

Tissue-specific turnover rates and trophic enrichment of stable N and C isotopes in juvenile Atlantic cod *Gadus morhua* fed three different diets

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ABSTRACT: A 150 d laboratory diet-shift study was conducted on juvenile Atlantic cod *Gadus morhua* L. to examine (1) stable nitrogen and carbon isotope turnover rates, (2) the tissue-specific relative contribution of metabolism and growth to isotopic change, and (3) diet–tissue enrichment of the isotopes in white muscle, heart, whole blood, and cranial bone collagen. Time- and growth-based models described the change in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of the tissues following a diet shift from pelleted food to either blue mussels *Mytilus edulis* L., sandeel *Ammodytes marinus* Raitt., or whiting *Merlangius merlangus* L. diets. Cod growth rates ranged from 1.5 to 2.0% d^{-1} , with the exception of whiting-fed cod that experienced a cessation in growth after Day 86. Isotope turnover rates, expressed as half-lives ($T_{1/2}$), differed little between tissues as expected in fast-growing fish. For $\delta^{15}\text{N}$, heart tissue had the shortest $T_{1/2}$ while muscle had the longest (30.7 to 34.8 versus 35.7 to 77.9 d^{-1} ; range among diets). Heart tissue also had the shortest $\delta^{13}\text{C}$ $T_{1/2}$, whereas blood had the longest (25.5 to 38.5 versus 49.5 to 60.3 d^{-1} ; range among diets). Diet-tissue enrichment varied between 0.8 and 5.1‰ ($\Delta\delta^{15}\text{N}$) and between 0.7 and 2.2‰ ($\Delta\delta^{13}\text{C}$). Growth accounted for most of the isotopic change, except in heart tissue of all cod and in all tissues of whiting-fed cod, where there was a significant effect of metabolism on isotopic change. Despite variability in enrichment among diets and tissues, fish feeding on the 3 diets could be distinguished based on their tissue-specific isotopic signatures.

KEY WORDS: Isotopic turnover · Diet shift · ^{15}N · ^{13}C · Nitrogen · Carbon · Growth · Metabolism

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INTRODUCTION

Ever since the natural abundances of stable isotopes of nitrogen and carbon in consumers were shown to reflect isotope abundances of their diets (DeNiro & Epstein 1978, 1981), stable isotopes have been widely used by ecologists to study food webs and trophic relationships in various ecosystems (Post 2002). The integrative nature of the signal makes it especially useful in connection with longer-term trophic dynamics. However, potential tissue specificity with regard to tissue–diet enrichment and isotopic turnover rate, and consequently the time to equilibrium, makes prior knowledge of the rate of enrichment and isotopic turnover in different tissues

essential in any isotope-based study of trophic dynamics.

The tissue–diet enrichment (Δ) between trophic levels is a result of discrimination among isotopes in biological reactions (fractionation). The ^{15}N trophic enrichment is primarily due to discrimination against the heavier ^{15}N during nitrogen excretion and thus depends on the excretory pathway, but also diet composition (e.g. amino acid composition; McClelland & Montoya 2002) and physiological stress (see meta-analysis by Vanderklift & Ponsard 2003). Following catabolism of proteins, lipids, and carbohydrates, discrimination against ^{13}C occurs during formation of CO_2 when acetyl groups are oxidized, leading to the release of ^{13}C -depleted CO_2 (Galimov 1985). This

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difference in the metabolic pathways of carbon and nitrogen is the basis for the lower trophic enrichment in ^{13}C compared to ^{15}N .

In many food web studies, fixed values of consumer–diet enrichments have been employed even though controlled feeding studies show considerable variation (McCutchan et al. 2003). Typical values of $\Delta(\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{diet}}) = 3.4\text{‰}$ and $\Delta(\delta^{13}\text{C}_{\text{consumer}} - \delta^{13}\text{C}_{\text{diet}}) = 1.0\text{‰}$ have been employed (DeNiro & Epstein 1978, Minagawa & Wada 1984). In fish, an average $\Delta\delta^{15}\text{N}$ of 2.79‰ (Sweeting et al. 2007a) and $\Delta\delta^{13}\text{C}$ of 1.5‰ (Sweeting et al. 2007b) have been reported.

Few studies of stable isotopes in fish have compared isotope dynamics of different tissues (MacAvoy et al. 2001, Trueman et al. 2005, Logan et al. 2006, Miller 2006, Guelinckx et al. 2007, Buchheister & Latour 2010), and these have found variable turnover rates and trophic enrichments among fish species and tissues, highlighting the need for specific data. Furthermore, studies trying to find the most appropriate fish tissue for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ analysis (e.g. Pinnegar & Polunin 1999, Miller 2006, Guelinckx et al. 2007) have usually employed a single diet shift, but the nutritional quality of diet may affect turnover rates and trophic enrichments (Gaye-Siessegger et al. 2003, Vanderklift & Ponsard 2003). We included a shift to 3 different diets in our study to examine the effect of different prey types.

Here we present the first laboratory diet-shift study on Atlantic cod *Gadus morhua*, L. (hereafter cod). Our aim was to quantify turnover rates and diet–tissue enrichment of stable isotopes in white muscle, heart, blood, and cranial bone tissue of juvenile cod fed 3 natural diets. We further estimated the tissue-specific relative contribution of metabolism and growth to isotopic change (turnover rates) and evaluate the ability and the associated temporal resolution of stable isotope signatures to discriminate between diets.

MATERIALS AND METHODS

Fish rearing

Cod were chosen as model organisms because of their opportunistic feeding and their ecological and commercial importance (Demain et al. 2011). Moreover, recruitment failure in some of the North Atlantic cod stocks has been linked to changes in the abundance of their preferred prey, which makes retrospective analyses of feeding behavior very relevant for this species (Marshall et al. 1999, Beaugrand et al. 2003).

Cod were hatched and reared to about 2.5 g wet weight (WW) and 5 cm total length (TL) at Austevoll Aquaculture Research Station in Norway and subsequently transferred to the North Sea Oceanarium at Hirtshals, Denmark. Prior to the feeding experiment, the juveniles were kept for 1 mo in 1.2 m³ (n = 3) tanks on a dry pellet diet. Tanks were supplied with filtered and temperature-controlled seawater from the North Sea (11°C and 33 psu). The light:dark ratio was 12:12 h. There was no difference in levels of NH_4^+ between inlet and outlet.

In March 2006, when the cod had obtained an average WW of 9.6 ± 0.6 g (SE), each tank containing about 200 fish was assigned to 1 of 3 experimental diets: blue mussel *Mytilus edulis* L., a phytoplankton filtrator; lesser sandeel *Ammodytes marinus* Raitt., a zooplanktivorous fish; or whiting *Merlangius merlangus* L., a piscivorous fish. The cod were fed to satiation during 1 daily feeding event throughout the next 150 d of the experiment.

Blue mussels and sandeel came from batches caught at the same time and place so that the diet type would be as homogeneous as possible. The mussels and the sandeel were cut into small pieces of about 1 cm in diameter prior to feeding. The whiting diet was composed of whole fish (TL: 35.2 ± 0.8 cm, SE) without guts. These fish were homogenized together in a mincer and the homogenate was spread into a thin layer and frozen (−20°C) to obtain a pellet structure. All food was kept in a freezer (−20°C) during the experiment.

Fish sampling and processing

Sampling took place at the diet shift and then after 31, 60, 86, 123, and 150 d, resulting in a total of 6 samplings. At each sampling occasion, 5 fish were randomly selected from each tank and the individual WW and TL were measured. From each fish, a sample of white muscle, heart, whole blood, and cranial bone were taken. Care was taken to collect samples in the same way for all individuals. Only the ventricle was sampled from the heart. All analyses on blood were performed on whole blood (hereafter referred to as blood) samples taken from the venae portae renalis with a syringe. White muscle tissue was sampled by first removing the skin from an area dorsal to the lateral line starting from the posterior end of the second dorsal fin and 1 cm posterior from this point. Then the underlying dorsal muscle was removed. In this dorso-caudal region there are no epical bones, and the muscle tissue

is easy to sample without the risk of collecting bones within the muscle sample. Sampling of bone was done by removing the whole cranium to get enough material for stable isotope analysis of the bone collagen.

Collagen from cranial bones was extracted by a method modified from DeNiro & Epstein (1981), Brown et al. (1988), Jørkov et al. (2007), and Skierka et al. (2007). First the adhering tissue was separated from the cranial bones by sonication in ultra pure MilliQ^R water for 1 h followed by manual sorting of bones. Remaining macroscopic adhesive tissue was removed by a second sonication step. For each liquid used in the procedure after removal of tissue adhering to bones, 1 h of sonication was followed by leaving the bones in a fume hood for 24 h until change of liquid the next day. The sequence of liquid used after MilliQ^R water was ethanol, acetone, and finally heptane for lipid removal. Samples were then demineralized in 2% HCl and rinsed in MilliQ^R water, 0.2 M NaOH, and a final rinse with MilliQ^R water. The sample was then freeze-dried.

Four to 5 diet subsamples from each diet type were selected randomly from the diet batches in order to determine diet isotope ratios. One subsample of blue mussel, sandeel, or commercial dry pellets consisted of 5 individuals or pellets homogenized in a blender. The whiting diet consisted of a mince of all available whiting, and therefore 5 subsamples were taken from this mince. No lipid extraction was performed on the diet samples. All tissues and diet samples were stored at -18°C and freeze-dried afterwards for at least 24 h.

Stable isotope analysis

Samples from all tissues and diets were homogenized by crushing them between fingertips using nitrile gloves. After this, a subsample of approximately 1 mg was collected and its mass determined to the nearest 1 μg in 5×9 mm tin cups. During analysis a few samples were lost, but for each sampling event at least 3 fish were analyzed for each combination of diet and tissue.

Stable isotope analysis was performed at The Scottish Crop Research Institute, Invergowrie, Scotland. Here the continuous flow–isotope ratio mass spectrometry (CF-IRMS) analysis was done using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyzer with ANCA-NT Solid/Liquid Preparation Module (Europa Scientific). For samples containing around 10% N, the CF-IRMS was operated in the

dual isotope mode, allowing $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ to be measured in the same sample. The analytical precision (SD, $n = 5$) was 0.2‰ for both N and C, estimated from standards analyzed along with the samples. Working standards were 1 mg leucine prepared by freeze-drying 50 μl of a 20 mg ml^{-1} stock solution into tin cups, and calibrated against ‘Europa flour’ and IAEA standards N1 and N2.

The stable isotope results are given in the international standard δ -notation:

$$\delta X(\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 100 \quad (1)$$

where X is either ^{13}C or ^{15}N , and R_{sample} is $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ in the sample, whereas R_{standard} contains the ratio for the given standards. The international standards are pure atmospheric nitrogen (N_2) for $\delta^{15}\text{N}$ and Vienna Pee Dee Belemnite for $\delta^{13}\text{C}$.

Modeling of isotopic turnover

Mean values are based on 5 individuals for each sampling event and tissue except for sampling just before the diet shift, which consisted of 15 fish.

Turnover as a function of time

The change in isotopic composition in an animal can be described as a function of time (Tieszen et al. 1983):

$$\delta_t = \delta_f + (\delta_i - \delta_f) \exp(vt) \quad (2)$$

Here, δ_t is the stable isotope composition of a tissue from a fish sampled at time t . δ_f represents the isotopic value at equilibrium with diet, δ_i is the initial value before the diet shift, t denotes the time since the start of the experiment, and v is a measure of the isotopic turnover rate. Two of the parameters, δ_f and v , were estimated using the Marquardt-Levenberg iterative non-linear least-squares model fitting. The tissue specific δ_i value was calculated as the mean value from the 15 fish sampled just prior to the diet shift.

Isotopic turnover rates are often expressed in terms of half-life ($T_{1/2}$) referring to the time it takes for the isotopic value to reach the midpoint between the initial δ_i and the value at equilibrium δ_f . By transformation of Eq. (2), the following expresses the tissue-specific half-life values (Tieszen et al. 1983):

$$T_{1/2} = \ln(0.5)/v \quad (3)$$

Turnover as a function of growth

The relative contribution of growth and metabolic turnover can be evaluated by the growth model of Fry & Arnold (1982):

$$\delta_t = \delta_i + (\delta_i - \delta_f)(W_t/W_i)^c \quad (4)$$

The δ_t , δ_f , and δ_i parameters represent the same here as in Eq. (2). W_t is the individual fish WW at time t after the diet shift, and the initial WW, W_i , is determined as the mean weight of the 15 fish sampled at the time of diet shift. The relative contribution of growth and metabolic turnover to the change in stable isotope composition can be estimated from the value of c . If $c = -1$, the isotopic change is solely due to growth by the addition of new tissue. A value of $c < -1$ indicates an increasing part of the change in isotopic composition due to metabolic turnover. Estimation of δ_f and c for this growth model was also done using the Marquardt-Levenberg fitting model.

Weight specific growth rates (SGR) were calculated as:

$$\text{SGR} = (e^g - 1) \times 100 \quad (5)$$

where $g = [\ln(W_2) - \ln(W_1)]/(t_2 - t_1)$.

Statistics

One-way analyses of variance (ANOVAs) were used to compare diet $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, and C/N ratios, as well as tissue-specific $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, and fish TL and WW prior to the diet shift. One-way analysis of covariance (ANCOVA) was used to compare growth of the cod reared on the 3 diets. Mixed, between-within subjects repeated measures ANOVAs were used to test for the effect of time and diet on $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$. Table-wide Bonferroni corrected t -tests were used to test for differences in enrichment among tissues and diets. t -tests were further used to test whether c was significantly different from -1 . We calculated 95% prediction intervals of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$

Table 1. Diet $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, and C/N ratios (mean \pm 95% CI, $n = 5$)

Diet	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	C/N ratio
Pellet	10.65 \pm 0.14	-21.60 \pm 0.11	5.15 \pm 0.40
Blue mussel	10.88 \pm 1.10	-19.46 \pm 0.71	4.55 \pm 0.36
Sandeel	12.49 \pm 0.75	-20.63 \pm 0.54	4.68 \pm 0.16
Whiting	15.57 \pm 0.20	-18.90 \pm 0.11	3.61 \pm 0.02

(Zar 1999) for each date, diet, and tissue in order to determine when a randomly chosen fish could be reliably assigned to 1 of the 3 diets. All tests were preceded by tests of their assumptions. Statistical significance was evaluated at a significance level of 0.05.

RESULTS

The pellets fed prior to the experiment and the diets given to the cod after the diet shift (Table 1) differed significantly from each other with regard to $\delta^{15}\text{N}$ (1-way ANOVA, $F_{3,15} = 106.9$, $p < 0.001$; Tukey's HSD) with the exception of blue mussels and the pellets. The $\delta^{13}\text{C}$ values of diets were significantly different except for mussels and whiting (1-way ANOVA, $F_{3,15} = 70.8$, $p < 0.001$, Tukey's HSD). C/N ratios indicating relative lipid content were similar between sandeel and mussel diets (Tukey's HSD, $p = 0.498$), while the whiting diet exhibited significantly lower ratios (1-way ANOVA, $p < 0.001$, both Tukey's HSD, $p < 0.001$).

Fish did not differ significantly among the 3 tanks with regard to tissue-specific $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, total length, and weight just prior to the diet shift (Day 1; all 1-way ANOVAs, $p > 0.132$). Therefore, the 15 fish sampled on Day 1 were pooled, representing the mean isotopic baseline just before the diet shift.

Cod growth was not significantly influenced by diet for the first 86 d of the experiment (Fig. 1; 1-way ANCOVA Day 0 to 86, $\ln[\text{mass}]$: $F_{2,56} = 1.6$, $p = 0.22$). During the second half of the experiment, cod fed whiting showed a remarkable decrease in appetite

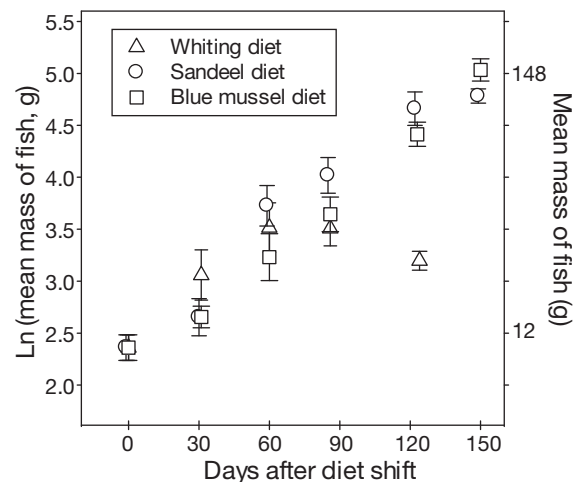


Fig. 1. *Gadus morhua*. Increase in mass with time after diet shift ($n = 5$, \pm SE)

and lost mass until Day 123. No whiting-fed cod survived until Day 150. The sandeel- and mussel-fed cod increased their weight an average of 14.4 times, while the whiting diet led to only an average of 2.6 times increase by the end of the experiment. The mass-specific growth rates and the increase in length from Day 0 to Day 150 was 2.01 % d⁻¹ and 0.88 mm d⁻¹ on sandeel diet, and 1.79 % d⁻¹ and 0.82 mm d⁻¹ on mussel diet. The mass-specific growth rate and the increase in length from Day 0 to Day 86 for the whiting diet was 1.43 % d⁻¹ and 0.66 mm d⁻¹.

Turnover and trophic enrichment of nitrogen

Fig. 2 shows a clear effect of time and diet on the development of $\delta^{15}\text{N}$ in cod tissues. This was confirmed by a mixed, between-within subjects repeated measures ANOVA, which showed a significant effect of time (all Wilk's lambda, $F_{4,9} > 13.7$, $p < 0.006$) and diet type (all Wilk's lambda, $F_{8,18} > 3.1$, $p < 0.006$) on $\delta^{15}\text{N}$ for all tissues. Most tissues did not reach a new equilibrium, defined here as the time at which the isotopic signal had changed 95% of the isotopic distance between diet and the estimated δ_t , within the 150 d. A noticeable exception was heart tissue $\delta^{15}\text{N}$ where equilibrium was reached within 130 d for cod fed sandeel and blue mussel diets (Fig. 2).

Isotopic turnover rates expressed as half-life varied between 30.7 and 77.9 d, depending on tissue and diet type (Fig. 3). The half-lives of $\delta^{15}\text{N}$ were ranked heart < bone collagen < whole blood < muscle for both mussel- and sandeel-fed cod, whereas in whiting-fed cod, half-lives ranked heart < muscle < blood < bone collagen. The half-life of the muscle tissue $\delta^{15}\text{N}$ varied considerably between diets (25.7 to 77.9 d), whereas the variation was less than 11 d in all other tissues.

Isotopic enrichment, $\Delta(\delta_t - \delta_d)$, between cod tissue (δ_t) and diet (δ_d) ranged between 0.76 and 5.1‰ (Fig. 3). There were significant differences in enrichment among tissues and diet types (Bonferroni corrected t -tests in Table 2). Overall, heart tissue showed the largest enrichment. The whiting-fed cod showed the smallest enrichment on average, but large differences existed between tissues. Tissue enrichment in ^{15}N of cod fed sandeel

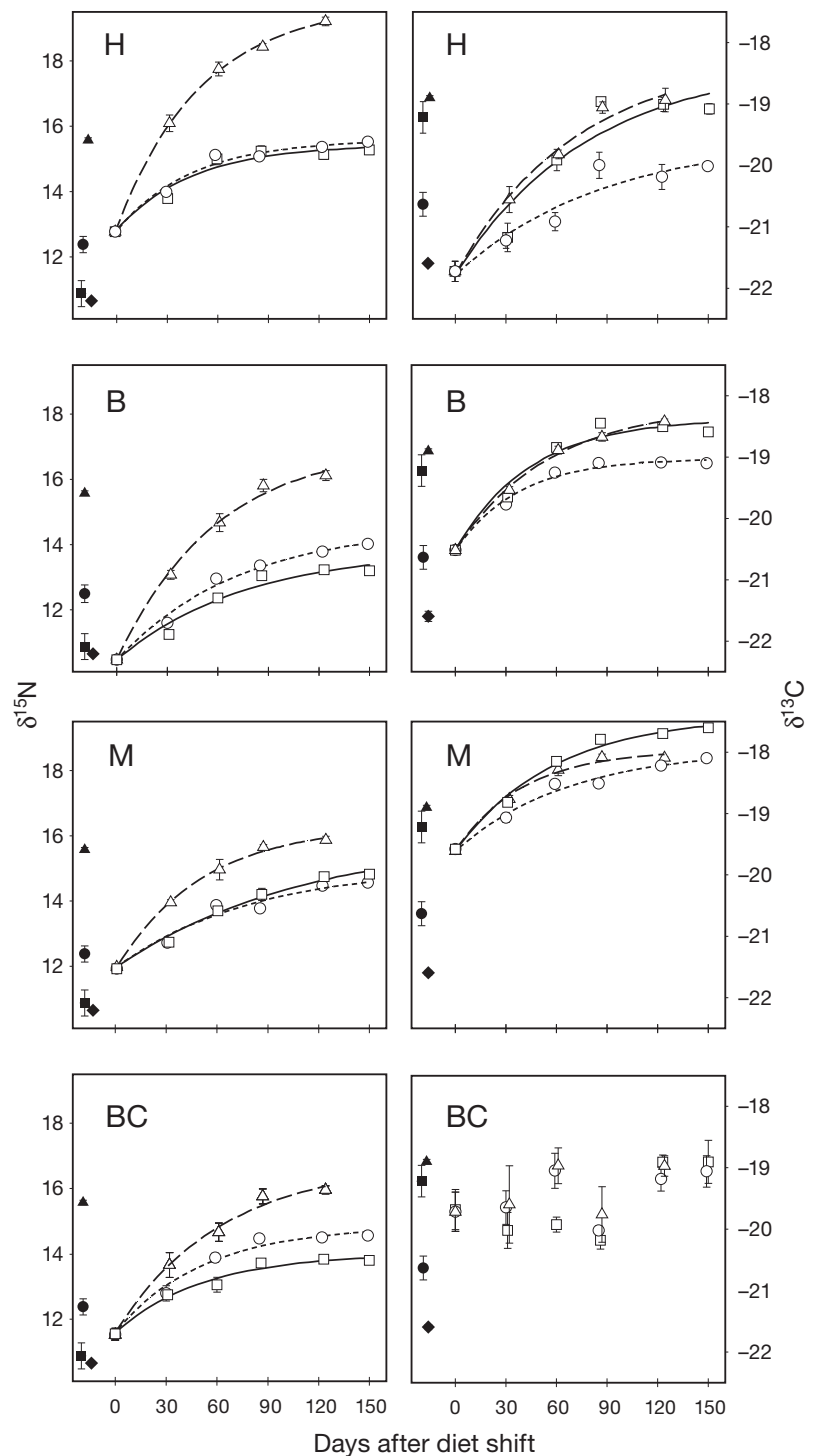


Fig. 2. *Gadus morhua*. Changes in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ with time since diet shift ($n = 5$, \pm SE) in heart (H), blood (B), muscle (M), and bone collagen (BC). (□) blue mussel diet; (○) sandeel diet; (△) whiting diet. The fitted model is a function of isotopic turnover: $\delta_t = \delta_d + (\delta_i - \delta_d)(\exp(-vt))$ (see 'Materials and methods'). δ_i is the mean of 15 fish sampled at $t = 0$. (—) blue mussel diet; (---) sandeel diet, (- - -) whiting diet. No significant fit was obtained for $\delta^{13}\text{C}$ in bone collagen. Mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of diet (\pm SE) are shown as filled symbols

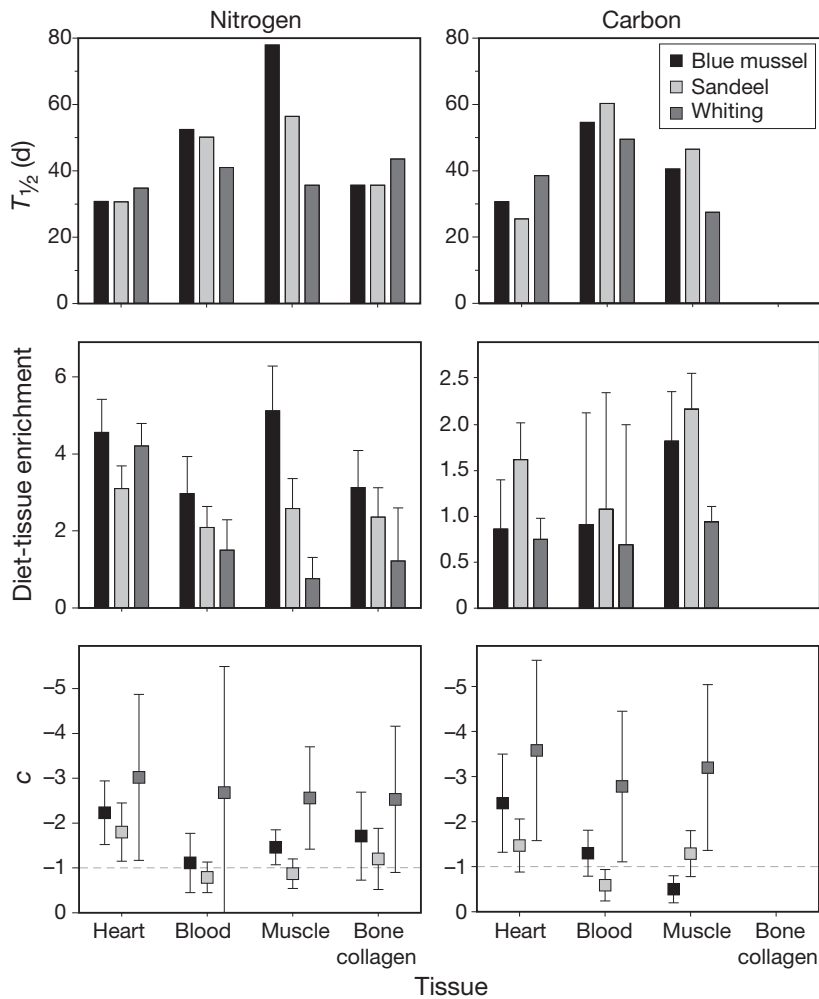


Fig. 3. *Gadus morhua*. Tissue- and diet-specific carbon and nitrogen isotopic half-lives ($T_{1/2}$), diet–tissue enrichment ($\pm 95\%$ CI), and relative contribution of growth and metabolism to isotopic turnover (c) ($\pm 95\%$ CI). Note inverted scale for c . When $c = -1$ (dashed line) no metabolic turnover occurs. $c < -1$ indicates increasing metabolic contribution to isotopic turnover

differed in the following order: heart > muscle > bone collagen > blood, in which only heart and blood were statistically distinguishable. For mussel-fed cod, the tissue enrichment was ranked muscle > heart > bone collagen > blood, and here differences were significant between all tissues except blood and bone collagen, and heart and muscle (Table 2). The enrichment ranking for whiting-fed cod was heart > blood > bone collagen > muscle, of which heart differed significantly from blood, muscle, and bone collagen. Significant diet effects on enrichment were observed for heart and muscle tissue. Especially in the muscle tissue, there was a large difference in enrichment between diets (0.76‰ for whiting diet to 5.1‰ for mussel). $\Delta\delta^{15}\text{N}$ of muscle tissue in whiting-fed cod was remarkably low

(0.76‰) compared to all other combinations of tissue and diet enrichments.

The data in Fig. 4 show a significant relationship (power function, all $F > 42.40$, $p < 0.001$) between growth and isotopic change in $\delta^{15}\text{N}$. For each combination of tissue and diet, growth could explain between 65 and 95% of the isotopic variation. The metabolic turnover contribution to isotopic change was dependent both on tissue and diet type (Fig. 3, Table 2). The data indicated that the change in $\delta^{15}\text{N}$ of bone and blood was a result of growth alone, since c did not differ significantly from -1 in any of the 3 diet groups (all $t < 1.90$, $df = 27\text{--}38$, $p > 0.07$). In contrast, metabolic turnover significantly accelerated isotopic change in both white muscle and heart (all $t > 2.22$, $df = 32\text{--}40$, $p < 0.04$), except for muscle tissue in cod fed with sandeel ($t = 0.81$, $df = 37$, $p = 0.42$).

Turnover and trophic enrichment of carbon

The effect of time on $\delta^{13}\text{C}$ was significant for muscle, heart, and blood (all Wilk's lambda, $F_{4,9} > 134.5$, $p < 0.001$) and diet (all Wilk's lambda, $F_{8,18} > 3.1$, $p < 0.022$; Fig. 2), and fitted exponential curves and parameter estimates were all statistically significant ($p < 0.001$) with the exception of bone collagen $\delta^{13}\text{C}$.

Table 2. *Gadus morhua*. Comparison of effects of diet on relative contribution of metabolism (c values) to $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ change and on diet–tissue isotopic enrichment (D) in the 4 different tissues. Significant difference (table-wide Bonferroni-corrected t -tests) between cod fed different diets is marked by < or > (BM: blue mussel, S: sandeel, W: whiting diet). A dash (–) indicates tissue samples that could not be fitted to the model: $\delta_t = \delta_i + (\delta_i - \delta_t)(W_t/W_i)^c$ (see 'Materials and methods'). ns: non-significant isotopic difference between the same tissue of fish fed 3 different diets

Tissue	$\delta^{15}\text{N}$		$\delta^{13}\text{C}$	
	c	D	c	D
Heart	ns	S<BM=W	ns	S>BM=W
Blood	ns	ns	S>BM	ns
Muscle	S<BM	S>BM>W	S<BM	ns
Bone collagen	ns	ns	–	–

Isotopic turnover rates of $\delta^{13}\text{C}$ varied among tissues and diet types with half-lives from 25.5 to 60.3 d (Fig. 3). Half-life ranking order was heart < muscle < blood for the sandeel and blue mussel diets. Tissues from whiting-fed cod ranked muscle < heart < blood. Heart tissue from fish fed mussel and sandeel equilibrated with the new diet within the 150 d experiment as did muscle tissue from fish fed whiting. Even though most tissues did not reach our definition of isotopic equilibrium with diet, isotopic values on Day 150 were all close to the estimated equilibrium value δ_f .

The isotopic enrichment between cod tissue (δ_i) and diet (δ_d) ranged between 0.69 and 2.17‰ (Fig. 3). The only significant difference between tissues was found for heart and muscle with muscle showing the highest enrichment. This difference was found in both sandeel- and mussel-fed cod (Table 2). Diet effects on enrichment was observed for heart and muscle, where $\Delta\delta^{13}\text{C}$ values were ranked sandeel > mussel > whiting. Blood isotopic enrichment followed the same pattern, but the differences were not significant. Bone collagen $\Delta\delta^{13}\text{C}$ showed a negative correlation with C/N values for cod (all diet groups) sampled after 31, 86, 123, and 150 d (All Spearman rank correlations, $r < -0.581$, $N = 10$ to 15, $p < 0.029$), suggesting that the demineralization step in the collagen extraction may have been insufficient.

There was a significant relationship between growth and isotopic change in all tissues except cranial bone collagen (power function, all $F > 42.40$, $p < 0.001$; Fig. 4). Metabolic turnover contribution ($c < -1$) to isotopic change was dependent both on tissue and diet type (Fig. 3, Table 2). For $\delta^{13}\text{C}$, only blood from mussel- ($t = 1.19$, $df = 36$, $p = 0.24$) and sandeel-fed cod ($t = 1.019$, $df = 36$, $p = 0.32$) and muscle from sandeel-fed cod ($t = 1.18$, $df = 36$, $p = 0.25$) showed change primarily due to growth. The remaining c values were all significantly different from -1 , indicating some degree of metabolic isotopic turnover. This was especially pronounced for heart tissue.

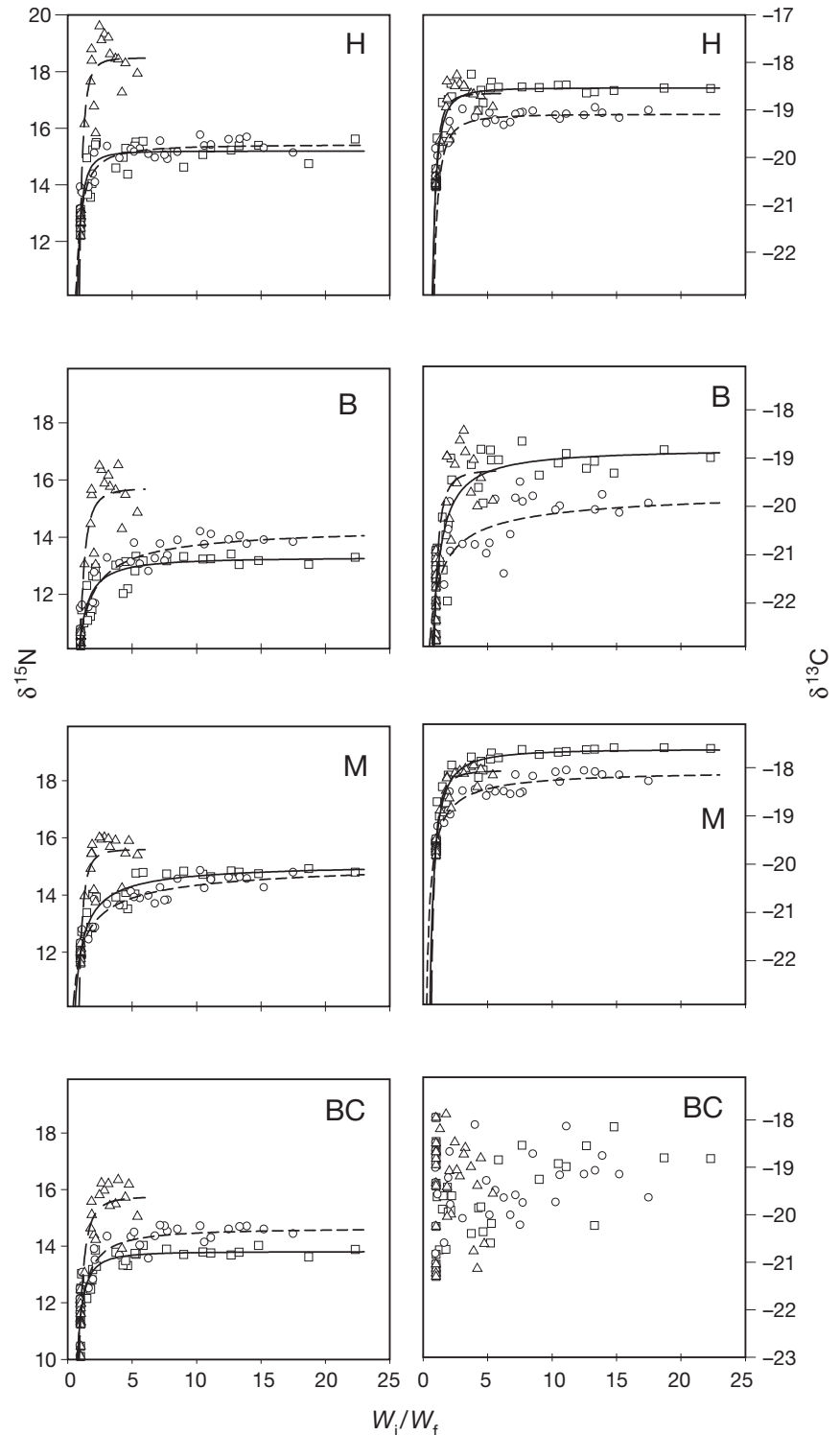


Fig. 4. *Gadus morhua*. Change in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ as a function of fish mass change since diet shift. The model $\delta_i = \delta_f + (\delta_i - \delta_f)(W_i/W_f)^c$ (see 'Materials and methods') is a function of both metabolic turnover and growth in heart (H), blood (B), muscle (M), and bone collagen (BC). (□) blue mussel diet; (○) sandeel diet; (△) whiting diet. δ_i is the mean of 15 fish sampled at $t = 0$. (—) blue mussel diet; (---) sandeel diet, (- - -) whiting diet. No significant fit was obtained for $\delta^{13}\text{C}$ in bone collagen

Table 3. *Gadus morhua*. Predictive power of different tissues indicated by the first date at which the 95 % prediction interval of $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ of cod reared on a specific diet was not overlapping with the prediction intervals of cod reared on one of the other diets (–: that cod could not be separated). BM: blue mussel, S: sandeel, W: whiting diet

Diet comparison	Heart		Blood		Muscle		Bone collagen	
	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$
BM vs. S	–	123	123	–	–	86	–	–
S vs. W	60	123	86	–	86	–	–	–
BM vs. W	60	–	86	–	123	123	86	–

Stable isotope signatures as indicators of diet

The ability to assign the cod samples to a specific diet based on stable isotope data was dependent on the tissue studied. Equilibrium (δ_f) $\delta^{15}\text{N}$ from the heart samples of whiting-fed cod differed significantly from sandeel- and blue mussel-fed cod (both $t > 13.7$, $df = 66$, $p < 0.001$). Muscle samples at equilibrium (δ_f) only differed significantly between the sandeel and whiting diet ($t = 2.25$, $df = 65$, $p = 0.002$). Blood and bone collagen (Fig. 2) were the only 2 tissues showing significant $\delta^{15}\text{N}$ differences among all 3 diets (all Student's t -tests, $t > 2.25$, $df = 61$ to 73, $p < 0.027$). The $\delta^{13}\text{C}$ distance at equilibrium between cod fed the different diet types was significant for

heart (δ_f ; sandeel < mussel and whiting) and muscle (δ_f ; sandeel < whiting < mussel).

Moreover, the ability to reliably assign individual cod to their diet at any of the sampling occasions was investigated by testing for overlap between the diet-specific 95 % prediction intervals of a single future sample of either $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ (Table 3). Individual fish fed whiting could be separated from fish on either a mussel or a sandeel diet from Day 60 using $\delta^{15}\text{N}$ of the heart, but fish on mussel and sandeel diets could not be separated. The prediction interval of $\delta^{15}\text{N}$ for blood was the most successful in assigning individual cod to their diet, although this separation occurred later in the experiment. Muscle tissue could also be used to separate whiting-fed fish from the fish on either of the

other 2 diets, but only after Day 86. Finally, cranial bone collagen $\delta^{15}\text{N}$ could only be used to separate between mussel and whiting diets. Overall, the use of $\delta^{13}\text{C}$ prediction intervals was less successful. $\delta^{13}\text{C}$ separated sandeel-fed cod from whiting- and mussel-fed cod after Day 123 using heart tissue and mussel-fed cod from fish fed sandeel and whiting after 86 and 123 d, respectively, using muscle tissue. $\delta^{13}\text{C}$ of the other tissues could not be used to separate between diets. By combining the information from $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, separation of fish according to diet could be achieved at a much earlier stage (Fig. 5). Diet-specific heart and muscle isotope trajectories were clearly separated from the first sampling date, and mussel- and sandeel-fed cod trajectories could be separated despite similar $\delta^{15}\text{N}$ values. Blood isotope trajectories overlapped for whiting- and sandeel-fed cod, and could not be separated without prior knowledge of sampling date. Bone collagen trajectories were strongly influenced by the biased $\delta^{13}\text{C}$ values and did not yield a clear separation by diet.

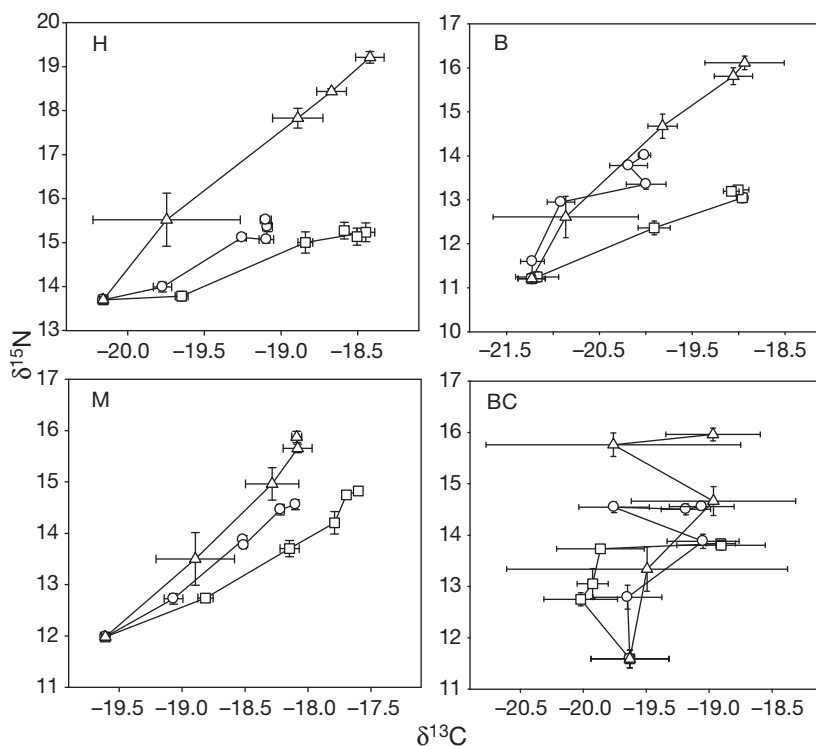


Fig. 5. *Gadus morhua*. Combined changes in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ over time in heart (H), blood (B), muscle (M), and bone collagen (BC). (□) blue mussel diet; (○) sandeel diet; (△) whiting diet ($n = 5$, $\pm 95\%$ CI)

DISCUSSION

Growth pattern of reared cod

Because addition of new tissue by growth is an important cause of isotopic shift in fish (Bosley et al. 2002, Trueman et al. 2005, Logan et al. 2006), growth rates in laboratory experiments should be similar to those of natural populations if turnover rates are to be used as an isotopic clock in natural populations. Previous experiments with reared cod fed dry pellets under similar temperature conditions showed an increase in size from 8.7 to 46–60 g during 82 d and SGRs of 2.14 to 2.48 % d⁻¹ (Fülberth 2008). The cod in the present experiment grew generally slower than this, with SGR = 1.43 to 2.12 % d⁻¹ during the first 86 d, probably due to the lower energy content of natural diets. Juvenile Baltic cod *Gadus morhua* similar in size range compared to our study (standard length: 4 to 15.3 cm, age 90 to 230 d) caught at the Gdansk Bay showed growth rates of 0.078 cm d⁻¹ based on individual size at catch and otolith back-calculation of age (Fey & Linkowski 2006), which is in the range of growth rates (length) experienced in the present study.

Differences in growth rate between cod fed the 3 diets could be explained by the whiting diet consisting of primarily muscle tissue, depleted in lipids and essential nutrients (from internal organs) relative to whole blue mussel and sandeel. Hence, whiting-fed cod represent an example of stable isotope change under nutritionally poor growth conditions.

Tissue-specific isotopic turnover rates

The isotopic turnover rate determines the speed at which a new equilibrium is reached after a diet shift, and hence strongly influences the temporal resolution of isotope-based reconstruction of trophic relationships (e.g. Herzka 2005).

An exponential increase in $\delta^{15}\text{N}$ towards an equilibrium value was observed for all diet types and tissues except $\delta^{13}\text{C}$ in bone collagen. In the literature, inconsistency exists in determining isotopic equilibrium. In the present study, the tissue was by definition in equilibrium when it had increased to 95 % of the isotopic distance between the starting value and the modeled equilibrium value. Even though many combinations of tissues and diets did not reach equilibrium, the data showed a close fit to the model used, and hence isotopic half-lives could be estimated from the model.

Isotopic turnover rates are determined by the combined effect of tissue-specific growth rates and metabolic turnover, and in fast-growing juvenile fish, growth dominates the isotopic turnover (Bosley et al. 2002, Trueman et al. 2005). Consequently, high growth rates decrease the relative importance of metabolism in isotopic change, which is the principal cause of difference in isotopic turnover rates among tissues (Reich et al. 2008). This is consistent with results from the present study. The metabolically active heart tissue had the lowest half-life with regard to both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, but the difference among tissues was relatively small, with only a doubling in turnover rate from muscle to heart. This is similar to Miller (2006) who found that heart tissue in Pacific herring *Clupea pallasii* had slightly higher $\delta^{15}\text{N}$ turnover rate than muscle, while a 4 times higher rate for heart was observed by Guelinckx et al. (2007) in slow-growing fish (ca. doubling in weight during 90 d). Heart exhibited the largest contribution of metabolism to observed isotopic change as expected for a highly physical and metabolically active tissue, whereas the contribution of metabolism was similar among the other tissues.

The $\delta^{15}\text{N}$ values of bone collagen showed a mean half-life value just above that of heart tissue. This finding is surprising, since bone is generally considered as a tissue with low metabolic turnover compared to e.g. heart tissue (Hobson & Clark 1992). This result suggests that the cranial bone development in early juvenile cod is rapid. Bone collagen $\delta^{13}\text{C}$ showed no significant change during the experiment, and the exponential model could not be applied to these data. This is likely due to variations in the amount of inorganic carbon remaining in the sample after demineralization. A higher C/N ratio indicates incomplete inorganic carbonate removal, and bone collagen $\delta^{13}\text{C}$ values were negatively correlated with C/N ratio, which explained 34 to 96 % of the variation. Inorganic bone carbon in fish probably originates from both carbonate in the surrounding water and from the diet as seen in biominerals from mollusk shells, where $\delta^{13}\text{C}$ values resemble those of the surrounding sea water (DeNiro & Epstein 1978).

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ turnover rates in whole blood were slower than the turnover in other tissues for most combinations of tissue and diet, and the changes were primarily due to isotopic dilution (c not different from -1). Whole blood not only contains the newly absorbed nutrients but also the 'old' catabolized and recycled amino acids, explaining the slow isotopic turnover of whole blood also found in other studies (Hobson & Clark 1993, Miller 2006). If separated into

blood plasma/serum and blood cells, blood has been shown to be able to track both isotopic changes integrated over months (cellular) and weekly (plasma) basis (Hobson et al. 1993, Buchheister & Latour 2010, German & Miles 2010). This finding is consistent with the metabolism and protein turnover of blood cells being slower than that of plasma constituents (Schoenheimer 1949, Waterlow et al. 1978). Separated blood samples can therefore play an important role in stable isotope measurement of endangered species, where nondestructive sampling is preferred. In principle, 1 blood sample followed by separation is enough to provide both long-term and short-term dietary and migratory information.

Prey type also influenced the c values of both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$. The reduced appetite and nutritional stress of whiting-fed cod decreased growth and most likely raised the level of amino acid and lipid catabolism and thereby the metabolic turnover rate in muscle (Hobson et al. 1993). This is consistent with muscle being the only tissue with a substantial difference in the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ turnover rates among diets. Higher metabolism due to catabolism induced by low food intake has been shown in Nile tilapia *Oreochromis niloticus* (Gaye-Siessegger et al. 2003). Minor, but consistent, differences in the metabolic contribution to N turnover were also found between blue mussel and sandeel diets, where fish on the mussel diet showed a larger contribution of metabolism.

Trophic enrichment

Knowing the amount of trophic enrichment is essential in identifying potential diets from isotopic signatures of consumers. Diet–tissue enrichments were within the reported range of $\Delta\delta^{15}\text{N}$ (–1 to 6‰) and $\Delta\delta^{13}\text{C}$ (–0.8 to 4‰; Logan et al. 2006, Caut et al. 2009), and overall close to the mean $\Delta\delta^{15}\text{N}$ of 2.79‰ and $\Delta\delta^{13}\text{C}$ of 1.5‰ reported for fish by Sweeting et al. (2007a,b). However, the diet–tissue enrichments varied among diets and tissues. In a recent review, Caut et al. (2009) demonstrated a significant relationship between $\Delta\delta^{15}\text{N}$ and $\Delta\delta^{13}\text{C}$ and the corresponding diet isotope ratios in a large number of animal taxa, including fish. Our results corroborate this general pattern. However, no mechanistic explanation for this effect was offered by Caut et al. (2009). Further, studies have shown a negative relationship between different measures of protein quality and $\Delta\delta^{15}\text{N}$ (Florin et al. 2011). The low $\Delta\delta^{15}\text{N}$ found in whiting-fed cod tissues, especially muscle, can be partly explained assuming that the whiting flesh

most closely matches the amino acid requirements of the juvenile cod, and hence can be routed directly to the tissue (McMahon et al. 2010).

The distribution of essential amino acids in the different tissues likely influences the observed tissue–diet enrichments (Gaebler et al. 1966). Essential amino acids are subject to less biochemical change and subsequent isotopic fractionation compared to the digestion and re-synthesis of the non-essentials (Pinnegar & Polunin 1999, McMahon et al. 2010). The presence of non-essential free taurine in large amounts in fish muscle and heart (Wilson & Poe 1974) could be a possible explanation for their large $\Delta\delta^{15}\text{N}$ values of up to 5.1‰. Similar high diet–tissue enrichment values of nitrogen isotopes have been reported for fish muscle (e.g. 5.1‰, Miller 2006; 3.1 to 5.4‰, Barnes et al. 2007). Blue mussel-fed cod showed highest trophic enrichment in all tissues. This could also be due to the even larger amounts of taurine in mussels (510 mg 100 g⁻¹ WW) compared to cod muscle (120 mg 100 g⁻¹ WW; Dragnes et al. 2009). In contrast, the low $\Delta\delta^{15}\text{N}$ in bone collagen can be due to a large proportion of glycine, which was found to exhibit low trophic enrichment (Bloomfield et al. 2011).

Heart $\Delta\delta^{15}\text{N}$ was significantly higher than whole blood, and in general blood and bone collagen had the lowest $\Delta\delta^{15}\text{N}$. Amino acids directly transported from the diet into the blood plasma are possibly only subject to limited fractionation, which would decrease $\Delta\delta^{15}\text{N}$ measures in blood plasma and whole blood samples. Muscle tissue showed a very large variation in $\Delta\delta^{15}\text{N}$ among diets with low values probably related to the apparently nutritionally poor, but protein-rich, whiting diet.

Heart tissue displayed $\Delta\delta^{15}\text{N}$ values similar to muscle for cod with stable growth (mussel and sandeel diet), while $\Delta\delta^{15}\text{N}$ in muscle of whiting-fed cod was surprisingly low (0.76‰) compared to heart tissue (4.21‰). Differences in $\Delta\delta^{15}\text{N}$ due to diet could be explained by the possible difference in amino acid composition of the diet. The low $\Delta\delta^{15}\text{N}$ measured for whiting-fed cod is consistent with decreasing tissue $\Delta\delta^{15}\text{N}$ with increasing protein gain. If protein is available in large amounts, catabolism is reduced and synthesis of new proteins directly from diet amino acids is likely stimulated. All cod fed the whiting diet had the highest contributions of metabolism (lowest c values) to the isotopic shift, and also the lowest enrichment. Heart tissue $\Delta\delta^{15}\text{N}$ was an exception to the low enrichment of whiting-fed cod, likely due to the nature of heart tissue as a highly metabolically active tissue, where differences in

somatic growth are less important to the isotopic turnover. Muscle $\Delta\delta^{15}\text{N}$ seemed to be most affected by the whiting diet, and it is evident that tissues with different metabolic rates react differently to nutritionally poor diets such as whiting.

$\Delta\delta^{13}\text{C}$ in muscle was considerably higher than in heart and blood. Similar differences among tissues were found by Sweeting et al. (2007) and Pinnegar & Polunin (1999). When lipid was extracted, Pinnegar & Polunin (1999) found that differences between $\Delta\delta^{13}\text{C}$ in white muscle, heart, red muscle, and liver became statistically indistinguishable. Relative lipid content of a tissue is reflected in the C/N ratios, and these were 5.06, 4.37, and 3.54 in the blood, heart, and muscle tissue, respectively, of sandeel-fed cod on Day 120. This suggests that the increased $\Delta\delta^{13}\text{C}$ could be due to lower lipid content in the muscle compared to heart and blood. Diet effects on $\Delta\delta^{13}\text{C}$ were only significant for heart tissue, but the pattern was consistent among tissues, and $\Delta\delta^{13}\text{C}$ values for cod fed the different diets were ranked in the same order as the C/N ratios of the diet. Hence, varying lipid content between diets most likely explained the diet effect on $\Delta\delta^{13}\text{C}$, but other factors may contribute to variability in $\Delta\delta^{13}\text{C}$, including food quality, feeding rate, and metabolism (Sweeting et al. 2007b).

Using isotope signatures to identify diets

Besides knowing the isotopic turnover rate and trophic enrichment, the ability to detect diet differences is related to the isotopic variation in the tissue, the relative influence of growth and metabolism on the isotope signature, and the isotopic difference between diets. Here, 3 different approaches were used to evaluate whether the isotope signatures could be used to distinguish among cod reared on 3 different diets.

The first approach was to look at isotopic differences at equilibrium. This models a steady-state system and is not influenced by differences in turnover rates. The data suggest that blood and bone collagen $\delta^{15}\text{N}$ are superior in distinguishing between diets at equilibrium. These 2 tissue types had no significant contribution of metabolic turnover to isotopic change. Therefore, the isotopic shift in whole blood and bone tissues is mainly caused by the simple dilution effect of synthesis of new tissue reflecting the new diet, which would explain why bone and blood show the clearest trophic shift in fast-growing fish such as these cod. In contrast, metabolically active tissues such as heart and muscle did not show signif-

icant differences between blue mussel and sandeel diets at equilibrium. However, the use of diet-specific differences in equilibrium values does not take into account the turnover rate, i.e. how fast differences can be established. Therefore, a second approach was to use prediction intervals to determine whether single individuals from 2 or more diet groups can be separated. Based on $\delta^{15}\text{N}$, significant separation was found in 8 out of 12 combinations of prey and tissue. Individual cod fed whiting could be separated from cod fed either mussels or sandeel from Day 60 using heart tissue and Day 86 or 123 using muscle and blood $\delta^{15}\text{N}$. Only blood was able to distinguish all diet combinations. Hence, this approach suggests that metabolically active tissue is able to distinguish between diets faster than e.g. bone collagen. Finally, the combination of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ from heart and muscle showed good separation among all 3 diets.

Other diet-shift studies with fish (Pinnegar & Polunin 1999) have argued that the most suitable tissue is the one with lowest isotopic variation within the tissue and found white muscle to express the least $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotopic variation when fish were fed a single homogeneous diet. In the present study, white muscle also showed the least carbon δ_f variation (SD) among the sampled tissues, but for nitrogen, the tissue variation in δ_f was lowest in heart and blood tissue, which is consistent with the superior ability of these 2 tissues to distinguish between diets using prediction intervals and equilibrium $\delta^{15}\text{N}$ values.

CONCLUSIONS

This study is the first to provide isotopic turnover and diet-tissue enrichment estimates for juvenile cod. The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ turnover rate differences among tissues were relatively small compared to other studies of fish, which supports the finding that fast growth decreases the difference in isotopic turnover among tissues. Differences in diet-tissue enrichment were primarily related to the different prey types, which may complicate reconstruction of diets. However, the main difference was due to low ^{15}N enrichment in the whiting diet, which is likely related to the nutritional quality of this prey. Further, the differences in ^{15}N enrichments were negatively related to diet $\delta^{15}\text{N}$, and it is possible to correct for this relationship (Caut et al. 2009). The $\delta^{13}\text{C}$ enrichment was higher in muscle than in the other tissues. Despite variability in enrichment among diets and tissues, fish feeding on the 3 diets could be distinguished based on their isotopic signatures. The pre-

sent study can enhance the application of stable isotope-based methods to field studies of fast-growing juvenile fish trophic dynamics by providing crucial species, life stage, and tissue-specific knowledge on turnover rates and trophic enrichment. Furthermore, the results suggest that analyses of trophic enrichment of stable isotope ratios can be used to optimize diets for fishes in aquaculture, under the assumption that an optimal amino acid profile in the diet will be evident as a low trophic enrichment. Finally, it is clear that further experiments on the fractionation of individual amino acids are necessary in order to harvest full potential of stable isotopes in ecological studies.

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