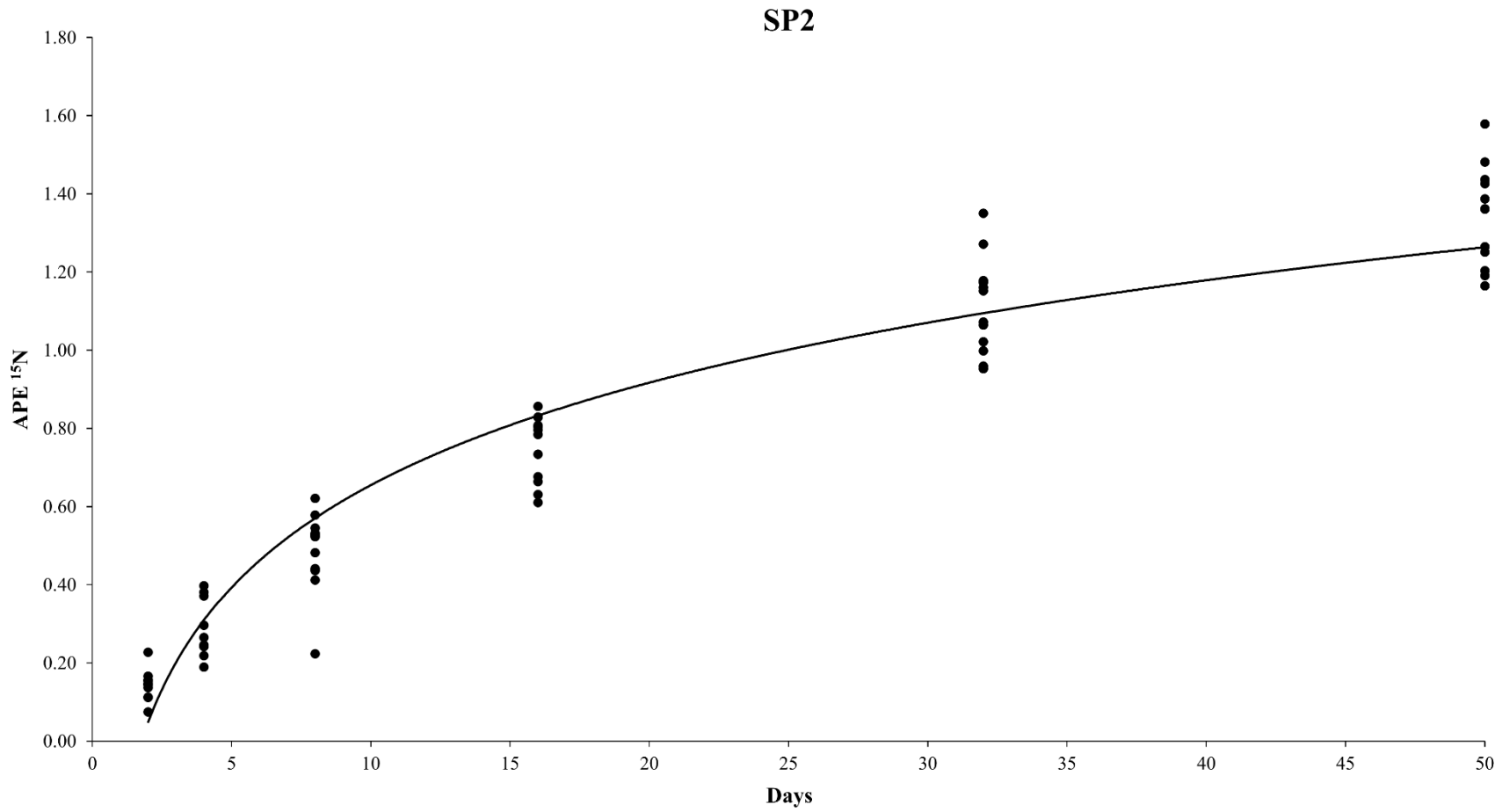

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520 **Figure 3.** Atom percentage excess (APE) ^{15}N and individual isotopic variation in muscle over time (n = 12 at 0, 2, 4, 8, 16, 32 and 50
521 days) in fish fed the SP2 (2% inclusion of ^{15}N) diet. The estimated regression line was: $y = 0.38\ln(X) - 0.22$, with $R^2 = 0.93$.