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Nitrogen stable isotope variability in tissues of juvenile tilapia *Oreochromis aureus*: empirical and modelling results

Yinping Wang[1,2], Xiaohong Gu[1]*, Qingfei Zeng[1], Zhigang Mao[1] and Wenxia Wang[1,2]

[1]State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, Nanjing 210008, P.R. China
[2]University of Chinese Academy of Sciences, Beijing 100049, P.R. China

RATIONALE: Studies on diet or trophic interactions of organisms based on stable isotopes require accurate estimates of how quickly stable isotope ratios change in the investigated tissues. However, rates of isotope turnover in fish tissues, especially in omnivorous species, are poorly understood.
METHODS: We conducted a diet-shift study using juvenile tilapia to (i) empirically estimate the isotopic turnover rates of nitrogen in the dorsal muscle, liver, fin and backbone; (ii) model the relative contributions of metabolism and growth to the total isotopic turnover in each tissue; and (iii) develop a non-lethal approach for estimating body nitrogen stable isotope ratios for threatened or endangered species. Isotopic analyses were performed using a Flash EA CN elemental analyser coupled to a ThermoFinnigan Delta Plus mass spectrometer.
RESULTS: Nitrogen isotopic turnover rates were consistently ranked in the order backbone > liver > muscle > fin due to the relatively lower metabolic rates of muscle and fin tissue. Backbone tissue turned over significantly faster than other tissues, suggesting the potential for a multiple-tissue stable isotope approach to the study of movement and trophic position over different time scales for omnivorous fish. However, fin tissue had the longest half-life, at 57.81 days, indicating that this tissue is more useful than muscle as a long-term dietary indicator.
CONCLUSIONS: The change in nitrogen isotope ratios in dorsal muscle was mainly regulated by somatic growth, but metabolic activity markedly stimulated the turnover rate of backbone. This study is one of a few to demonstrate significant variation in the $\delta^{15}N$ turnover rates among multiple tissues of a single organism, especially for omnivorous fish. Our results, to some extent, also indirectly contribute to the conservation of threatened or endangered species. Copyright © 2016 John Wiley & Sons, Ltd.

Nitrogen stable isotope analysis is widely used to make inferences regarding the food web structure and the phenology of consumer diet conversions, applications that require a precise isotopic interpretation of trophic resources to avoid biased inferences of feeding relationships.[1] Coupling the known spatial variation of stable isotopes with tissue-specific temporal variation allows researchers to estimate trophic positions and to determine movement patterns.[2] In addition, stable isotopes can reflect animal dietary patterns over longer periods of time than a direct analysis of stomach contents.[3,4] Therefore, knowledge of tissue-specific turnover and nitrogen discrimination in study organisms is important for an accurate interpretation of field results, such as tissue-specific rates of change as a measure of 'recent' and 'long-term' diets.[5] Discrimination represents the discrepancy between isotope values for a diet and fully equilibrated consumer tissue.[6] Nitrogen stable isotope ratios, which typically discriminate approximately 3.4‰ per trophic level,[7] are often used to determine the trophic position, nutrient partitioning and migration of an organism.[8]

* *Correspondence to:* X. H. Gu, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, 73 East Beijing Rd., Nanjing 210008, P.R. China.
E-mail: xhgu@niglas.ac.cn

For aquatic ectotherms, such as fish, the temporal integration of isotopes has been tested in juvenile stages, during which isotopic turnover was almost completely due to growth rather than to metabolic turnover.[9,10] Of the studies determining isotopic integration in fast-growing organisms, most have focused on white muscle and inner organs, such as the liver, heart, blood, gill and digestive gland,[11,12] whereas empirical measurements of fractionation values for hard tissue, such as the backbone and fin, are lacking. Hard tissues that are regenerated could be used for the measurement of isotope ratios instead of sacrificing the experimental organism, providing an additional tool to help researchers study threatened or endangered species.

The present study uses the juvenile tilapia *Oreochromis aureus* to investigate the turnover rates of nitrogen stable isotopes in conventional tissues (dorsal muscle and liver) and hard tissues (fin and backbone). Tilapias are among the most commercially cultured species and constitute the third largest group of farmed finfish worldwide, especially in tropical and subtropical regions.[13,14] Many attempts using various methodologies have been made to understand nutrient assimilation and growth efficiency in tilapia. Some researchers have reported changes in the stable isotope ratio of nitrogen in particular tissues of tilapia, but studies on nitrogen isotopic discrimination among tissues are relatively rare.

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The main purpose of this study was to empirically estimate the isotopic turnover rates of nitrogen in the dorsal muscle, liver, fin and backbone of juvenile tilapia after a diet switch. In addition, the relative contributions of metabolism and growth to the total isotopic turnover in each tissue were determined. Furthermore, a non-lethal approach to estimating body nitrogen stable isotope ratios for threatened or endangered species was assessed. Finally, this study may shed light on the differences in trophic enrichment among different tissues and organs.

EXPERIMENTAL

Fish collection and husbandry

On 1 July 2014, tilapia were sampled with a 2 m beam trawl in a pond adjacent to Meiliang Bay, close to the Taihu Laboratory for Lake Ecosystem Research, Chinese Academy of Sciences (TLLER, CAS), where this experiment was conducted in a recirculating aquaculture system. All the individuals were transferred to five large polyethylene containers to acclimatise to experimental conditions. To maximise growth rates for the experiment, only juvenile tilapia were selected. Six fish were selected at random, blotted dry, measured to the nearest mm in total length, weighed (0.01 g) and sacrificed to determine the initial $\delta^{15}N$ value of each tissue before the diet switch. The variations among tissues were taken into consideration when determining the expected values.

After a week of acclimation, the fish were transferred to three aquaria (capacity 60 L) with a flow of ambient pond water for 100 days, with 15 fish per aquarium. The aquaria had a 2–3 cm sand layer and a fine-meshed gauze cover to prevent tilapia escape. Cyanobacterial pellets (Table 1) were fed to the tilapia twice per day. To ensure the isotopic homogeneity of the food source, the cyanobacteria were lyophilised and ground to a homogeneous powder prior to forming cyanobacterial pellets. The feeding procedures were as follows: (1) feed at 1.5% of the biomass every day at 8:30 and 16:30; (2) feed to satiation (until 5–10 pellets are left on the bottom). Fish faeces and the remaining food were siphoned away daily.

Table 1. Approximate composition (dry weight %) of cyanobacterial pellets in the experiment

Material composition		Energy composition	
Cyanobacteria	75%	Crude protein	44.1%
Wheat meal	15%	Crude lipid	4.41%
Premix powder[a]	2%	Crude fibre	0.465%
Cellulose	1.5%	Ash	7.605%
Attractants	1%	Total energy (MJ/kg)	18.07
Oils[b]	5%	-	-
Adhesive	0.5%	-	-

[a]The compound premix provides the following minerals and vitamins per kg of diet: vitamin A, 40 000 I.U.; vitamin D_3, 25 000 I.U.; vitamin E, 600 mg; vitamin K_3, 120 mg; vitamin B_1, 180 mg; vitamin B_2, 640 mg; vitamin B_6, 180 mg; vitamin B_{12}, 1.6 mg; vitamin C, 600 mg; niacin, 100 mg; calcium pantothenate, 150 mg; folic acid, 40 mg; inositol, 450 mg; biotin, 8 mg; choline chloride, 12 mg; Fe, 24.6 mg; Cu, 1.57 mg; Mn, 1.68 mg; Zn, 3.52 mg; K, 1.54 mg; and Na, 0.322 mg.
[b]The ratio of fish oil to maize oil is 1:1.

Three random tilapias were sampled at 5, 11, 18, 25, 35, 45, 60, 75 and 90 days after the diet switch to determine their tissue nitrogen stable isotope ratios. To minimise the sampling error, the fish were transferred to polyethylene buckets for 24 h to allow for gut evacuation and then blotted dry, measured to the nearest mm in total length, weighed (0.01 g) and sacrificed for tissue sampling. To avoid ecological variability,[11] the dorsal muscle, liver, fin and vertebra of three fish from each aquarium were used for stable isotope measurements. Tissues from 33 tilapias were analysed for their $\delta^{15}N$ values.

Stable isotope analysis

After the fish had been sacrificed, the tissues were quickly rinsed with deionised water, dried to a constant weight at 60°C and crushed to a fine powder using a mortar and pestle. Sub-samples were then weighed to the nearest 0.001 mg and packed in tin capsules for isotopic analysis. Cyanobacterial pellets and tissues from each sampling event were analysed in triplicate. Isotopic analyses were performed at the Nanjing Institute of Geography and Limnology using a Flash EA CN elemental analyser coupled to a ThermoFinnigan Delta Plus mass spectrometer (both from Thermo Fisher Scientific, Bremen, Germany). Stable isotope ratios were expressed in the conventional δ notation as parts per thousand (‰), according to the following equation:[15,16] $\delta^{15}N = [\frac{R_{sample}}{R_{standard}} - 1]$, where R is the ratio of heavy and light isotopes in a sample, $^{15}N/^{14}N$. The R standard value was based on atmospheric N_2. The precision of the isotopic analyses was ±0.1‰.

Turnover modelling

An equation developed by Tieszen *et al*. was fitted to the dorsal muscle, liver, fin and backbone isotope data.[17] The Tieszen equation predicts the tissue isotopic signature as a function of time: $\delta_t = \delta_f + (\delta_i - \delta_f)e^{vt}$, where $\delta_t = \delta^{15}N$ value of a tissue at the time of fish sacrifice, $\delta_f = \delta^{15}N$ value in equilibrium with a new diet, δ_i = initial $\delta^{15}N$ value, t is the sampling time in the experiment (days), and v is a measure of the turnover rate (day^{-1}). δ_f and v were determined by fitting each equation using an iterative, non-linear least-squares regression. The initial $\delta^{15}N$ value was determined using the mean value of the six individuals before the diet switch. The half-life of isotopic turnover was estimated using the following equation:[17] $t_{\frac{1}{2}} = \frac{\ln(0.5)}{v}$.

The relative contributions of growth and metabolic turnover to the observed isotopic turnover rates were estimated using the equation developed by Fry and Arnold.[18] The Fry–Arnold equation predicts tissue isotopic signature as a function of growth: $\delta_t = \delta_f + (\delta_i - \delta_f) \times M_R^c$, where δ_t, δ_i and δ_f have the same meaning as in the time model; M_R= mass ratio = final mass/initial mass; and c = curve-fitted turnover rate. When $c = -1$, the rate of change in isotopic composition was due only to growth (simple dilution), whereas $c < -1$ represents proportionately greater contributions of metabolic turnover to overall isotopic

change.[18] δ_f and c were determined by fitting each equation using an iterative, non-linear least-squares regression. The weight gain ratio (WGR, %) was calculated for individual fish: WGR = 100 × $(W_t–W_i)/W_i$, where W_i and W_t indicate the weight of an individual at the beginning and at the time of sampling in the experiment, respectively, and t has the same meaning as in the turnover model.

Statistical analysis

The equation curve fitting was performed using STATISTICA version 10 (StatSoft, Tulsa, OK, USA). Again, for the curve-fitting procedure, δ_i was regarded as the mean initial value. The differences in isotopic composition among tissues were tested using one-way analysis of variance (ANOVA); moreover, curve-fitted c values were statistically compared using a one-tailed t-test to a c value of −1. A significance level of $\alpha = 0.05$ was chosen for all of the statistical tests. Statistical analyses were performed using SPSS 22.0 (IBM Corp, Armonk, NY, USA). The data are expressed as the mean ± standard deviation (SD).

RESULTS

The tilapia had quadrupled their biomass by the end of the experiment; the starting (pre-diet-switch) fish weight was 15.23 ± 1.75 g (n = 33), and the final weight was 59.22 ± 3.14 g (n = 3) after 90 days. In addition, the tilapia weight varied significantly with time (r^2 = 0.83) (Fig. 1). The mean growth rate in the present study was 0.018 g·day^{-1}, and individual weight growth rates ranged from a 5.96% to 78.86% change in g per day (17.46 ± 4.48% day^{-1}; n = 27). The mass ratio (W_t /W_i) changed almost linearly with time, with a final value of 4.04 ± 0.37 (n = 3) at day 90 (Fig. 1).

All the estimates and models were statistically significant ($P < 0.05$), and they explained 84 to 98% of the variation. The δ^{15}N values of all the tissues changed immediately after the shift to the pellet diet (Fig. 2). The rate of isotopic change for nitrogen differed considerably among tissues. The highest isotopic turnover rate was observed in backbone tissue (2.24 days), whereas fin tissue had the slowest turnover rate, with a half-life of 57.81 days. Surprisingly, the liver δ^{15}N value exhibited a turnover rate (6.5 days) similar to that of the dorsal muscle δ^{15}N value (10.47 days). There were differences between the $\delta_{f\ \mathrm{observed}}$ and $\delta_{f\ \mathrm{predicted}}$ values for each tissue, but the differences were small (0.44‰, 0.41‰, 0.69‰ and 0.05‰ for muscle, liver, fin and backbone, respectively; Table 2). These results demonstrate that all the tissues were extremely close to equilibrium by the end of the experiment.

Values for the coefficient of metabolic decay (c) indicated that isotopic turnover rates were attributable to both biomasses gain and metabolic turnover (Fig. 3, Table 3). The exponent of metabolic decay (c) was not significantly different from −1 for δ^{15}N values in dorsal muscle and liver tissue, whereas the c values for fin and backbone differed notably from −1 ($P < 0.01$; Table 3). The isotopic turnover rates varied among all tissues; the estimated c values for the Fry–Arnold equation as a function of growth were −1.43 ± 0.19 for dorsal muscle, −1.73 ± 0.25 for liver, −2.81 ± 0.29 for fin and −10.4 ± 1.17 for backbone ($P < 0.01$; Table 3).

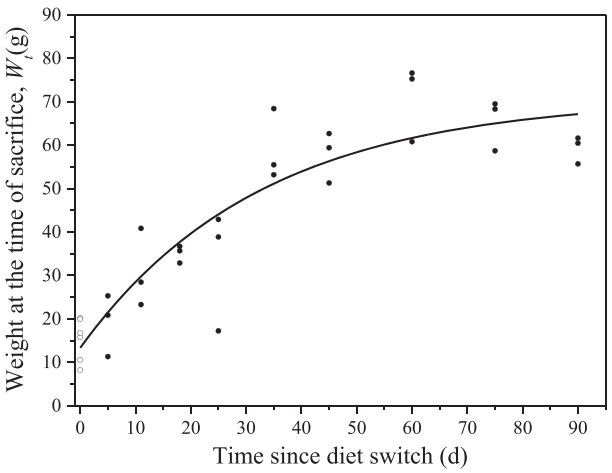

Figure 1. Tilapia (*Oreochromis aureus*) growth following a diet switch. Growth is represented as weight at the time of sampling (W_t); the open circles represent the initial weight.

DISCUSSION

The data from the present study make a small contribution to the literature of isotopic variation dynamics in the early life stages of fish.[19] These fish exhibit a rapid growth rate during the initial stages of their lives, and their turnover rate processes rely heavily on environmental variables.[20,21] The liver and fin tissues of juvenile tilapia reached or approached equilibrium with the new diet by the time that a 2-fold biomass gain was achieved and within the duration of the dietary switch experiment (90 days), and a biomass gain of greater than 5-fold was reached when dorsal muscle approached equilibrium, within 1 month. Moreover, a relatively short time (approximately 10 days) was required for backbone tissue to reach equilibrium, accompanied by an approximately 1.5-fold increase in mass. These results indicate that variations in growth rate and/or metabolic turnover between individuals following a diet switch will result in differences in isotopic composition and will lead to within-population variation.[22]

The δ^{15}N values of all the tissues changed rapidly following the shift to an artificial pellet diet (Fig. 2). The final value of δ^{15}N was distinct among tilapia tissues, presumably related to the variations in relative abundances of amino acids in the different tissues. Pinnegar and Polunin[23] reported that the isotopic composition of essential amino acids showed no evident change during assimilation. Nevertheless, protein synthesis and turnover may produce alterations in δ^{15}N values to some extent, depending on the differential metabolic activities.[23] Liver protein has a low proportion of essential amino acids, which might explain the lower δ^{15}N value observed for liver.[21,24] The δ_f values of dorsal muscle, fin and backbone are higher than that of liver tissue (Fig. 2). These results are consistent with those of Sweeting *et al.*[9] and Carleton and Del-Rio,[20] who demonstrated that digestive organs, such as liver and intestine, generally showed higher isotopic turnover rates than other tissues.

The nitrogen turnover rates estimated based on time- and growth-based models consistently decreased in the order backbone > liver > muscle > fin. Due to the different turnover

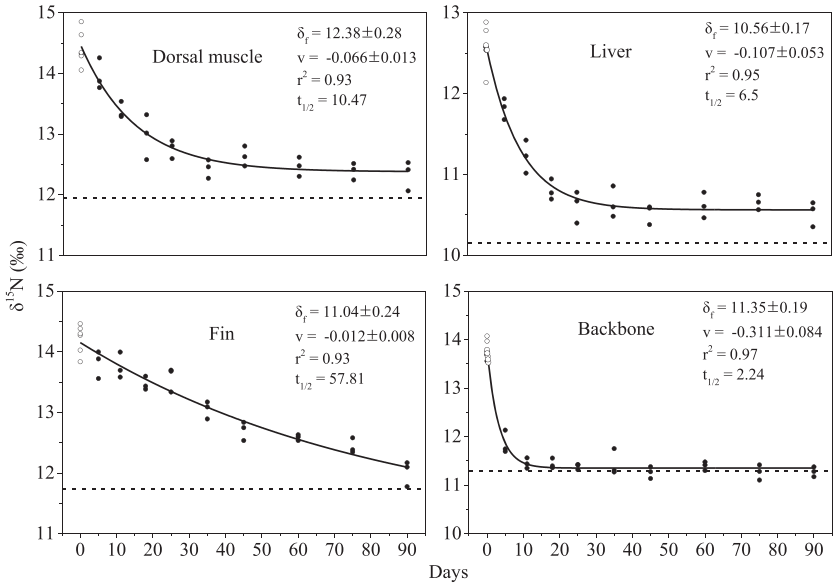

Figure 2. Changes in nitrogen stable isotope ratios as a function of time (t) for muscle, liver, fin and backbone tissues. Continuous lines: best fit through data following $\delta_t = \delta_f + (\delta_i - \delta_f)e^{vt}$; δ_f (‰) and v were estimated with the model (± SE); $t_{1/2}$ values (days (d)) are also shown. Dashed lines: expected final values when in equilibrium with the pellet diet (Table 2); the open circles represent the initial δ^{15}N values. For muscle tissue, expected values were calculated by adding 3.4‰ to the pellet δ^{15}N values; for liver, fin and backbone tissues, initial differences in isotopic ratios from those of muscle tissue were considered.

Table 2. Turnover modelling equation as a function of time and the expected δ^{15}N value of tilapia (*Oreochromis aureus*) dorsal muscle, liver, fin and backbone tissues

Tissue	Tieszen equation	*df*	r^2	Initial δ^{15}N (‰)	Expected δ^{15}N (‰)
Dorsal muscle	$\delta t = 12.38 + 2.07e^{-0.066t}$	33	0.93	14.42 ± 0.28	11.94
Liver	$\delta t = 10.56 + 2.04e^{-0.11t}$	33	0.95	12.63 ± 0.17	10.15
Fin	$\delta t = 11.04 + 3.11e^{-0.012t}$	33	0.93	14.21 ± 0.24	11.73
Backbone	$\delta t = 11.35 + 2.43e^{-0.31t}$	33	0.97	13.78 ± 0.19	11.3
Pellet diet	–	–	–	8.54 ± 0.22	–

rates, backbone had the shortest half-life (2.24 days), suggesting that it had the greatest potential to indicate the most recent change in dietary isotopes. In contrast, fin was significantly slower to respond to diet switch (Fig. 2, Table 2). Surprisingly, liver had the second shortest half-life (6.5 days), consistent with the results of Guelinckx *et al.*[11] but different from those of MacNeil *et al.*[25] and Malpica-Cruz *et al.*,[26] who demonstrated a higher turnover rate in liver tissue than in cartilage. The turnover rate of nitrogen was higher in liver tissue than in dorsal muscle, which was consistent with results found in most other fish,[27] although some studies have demonstrated little or no difference in isotopic turnover rates among the tissues of each specific fish.[9,28] In the present study, tilapia fin tissue showed a longer half-life than those of muscle and other tissues (Fig. 2), indicating a relatively low turnover rate in fin tissue; the same result was observed in catfish.[28] In contrast, blue cod fin tissue exhibited a higher nitrogen isotopic turnover rate than muscle.[29] This difference suggests that any exploratory trial on fin tissue must undertake species-specific correction for comparisons with data from muscle tissue. The present results contrast with those of some reports exploring fin tissue as a muscle isotopic proxy[30] but agree with others.[31]

The differences in the turnover rate observed among tissues may be partly due to temperature variation. Bloomfield *et al.*[32] indicated that high temperature could partially explain the high metabolic turnover in digestive tissue, but this was not the case for the other tissues. A remarkable increase in the efficiency of digestive organ protein turnover but not in that of non-digestive tissue protein was observed in rainbow trout raised at elevated temperature, suggesting an increase in protein synthesis for liver at higher temperatures.[27] Nevertheless, the temperature in our study was approximately the same as that of the tilapia's natural environment; thus, differences in isotope turnover among tissues could not be linked to a change in temperature. Some researchers have also reported that isotopic turnover rates varied with body size, ontogenetic state and other environmental factors,[33–35] which were not responsible for the turnover variation in our study. We deduced that the

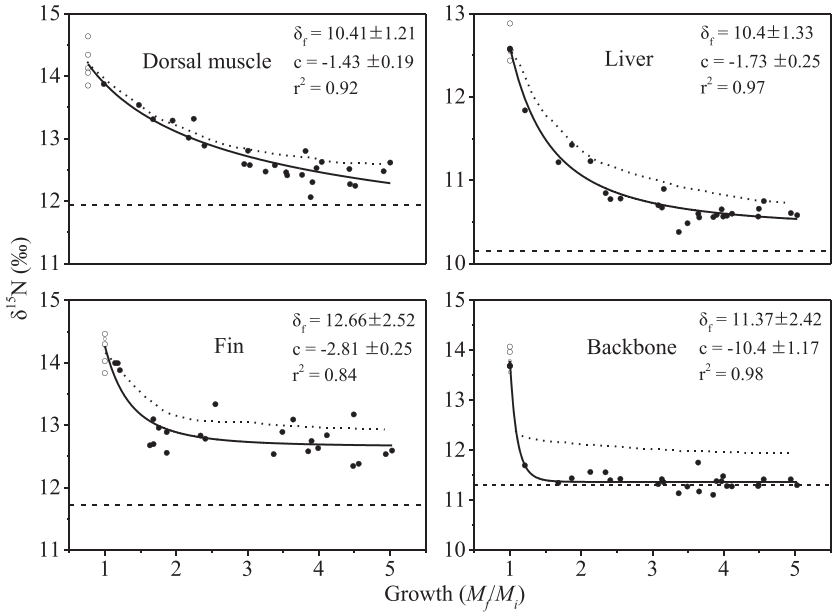

Figure 3. $\delta^{15}N$ values in tilapia (*Oreochromis aureus*) muscle, liver, fin and backbone tissues relative to growth, defined as final mass (M_f) divided by initial mass (M_i). Dotted lines: change in isotopic composition due to dilution only ($c = -1$). Dashed lines: expected final values when in equilibrium with pellet diet (Table 2); the open circles represent the initial $\delta^{15}N$ values.

Table 3. Values of *c* for tilapia (*Oreochromis aureus*) muscle, liver, fin and backbone tissues

Tissue	Fry-Arnold equation	*c* value (± SD)	*df*	r^2
Dorsal muscle	$\delta_t = 10.41 + 4.01 \times M_r^{-1.43}$	–1.43 ± 0.19	33	0.92
Liver	$\delta_t = 10.4 + 2.23 \times M_r^{-1.73}$	–1.73 ± 0.25	33	0.97
Fin	$\delta_t = 12.66 + 1.55 \times M_r^{-2.81}$	–2.81 ± 0.29	33	0.84
Backbone	$\delta_t = 11.37 + 2.41 \times M_r^{-10.4}$	–10.4 ± 1.17	33	0.98

variation in isotopic turnover rates was mainly attributable to the different biochemical compositions of the tissues. Furthermore, differences in composition among tissues might explain isotopic routing, meaning that dietary nutrient components are allocated differentially to specific tissues and tissue components.[11]

The coefficient of metabolic decay (*c*) provides a new way to evaluate the relative contribution of growth and metabolic replacement to turnover rate. The decreased *c* value represents an increase in the relative contribution of metabolic replacement to isotopic change. *c* was significantly different from −1 for $\delta^{15}N$ values in backbone ($c = -10.4$) and fin ($c = -2.81$) tissues (Fig. 3, Table 3), indicating that metabolic activity stimulates the change in isotopic turnover rate and results in tissue-specific isotopic turnover rates. The turnover rate of organism appendages, such as fish fins, was positively related to metabolic replacement, but no obvious connection was observed with growth.[36] In addition, the turnover rate in fin is likely to be variable and strongly species dependent. Malpica-Cruz *et al.*[26] reported that metabolic replacement made a limited contribution to the isotopic turnover rate ($c = -1.7$) for large sharks, whereas Willis *et al.*[29] demonstrated no consistency in the contribution of growth and/or metabolic activity to isotopic turnover. Furthermore, *c* may vary greatly with body size, temperature, species and ontogeny.[26,32,37] The shortest half-life was observed in backbone tissue, meaning that growth is only responsible for the isotopic turnover rate for a relatively short time, approximately 5 days. Thus, metabolic replacement exerted a remarkable influence on nitrogen turnover, resulting in the smallest *c* value (−10.4). The liver is a digestive organ with a continuous protein turnover rate that surpasses that in muscle tissue,[38] consistent with the shorter half-life of $\delta^{15}N$ in liver than in muscle. Dorsal muscle is considered to be representative of growth, rather than of regulatory tissue, when considering protein synthesis and decomposition.[38] This pattern might explain the finding that the estimated value of *c* (−1.43 ± 0.19, mean ± SD) was not significantly different from −1; in other words, metabolic activity is not important for nitrogen isotopic change in juvenile tilapia muscle.

CONCLUSIONS

The results presented here demonstrate notable differences in turnover rate among tissues and significant metabolic contributions to the nitrogen turnover rate of tilapia backbone and fin tissue. Our data suggest that fin tissue cannot be used

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to predict body isotope ratios because there are marked differences between the isotope turnover rates of fin and other tissues. However, our results provide theoretical support for non-lethal tissue substitution for small, rapidly growing fish and aid in research on large or long-lived fish species. Furthermore, the present results allow for a more precise interpretation of field data and markedly improve the ability to use stable isotopes for studying migration patterns and trophic ecology in the wild.

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