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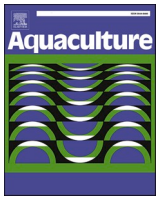

# Isotope fractionation in juvenile and large rainbow trout (*Oncorhynchus mykiss*): Repeatability of stable isotope measures and their relationship to growth rate

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ABSTRACT

The study aimed to assess the repeatability of isotope ratios, $\delta^{13}C$ and $\delta^{15}N$ in four tissues (muscle, liver, adipose fin, and visceral adipose) and the relationship between growth rate and these measures in rainbow trout (*Oncorhynchus mykiss*). Tissue samples were from two experiments with, respectively, juvenile ($n$ = 10; Experiment 1, one tank) and large ($n$ = 120; Experiment 2, three tanks) rainbow trout, fed commercial diets. In Experiment 2, the estimated repeatability was high for both $\delta^{13}C$ and $\delta^{15}N$ in muscle and liver, making one measure suffice ($r$ = 0.70–0.78 and $r$ = 0.80–0.91, respectively). Using regression analysis, bioaccumulation of $\delta^{13}C$ in muscle and $\delta^{15}N$ in liver (over life), through fractionation (without feed enrichment or depletion strategies) was found to be, respectively, negatively and positively associated with growth rate. Even though individual feed intake was not recorded, the significant relationship between GR (that correlates with feed efficiency) and, respectively, MC and LN, suggests isotopic fractionation as potential biomarkers for individual cumulative (over-life) feed efficiency in fish.

## 1. Introduction

The Norwegian aquaculture industry has grown significantly since its initiation in the 1970s, with 2 million tons of feed used in 2020 (Directorate of Fisheries, 2020). Moreover, during the grow-out phase, the Directorate of Fisheries (2020) states that 46% of the production cost in the sea comes from feed, with an economical feed conversion ratio of 1.32. Thus, developing sustainable aquaculture to lower this ratio is considered imperative since it will reduce production costs per kilo of fish produced and the environmental footprint (Besson et al., 2016; de Verdal et al., 2011). Consequently, it is crucial to assess how to best select for feed efficiency in aquaculture breeding programs (Gjedrem, 2005).

In practice, selection for improved feed efficiency has been carried out by indirect selection for increased growth rate, since individual body weight is easy to record, and all fish are harvested at the same age (e.g., Kristjánsson et al., 2020). Faster-growing fish will reach appropriate harvest weight at a younger age and is expected to reduce feed costs for body maintenance, improving FCR, but direct selection should have advantages since the genetic correlation to growth is not perfect (Dvergedal et al., 2019b; Henryon et al., 2002; Kinghorn, 1983). However, a complication in salmon farming is that fish are typically kept in large sea-cage units and fed communally by dispersing feed into the water, making individual recording of feed intake under commercial-like conditions practically impossible with current methods. An alternative is to decompose feed efficiency into digestive, metabolic, and deposition efficiency (Dvergedal et al., 2019b, 2019c, 2022, respectively), with indicators for each component being developed. Digestive efficiency is the fraction of feed nutrients being absorbed. Metabolic efficiency of growing animals can be defined as the fraction of turnover allocated to growth, while deposition efficiency is the efficiency of the growth process itself, i.e., efficiency of metabolite conversion into body tissues (fat vs. protein deposition).

Regarding metabolic efficiency phenotyping through, the use of stable isotope profiling and diet-switch experiments were used initially (Dvergedal et al., 2019a, 2019b). In the latter experiments and since all organic compounds contain carbon, while nitrogen is common to all amino acids the feed was both artificially enriched with $^{15}N$ and $^{13}C$ labeled Spirulina. The $^{15}N$ and $^{13}C$ concentrations were set so high

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(~1000‰, i.e., for $\delta^{13}C = \left(\frac{\frac{^{13}C}{^{12}C}Sample}{\frac{^{13}C}{^{12}C}Standard} - 1\right)1000$, and correspondingly for $\delta^{15}N$; for details see 2.4) that the effect of individual variation in isotope ratio prior to the experiment (e.g., 1–2.5‰) would become negligible. However, the cost of $^{15}N$ and $^{13}C$ labeled Spirulina was very high, and a more cost-effective alternative based on feedstuffs with a higher natural abundance of the isotopes (enriched with $^{13}C$ from corn gluten) was used in a subsequent experiment (Dvergedal et al., 2022). In these experiments, as mentioned, it was assumed that the results were not significantly affected by individual variation in isotope ratio prior to the experiments, denoted isotope fractionation. Fractionation is the preferential metabolic retention of the heavier isotopes, meaning that the heavy isotopes $^{15}N$ and $^{13}C$ tend to bioaccumulate, whereas the light isotopes $^{14}N$ and $^{12}C$ become preferentially excreted (Gamboa-Delgado, 2021; Fry, 2006). This causes an animal-diet fractionation, i.e., a contrast (Δ) between δ-values of the heavy isotopes in the animal to that in the diet (taking carbon as an example): $\Delta^{13}C = \delta^{13}C_{animal} - \delta^{13}C_{diet}$. However, if all animals in a group are fed an identical diet (as is the case here), the isotope ratio of the diet will be reduced to a constant, and fractionation assumed equal to the isotope ratio of the tissue.

According to the literature, an efficient fish will due to reduced animal-diet isotopic fractionation have an isotope profile closer to the fed diet (Cantalapiedra-Hijar et al., 2015, 2016; Cheng et al., 2013, 2015; Meale et al., 2018; Nasrollahi et al., 2020; Wheadon et al., 2014). The fractionation is due to excretion of waste products (ammonia, urea, and $CO_2$), expected to increase with the fraction of feed carbon and nitrogen being excreted. The above literature proposes isotope fractionation as an indicator trait for feed efficiency in livestock, but it has also been suggested for fish (Gaye-Siessegger et al., 2004; Martin-Perez et al., 2013; Trueman et al., 2005), and isotopic fractionation in growing animals may be considered an indicator trait (biomarker) for cumulative efficiency over the entire lifespan of the animal. Thus, one aim of this study was to assess the repeatability of recorded stable isotope profiles of $\delta^{13}C$ and $\delta^{15}N$ in various tissues of both juvenile and large rainbow trout (*Oncorhynchus mykiss*). Another aim was to examine the relationship between growth (as an indirect trait for feed efficiency) and isotopic fractionation in muscle, liver, adipose fin, and visceral adipose tissue. At least in the muscle, such an association between relative growth rate and isotope fractionation was expected to the extent that growth improves feed efficiency, and that animal-diet fractionation is an indicator for feed efficiency. The logic is that a fish using less energy for maintenance will have the potential to allocate these resources to growth and become more feed efficient. Thus, a relationship is expected between growth and isotopic fractionation and we, therefore, used growth rate as a proxy for feed efficiency.

## 2. Materials and methods

### 2.1. Samples

The fish were sampled at the Center for Sustainable Aquaculture at the Norwegian University of Life Sciences (NMBU), Aas, Norway, following the laws and regulations for experiments on live animals in the EU (Directive 2010/637EU) and Norway (FOR-2015-06-18-761). In Experiment 1, 10 rainbow trout with an average body weight of 205.5 g, kept in one tank, were sampled on the same day, while the number of fish in Experiment 2 was 120 with an average body weight of 1546.1 g, sampled from three tanks over two consecutive days and the third sampling one week later. In both experiments, fish had been kept in freshwater. They were of both sexes from the breeding company AquaGen AS. The fish were fed commercial diets from Skretting AS (Experiment 1: Nutra Olympic 3 mm with 46–50% protein, 23% fat, $\delta^{13}C$ of −24.4, and $\delta^{15}N$ of 7.6; Experiment 2: Select Rakfisk 80A 4 mm with approximately 41% protein, 25% fat, $\delta^{13}C$ of −24.2, and $\delta^{15}N$ of 6.9) by automatic feeders. The feeding level equaled 1% of the estimated biomass in the tank. The squared tanks (height = 1.0 m, length, and width = 2 × 2 m) with a 3000 l capacity were supplied with fresh water from the recirculating aquaculture system (RAS), at a flow rate of 8 l $min^{-1}$, and an average temperature of 15 °C. The fish were kept under a 24 h light regime. The tanks were controlled by OxyGuard water quality monitoring and control systems for aquaculture (OxyGuard International AS, Denmark), and the water quality was within legal legislation (Experiment 1: <0.05, <0.02, and NA mg $l^{-1}$ for ammonium ($NH_4$+), nitrite ($NO_2$-), and nitrate ($NO_3$-), respectively; Experiment 2: <0.05, 0.03, and 8.15 mg $l^{-1}$ for ammonium, nitrite, and nitrate). Dissolved oxygen was measured daily in the outlet water (Handy Delta, OxyGuard® AS, Farum, Denmark) and maintained above 7.5 mg $l^{-1}$.

### 2.2. Tissue sampling

Fish were anesthetized with Finquel vet. (Tricaine methanesulfonate; Scanvacc; 0.5 g $l^{-1}$ water) and killed with a sharp blow to the head prior to dissection. The sampling of tissues from muscle, liver, and adipose fin was sampled in Experiments 1 and 2, and additionally, visceral adipose tissue was sampled in Experiment 2. Tissue samples were snap-frozen in liquid nitrogen and stored at −20 °C until stable isotope analysis. Tissue sampling was standardized; Muscle was sampled in front of the dorsal fin on the left side of the filet (1 × 1 cm cube), the liver was divided into four small pieces, the whole adipose fin was frozen but only the tip of the adipose fin was utilized for stable isotope analysis in Experiment 2, and the adipose tissue deposited around the gut from the pyloric ceca until the distal intestine was sampled.

### 2.3. Muscle lipid extraction

In the diet, lipids are typically depleted in $^{13}C$ relative to protein and carbohydrates, and individual variation in fat content can thus be confounded with the incorporation of $^{13}C$ from protein and carbohydrates (Post et al., 2007). Thus, lipid extraction was carried out in the muscle samples in Experiment 2 (larger fish) because this tissue was our main tissue of interest for efficiency. To deal with this, several solutions have been suggested (Wessels and Hahn, 2010); one is chemical extraction of lipids from samples (Logan et al., 2008; Post et al., 2007; Wessels and Hahn, 2010). Thus, in Experiment 2, the muscle sample was split into two samples. In one subsample, stable isotopes were analyzed directly (resulting variable denoted MC, below) while in the other subsample (denoted MCP, below), we extracted lipids from the muscle by adding 1000 μl of 10:5:4 methanol:chloroform:water to a finely ground sample (21.6–379.9 mg) and vortexed the mixture before centrifugation (Bligh and Dyer, 1959; Pinnegar and Polunin, 1999). The mixture was spun down at 5000 x*g* for 10 min, and after centrifugation, the supernatant was discarded. The procedure was repeated three times (repeated until the supernatant was blank). On the final run, 1000 μl of methanol was added, and the solution was centrifuged at 5000 x*g* for 10 min. The sample was left in the fume hood for 10 min for evaporation of the methanol. The pellet retained was dried at 60 °C overnight.

### 2.4. Stable isotope analysis

Tissue samples (muscle, liver, adipose fin, and visceral adipose tissue) were freeze-dried and homogenized (except adipose fin), and approximately 1 mg per sample was weighed into small tin capsules (8 × 5 mm, Elemental Microanalysis, Devon, UK). Samples were analyzed for C- and N-isotope compositions using a Nu Horizon isotope-ratio mass spectrometer (IRMS) (Nu Instruments, Wrexham, UK) coupled to a Eurovector element analyzer (EA) 3028 (Eurovector S.p. A, Redavalle, Italy) at the Institute for Energy Technology (Kjeller, Norway). Both $\delta^{13}C$ and $\delta^{15}N$ (taking $\delta^{13}C$ as an example) were calculated as follows (Fry, 2006):

$$\delta^{13}C = \left( \frac{\frac{C_{13}}{C_{12}} Sample}{\frac{C_{13}}{C_{12}} Standard} - 1 \right) 1000,$$

where the two ratios are the proportions of $^{13}C$ or $^{15}N$ divided by the proportion of $^{12}C$ or $^{14}N$, in the sample and the reference standard respectively, per ‰: Vienna Pee Dee Belemnite for carbon, VPDB; $\delta^{13}C_{Standard} = 0.0112372$ (Craig, 1957), and air for nitrogen; $\delta^{15}N_{Standard} = 0.003676$ (Coplen et al., 1992).

The linearity calibration of $^{13}C$ and $^{15}N$ was performed by plotting all average values from triplicate analysis of international certified reference materials from the United States Geological Survey, USGS64 (average $^{15}N = 1.76$ and $^{13}C = -41.81$) and USGS66 (average $^{15}N = 40.83$ and $^{13}C = 0.67$). For every 25 samples, triplicate analysis of USGS65 was carried out and the average was used to perform operation correction. In addition, a correction was done for variation in the δ-values as a function of the peaked value/amount of sample.

### 2.5. Phenotypes analyzed

Final weight (FW) was recorded for each fish as well as their age (Age, as the number of days from hatching), and from this, the growth rate over life was calculated for each fish as:

$$GR = \frac{FW}{Age}.$$

From the tissue samples, the following individual variables were available for all fish: $\delta^{13}C$ and $\delta^{15}N$ in muscle (MC and MN), $\delta^{13}C$ and $\delta^{15}N$ in liver (LC and LN), and $\delta^{13}C$ and $\delta^{15}N$ in adipose fin (FC and FN). Furthermore, in the large rainbow trout (Experiment 2) $\delta^{13}C$ and $\delta^{15}N$ in lipid-extracted muscle (MCP and MNP) as well as $\delta^{13}C$ in visceral adipose tissue (AC) were available.

### 2.6. Repeatability of stable isotope analysis

Repeatability was based on all fish for juveniles ($n = 10$, Experiment 1) and a random sample of large fish in Experiment 2 ($n = 41$), to reduce costs of analyses. However, note that repeated measures of MCP and MNP were not made in Experiment 2, because this was done *posterior* to the repeated analysis of individual samples. In juvenile fish (Experiment 1), the samples from muscle were analyzed three times for isotopes per fish, and tissues from liver and adipose fin were correspondingly analyzed four times (one fish had three analyses for the adipose fin, and one fish had five analyses for all three tissues). The repeated measures of the isotope variables were exposed to the following statistical model:

$$Y_{ij} = \mu + a_i + \varepsilon_{ij},$$

where $Y_{ij}$ is the $j^{th}$ isotope measurement for fish $i$, $\mu$ is the intercept, $a_i$ is the random effect of the $i^{th}$ fish ($N \sim 0$, $\sigma_f^2$), and $\varepsilon_{ij}$ is a random residual for the $j^{th}$ observation ($N \sim 0$, $\sigma_e^2$).

In large fish (Experiment 2), samples from muscle, liver, adipose fin, and visceral adipose tissue were analyzed four times for isotopes per fish and tissue (except for one fish, where only muscle was analyzed three times). Prior to the analysis, the data was visually inspected for outlier detection, and one observation was removed for MC, FC, and FN, while two for MN, and five for AC. Then, the repeated measures of the isotope variables were scrutinized by use of this analysis of variance model:

$$Y_{ijk} = \mu + a_i + tank \times day_j + \varepsilon_{ijk},$$

where $Y_{ijk}$ is the $k^{th}$ isotope measurement for fish $i$, $\mu$ is the intercept, $a_i$ is the random effect of the $i^{th}$ fish ($N \sim 0$, $\sigma_f^2$), $tank \times day_j$ is the random effect of the $j^{th}$ $tank \times day$ ($N \sim 0$, $\sigma_{td}^2$), and $\varepsilon_{ijk}$ is a random residual for the $k^{th}$ observation ($N \sim 0$, $\sigma_e^2$).

The analyses were conducted using ASReml-R V.4 (Butler et al., 2018), and repeatability ($r$) was calculated as:

$$r = \frac{\sigma_f^2}{\sigma_p^2},$$

where $\sigma_p^2$ is the phenotypic variance.

In both models, the significance of the variance component of fish (which is a test of whether $r$ is significantly larger than 0) was tested using a likelihood-ratio ($LR$) test-statistic, comparing a single-trait model with effect of fish ($H_1$) to a model without ($H_0$):

$$LR = 2\left( (logL|\widehat{\theta}_{H_1}) - \left(logL|\widehat{\theta}_{H_0}\right) \right),$$

where $\widehat{\theta}$ denotes the parameters estimated under both models. The effect of fish was considered significant if $LR > \chi^2_{(\alpha=0.05;\ df=1)}$, $\alpha$ being the test statistics for one degree of freedom (df).

### 2.7. Associations between isotope and growth rate in juveniles

In Experiment 1, the regression of, in turn, each of the explanatory variables, utilizing one randomly sampled record per fish for variables with repeated measures (because GR was observed only once): MC, MN, LC, LN, FC, and FN ($X_i$'s) on GR ($Y_i$) were estimated with the following regression model:

$$Y_i = \beta_0 + \beta_1 X_i + e_i,$$

where $\beta_0$ is the intercept, $\beta_1$ is the regression coefficient of one of the isotope-derived variables $X_i$, and $e_i$ is the residual error term. The analysis was carried out by SAS®, V.9.4 (SAS, Inst. Inc., Cary, NC), and the PROC REG procedure. In this analysis, the coefficient of determination of prediction was computed as:

$$\widehat{R}^2 = 1 - \frac{PRESS}{SS_{tot}},$$

where $PRESS = \sum \left(y_i - \widehat{y}_i\right)^2$, with $\widehat{y}_i$ being the predicted individual phenotype using data from all other individuals in the analysis, and $SS_{tot}$ is the total sum of squares. The $\widehat{R}^2$ is an estimate of the fraction of variance in GR explained by the model in the prediction of missing observations under leave-one-out cross-validation.

**Table 1**
Descriptive statistics of observed variables in juvenile (Experiment 1) and large rainbow trout (Experiment 2).

| Experiment | Variable | N | Mean | Min | Max | SD |
|---|---|---|---|---|---|---|
| 1 | FW, g | 10 | 205.5 | 173.9 | 243.1 | 22.4 |
| | GR, g/day | 10 | 0.8 | 0.7 | 1.0 | 0.9 |
| | MC, ‰ | 10 | −23.4 | −24.5 | −22.6 | 0.6 |
| | MN, ‰ | 10 | 8.9 | 8.5 | 9.3 | 0.3 |
| | LC, ‰ | 10 | −23.1 | −24.0 | −22.7 | 0.4 |
| | LN, ‰ | 10 | 8.6 | 8.1 | 9.2 | 0.3 |
| | FC, ‰ | 10 | −20.8 | −21.1 | −20.5 | 0.2 |
| | FN, ‰ | 10 | 8.5 | 8.0 | 9.0 | 0.4 |
| 2 | FW, g | 120 | 1546.1 | 542.0 | 2195.0 | 326.4 |
| | GR, g/day | 120 | 3.8 | 1.3 | 5.5 | 0.8 |
| | MC, ‰ | 120 | −24.3 | −26.8 | −21.5 | 0.9 |
| | MCP, ‰ | 116 | −24.3 | −29.0 | −22.1 | 0.9 |
| | MN, ‰ | 120 | 9.5 | 7.7 | 12.5 | 0.7 |
| | MNP, ‰ | 116 | 10.6 | 9.2 | 11.6 | 0.4 |
| | LC, ‰ | 120 | −24.0 | −25.6 | −15.7 | 1.1 |
| | LN, ‰ | 120 | 9.6 | 7.8 | 12.4 | 0.8 |
| | FC, ‰ | 120 | −22.1 | −25.4 | −19.2 | 1.2 |
| | FN, ‰ | 120 | 8.8 | 6.5 | 11.3 | 0.7 |
| | AC, ‰ | 120 | −27.2 | −28.6 | −24.5 | 0.4 |

FW = Final weight; GR = Growth rate; MC = $\delta^{13}C$ in muscle; MCP = $\delta^{13}C$ in muscle protein; MN = $\delta^{15}N$ in muscle; MNP = $\delta^{15}N$ in muscle protein; LC = $\delta^{13}C$ in liver; LN = $\delta^{15}N$ in liver; FC = $\delta^{13}C$ in fin; FN = $\delta^{15}N$ in fin; AC = $\delta^{13}C$ in visceral adipose tissue.

**Table 2**
The number of records and estimated variance components for individual ($\sigma_f^2$, with *P*-value of being different from zero), the estimated repeatability of $\delta^{13}C$ and $\delta^{15}N$ in muscle (MC and MN), liver (LC and LN), adipose fin (FC and FN), and for $\delta^{13}C$ in the visceral adipose tissue (AC) (*r*, with their standard errors, *SE*) in juvenile (Experiment 1) and large rainbow trout (Experiment 2).

| Experiment | Variable | *N* | $\sigma_f^2$ | *P* | *r* | *SE* |
|---|---|---|---|---|---|---|
| 1 | MC | 31 | 0.30 | $2.3 \times 10^{-12}$ | 0.94 | 0.14 |
| | MN | 31 | 0.02 | 0.01 | 0.49 | 0.01 |
| | LC | 41 | 0.16 | $9.2 \times 10^{-19}$ | 0.95 | 0.07 |
| | LN | 41 | 0.04 | 0.0001 | 0.54 | 0.02 |
| | FC | 40 | 0.01 | 0.16 | 0.21 | 0.01 |
| | FN | 40 | 0.02 | 0.01 | 0.38 | 0.02 |
| 2 | MC | 163 | 0.57 | $3.8 \times 10^{-60}$ | 0.70 | 0.14 |
| | MN | 160 | 0.32 | $1.2 \times 10^{-28}$ | 0.80 | 0.08 |
| | LC | 160 | 0.30 | $4.7 \times 10^{-68}$ | 0.78 | 0.07 |
| | LN | 160 | 0.43 | $5.4 \times 10^{-58}$ | 0.91 | 0.10 |
| | FC | 159 | 1.02 | $1.2 \times 10^{-27}$ | 0.70 | 0.26 |
| | FN | 159 | 0.27 | $1.6 \times 10^{-32}$ | 0.49 | 0.07 |
| | AC | 154 | 0.04 | $2.0 \times 10^{-18}$ | 0.59 | 0.01 |

$N = 10$ and 40 fish with repeated measures in Experiment 1 and 2, respectively; $P$ = Likelihood-ratio test with one degree of freedom.

### 2.8. Associations between isotope and growth rate in large fish

In Experiment 2, the relationship between GR and the eight explanatory isotope variables analyzed (jointly, as regression variables, utilizing one randomly sampled record per fish for variables with repeated measures) in addition to the random effect of *tank* × *day* was explored by use of PROC MIXED in SAS®, V.9.4 (SAS Inst. Inc., Cary, NC) utilizing this model:

$$Y_{jk} = \mu + \sum_{l=1}^{8} b_l X_l + tank \times day_j + e_{jk},$$

where $Y_{jk}$ is the $k^{th}$ isotope measurement, $\mu$ is the intercept, $tank \times day_j$ is the random effect of the $j^{th}$ *tank* × *day* ($N \sim 0$, $\sigma_{td}^2$), and $\varepsilon_{jk}$ is a random residual for the $k^{th}$ observation ($N \sim 0$, $\sigma_e^2$).

## 3. Results

In juvenile fish (Experiment 1), the average FW was 205.5 ± 22.4 g, while in large fish (Experiment 2) the average FW was 1546.1 ± 326.4 g (Table 1). Regarding the isotopes, Experiment 2 had the consistent lowest mean $\delta^{13}C$ values per tissue and correspondingly the highest values of $\delta^{15}N$. The numerically largest mean value of $\delta^{13}C$ was in the adipose fin (FC, both experiments) and the least values were in the muscle (MC) and the visceral adipose tissue (AC) (Experiments 1 and 2, respectively). Correspondingly for $\delta^{15}N$, the lowest mean value was in the adipose fin (FN, both experiments), and the largest values were in the muscle (MN) and muscle protein (MNP) (Experiments 1 and 2, respectively). Moreover, extraction of lipids from the muscle in Experiment 2 had numerically no effect on mean value and standard deviation of $\delta^{13}C$ (comparing MC with MCP), but a larger effect on $\delta^{15}N$ in muscle (MN vs. MNP).

The repeatability estimates of $\delta^{13}C$ and $\delta^{15}N$ in Experiments 1 and 2 were all estimated 0.49 or larger (except for the adipose fin in Experiment 1) (Table 2), implying a high correlation between repeated measures for the same fish in a specific tissue. Except for this latter correlation, the variance components of fish were significantly larger than 0 for all variables, in both experiments. Moreover, the Pearson correlation between MN and MNP (0.27; Table 3) was estimated low, i.e., that the realized repeatability of muscle $^{15}N$ was affected by lipid extraction. Therefore, MNP was removed from further analysis. Moreover, the corresponding correlation between MC and MCP was positive and significant ($r = 0.50$).

Table 3 shows estimates of negative and significant correlations between GR and both MC and MCP ($r = -0.45$ and $-0.44$, respectively). In contrast, MN and MNP were estimated with no significant correlation to GR, while LN correlated moderate positive and significantly ($r = 0.33$). Note that LN, MN, and FN all were estimated with internally positive correlations ($r = 0.54$–$0.67$; Table 3). Moreover, weak and positive correlations were found between AC and carbon and nitrogen metabolism in muscle, liver, and adipose fin ($r = 0.20$–$0.34$; Table 3).

When regressing each of the stable isotope variables on GR in Experiment 1, the slope estimated for MC was found significant ($P = 0.004$) and negative (−0.13, Table 4; Fig. 1a). The coefficient of determination ($R^2$) of the model was 0.66, while $\widehat{R^2}$ under leave-one-out cross-validation was 0.49, indicating a rather close relationship between growth and carbon fractionation. This relationship was again supported in Experiment 2, since MC was the isotope variable with the largest effect on GR ($F = 10.83$, Table 5). Also here, the regression coefficient of MC on GR was negative (−0.32, Table 4), meaning that fish with a high growth rate had a lower level of $\delta^{13}C$ in muscle (Fig. 1b). In addition, LN was found with a significant ($P = 0.04$) and positive (0.25) regression coefficient to GR.

**Table 4**
Significant ($P < 0.05$) simple linear regression coefficient estimates ($\widehat{\beta}$) when, in turn, regressing the measured stable isotope variables in Experiment 1 in juvenile rainbow trout on growth rate (GR), utilizing one randomly sampled record per fish because GR was only observed once.

| Dependent variable | Effect | $\widehat{\beta} \pm$ SE | *P* | *F* | $R^2$ | $\widehat{R}^2$ |
|---|---|---|---|---|---|---|
| GR | Intercept | −2.10 ± 0.75 | 0.023 | 15.58 | 0.66 | 0.49 |
| | MC | −0.13 ± 0.03 | 0.004 | | | |

$P$ = $P$-value of estimates being different from zero; $F$ = $F$-value of model; $R^2$ = Coefficient of determination of model fit; $\widehat{R}^2$ = Coefficient of determination of model prediction; MC = $\delta^{13}C$ in muscle.

**Table 3**
Pearson correlation coefficients between variables in large rainbow trout (Experiment 2).

| | MC | MCP | MN | MNP | LC | LN | FC | FN | AC |
|---|---|---|---|---|---|---|---|---|---|
| GR | **−0.45** | **−0.44** | 0.12 | 0.12 | 0.06 | **0.33** | −0.10 | **0.23** | 0.02 |
| MC | | **0.50** | 0.16 | −0.02 | 0.14 | −0.08 | **0.21** | 0.03 | **0.28** |
| MCP | | | −0.00 | **−0.20** | −0.03 | **−0.18** | 0.13 | **−0.21** | 0.10 |
| MN | | | | **0.27** | 0.06 | **0.54** | **0.18** | **0.64** | **0.32** |
| MNP | | | | | **0.21** | **0.35** | 0.05 | **0.39** | **0.42** |
| LC | | | | | | 0.14 | 0.14 | 0.07 | **0.25** |
| LN | | | | | | | −0.03 | **0.67** | **0.26** |
| FC | | | | | | | | 0.12 | **0.20** |
| FN | | | | | | | | | **0.34** |

GR = Growth rate; MC = $\delta^{13}C$ in muscle; MCP = $\delta^{13}C$ in muscle protein; MN = $\delta^{15}N$ in muscle; MNP = $\delta^{15}N$ in muscle protein; LC = $\delta^{13}C$ in liver; LN = $\delta^{15}N$ in liver; FC = $\delta^{13}C$ in fin; FN = $\delta^{15}N$ in fin; AC = $\delta^{13}C$ in visceral adipose tissue; Significance levels: Bold = $P \leq 0.05$.

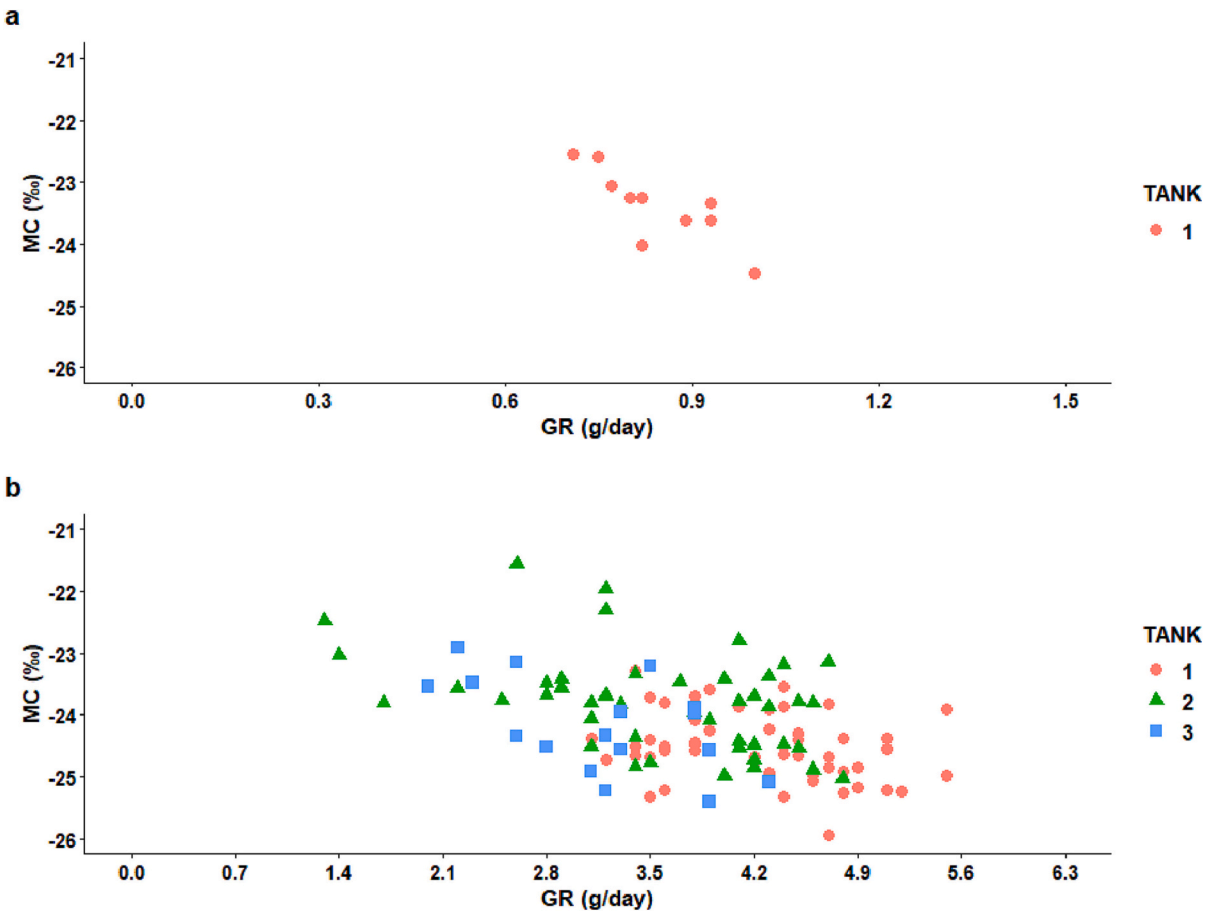


**Fig. 1.** Scatter plots of the relationship between $\delta^{13}C$ in the muscle (MC) and individual growth rate (GR), in (a) juvenile rainbow trout ($n = 10$, Experiment 1) and (b) large rainbow trout ($n = 120$, Experiment 2).

**Table 5**
Estimated regression coefficients ($\widehat{\beta}$) for isotope variables on growth rate (GR) in large rainbow trout (Experiment 2), utilizing one randomly sampled record per fish because GR was only observed once.

| Dependent variable | Effect | $\widehat{\beta} \pm$ SE | *F* | *P* |
|---|---|---|---|---|
| GR | Intercept | −3.23 ± 5.80 | | |
| | MC | −0.32 ± 0.10 | 10.83 | 0.001 |
| | MCP | −0.13 ± 0.08 | 2.49 | 0.12 |
| | MN | 0.05 ± 0.12 | 0.16 | 0.69 |
| | LC | 0.07 ± 0.06 | 1.36 | 0.25 |
| | LN | 0.25 ± 0.12 | 4.12 | 0.04 |
| | FC | 0.002 ± 0.05 | 0.00 | 0.97 |
| | FN | −0.09 ± 0.16 | 0.32 | 0.57 |
| | AC | 0.15 ± 0.20 | 0.62 | 0.43 |

*F* and *P* = Respectively *F* and *P* statistics of estimates being different form zero; MC = $\delta^{13}C$ in muscle; MCP = $\delta^{13}C$ in muscle protein; MN = $\delta^{15}N$ in muscle; LC = $\delta^{13}C$ in liver; LN = $\delta^{15}N$ in liver; FC = $\delta^{13}C$ in fin; FN = $\delta^{15}N$ in fin; AC = $\delta^{13}C$ in visceral adipose tissue.

## 4. Discussion

Generally, the repeatability of the isotope measures was estimated numerically highest in liver and muscle tissues (Table 2). Moreover, in Experiment 2, the repeatability of $\delta^{15}N$ was higher than those for $\delta^{13}C$, which might be explained by the mentioned confounding between levels of $\delta^{13}C$ in lipids relative to protein and carbohydrates (Post et al., 2007), and which lead us to extract lipids from the muscle samples. The low repeatability in Experiment 1, for the adipose fin can be explained by the homogenization not being applicable since this tissue was too hard and firm. Therefore, in Experiment 2, the sampling position of the fin was standardized, which increased the repeatability of the measurements, but the standard error was large which can be explained by intra-fin variability (Hayden et al., 2015). This points to the need for an appropriate method for homogenization of the fin, since homogeneity of the sample is known to limit the precision of Isotope Ratio Mass Spectrometry (Carter and Fry, 2013). Alternatively, other fins might be easier to homogenize (e.g., the caudal; Cano-Rocabayera et al., 2015) and should be investigated for this purpose. On the other side, liver and muscle tissues are more homogenous and easier to homogenize, explaining the higher repeatability estimates relative to the fin, but also to the visceral adipose tissue. For the latter tissue, some samples were however contaminated and removed prior to the analysis, which resulted in a repeatability estimate of 0.59. This can point to the need for an improved homogenization of the visceral adipose tissue. Of the above tissue samples, only the adipose fin would allow phenotyping of the selection candidates themselves, while the remaining tissue samples normally (no biopsy allowed) requires sampling in a sib-test to be used through genomic selection.

After lipid extraction, MC and MCP correlated positively and significantly ($r = 0.50$; Table 3), somewhat lower than that obtained by Dvergedal et al. (2022), also in large rainbow trout. The intent of lipid extraction was to remove the carbon structures for fat stored in the muscle tissue (leaving those from amino acids), but the extraction also affected the isotopic nitrogen measures in the muscle, resulting in a low correlation between MN and MNP ($r = 0.27$; Table 3), which is in accordance with Post et al. (2007). A *priori* our expectation was that this correlation would be similar to the estimated repeatability (0.80; Table 2) if only fat was extracted from the tissue. However, since this

was not what was estimated, the MNP variable was removed from further analysis, and we advise caution to be taken also for MCP for the same reason.

In this study, no correlation was found between GR and MN, in accordance with e.g., Ramseyer (2002), but a negative and significant ($P < 0.05$) correlation was estimated between GR and MC (−0.45; Table 3). These results correspond with Gaye-Siessegger et al. (2004) who showed a decrease in C-isotope fractionation in Nile Tilapia with higher protein retention, but not with Trueman et al. (2005) and Martin-Perez et al. (2013) who found a negative correlation between growth rate and muscle tissue-diet isotopic differences for $^{15}N$, in Atlantic salmon and Gilthead Sea Bream, respectively. Our results for MC can be explained by the faster-growing fish having, on average, higher feed efficiency, with a larger fraction of feed nutrients being retained in body tissue, implying a lowered fractionation. This association between growth and MC might, however, be impaired by muscle composition (fat vs. protein), but the correlation between GR and MCP, was as to MC (Table 3), indicating that the association between growth rate and carbon fractionation is primarily driven by C-use efficiency, which, in turn, affects fractionation. It should be noted that MC is expected to reflect C-use (contained in any organic compound) efficiency, while MN is expected to reflect N-use (protein) efficiency. In consequence, feed protein used as a carbon source in *de novo* synthesis of fatty acids will likely impact N- and C-use efficiencies differently. Moreover, the correlation between GR (over life) and MC was not perfect since growth rate is not expected to explain all variation in feed efficiency, because it will also be explained by feed intake (Dvergedal et al., 2022). In fact, a more extreme (negative) correlation between these two traits would, in case, have indicated that MC contains little information about feed efficiency that is not already explained by growth. Note, also that the correlations between GR and respectively LN, and FN were significantly positive, and internally the correlation between the latter variables was high ($r = 0.67$; Table 3). We interpret this to be due to growth since Dvergedal et al. (2019b and 2020), in Atlantic salmon, have shown a positive correlation between growth and nitrogen isotopes in both liver and muscle.

The results from the regression analysis for the relationship between GR and the isotope variables were in accordance with the correlation pattern referred to above; In Experiment 1, MC was found with a negative regression coefficient to GR (Table 4; Fig. 1a) and in Experiment 2 that the regression coefficient for MC was negative (Table 5; Fig. 1b), but correspondingly positive for LN (Table 5). The former result, for MC, corresponds with the negative correlation of −0.45 between GR and MC, discussed above (Table 3), and for LN the significant ($P < 0.04$, Table 5) association to growth is in harmony with findings of Dvergedal et al. (2020). This suggests primarily that growth-efficient fish synthesize new amino acids by using the carbon skeleton derived from carbohydrates and lipids in feed and recycled amino groups from degraded body protein, which result in higher deamination and transamination rates with enrichment of $^{15}N$ (Gaye-Siessegger et al., 2004). Yet another explanation for the very different correlations of MC and MN to GR comes from assuming variable fat deposition among fish; that being synthesized from amino acids with different impacts on N- and C-use efficiencies. Moreover, the MN indicator will not discriminate between amino acids used as an energy source (e.g., in fish having high maintenance relative to feed intake) and amino acids used to synthesize fat (e.g., in fish having low maintenance relative to feed intake), as both processes imply loss of nitrogen. However, MC may distinguish between these processes (using amino acids for energy gives more fractionation in MC than converting them to fat). However, if fat deposition has limited variance, the MN may have a closer relationship with growth.

## 5. Conclusion

The repeatability of the $\delta^{13}C$ and $\delta^{15}N$ measures were high in muscle and liver which should make one measurement suffice. Even though individual feed intake was not recorded, the significant relationship between GR (that correlates with feed efficiency) and, respectively, MC and LN, suggests isotopic fractionation as potential biomarkers for individual cumulative (over-life) feed efficiency in fish.

## Submission declaration

All authors read and approved the final manuscript for submission which has not been published or submitted for publication elsewhere.

## Authors' contributions

H.D., J.Ø., T.F.G., and G.K. designed the experiments. H.D. carried out the experiments, the samplings, and prepared samples for stable isotope analysis. H.D. and G.K. conducted the statistical analysis. H.D. wrote the first draft, and all authors have read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare no interest.

## Data availability

Data will be made available on request.

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