Turnover rates of nitrogen stable isotopes in the salt marsh mummichog, *Fundulus heteroclitus*, following a laboratory diet switch

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Abstract Nitrogen stable isotopes are frequently used in ecological studies to estimate trophic position and determine movement patterns. Knowledge of tissue-specific turnover and nitrogen discrimination for study organisms is important for accurate interpretation of isotopic data. We measured $\delta^{15}N$ turnover in liver and muscle tissue in juvenile mummichogs, *Fundulus heteroclitus*, following a laboratory diet switch. Liver tissue turned over significantly faster than muscle tissue suggesting the potential for a multiple tissue stable isotope approach to study movement and trophic position over different time scales; metabolism contributed significantly to isotopic turnover for both liver and muscle. Nitrogen diet-tissue discrimination was estimated at between 0.0 – 1.2 ‰ for liver and –1.0 – 0.2 ‰ for muscle. This is the first experiment to demonstrate a significant variation in $\delta^{15}N$ turnover between liver and muscle tissue in a fish species.

Keywords Discrimination · Liver · Metabolism · ^{15}N · Trophic level

Introduction

Nitrogen stable isotopes provide natural markers that are increasingly used to study food webs and movement patterns (Hobson 1999). The isotopic signatures of organisms reflect the stable isotope ratios of their diets offset by a discrimination factor. Discrimination represents the difference between isotope values for diet and fully equilibrated consumer tissue (Martínez del Rio and Wolf 2004). Nitrogen, which typically discriminates 2 to 4 ‰ (DeNiro and Epstein 1981; Minagawa and Wada 1984; Gannes et al. 1998; Post 2002), is often used to determine trophic position (Fry and Sherr 1984; Peterson and Fry 1987). Isotopic analysis of multiple tissue types with different turnover times can potentially be used to determine diet (Kurle and Worthy 2002) or movement patterns (Fry et al. 2003) over a range of time scales by linking isotopic values to specific food or habitat types.

Among ectothermic organisms, isotopic change is generally attributed to growth rather than metabolism. Most laboratory diet-switch experiments (Hesslein et al. 1993; Herzka and Holt 2000; MacAvoy et al. 2001; Bosley et al. 2002; Tominaga et al. 2003) and field studies (Vander Zanden et al. 1998; Maruyama et al. 2001) show that growth is the primary factor causing stable isotopic change in fish following a diet shift. One field study of larval red drum has found significant metabolic turnover, and the authors suggest that differences between larval energetic requirements in the laboratory and in the field may account for variation between lab and field results (Herzka et al. 2001).

In endothermic organisms, isotopic turnover varies significantly among tissues in relation to the tissue's relative metabolic activity; turnover is faster in liver than muscle tissue (Tieszen et al. 1983; Hobson and Clark 1992). While isotopic turnover rates in

ectotherms should theoretically also vary according to relative tissue metabolic activity, experimental results have not been able to show significant variation amongst tissues in ectotherms (Hesslein et al. 1993; Johnson et al. 2002).

This study uses the salt marsh mummichog, *Fundulus heteroclitus*, to examine turnover rates of nitrogen stable isotopes in liver and muscle. The salt marsh mummichog is an ecologically-important, estuarine species, which is abundant along the east coast of North America (Robins and Ray 1986). Stable isotopes have been used to determine the placement of mummichogs in food webs (Deegan and Garritt 1997) and estimate habitat use (Currin et al. 2003). Knowledge of species-specific and tissue-specific turnover and nitrogen discrimination is important for accurate interpretation of isotopic data because past studies have demonstrated variation between species (Hesslein et al. 1993; Herzka and Holt 2000; MacAvoy et al. 2001; Bosley et al. 2002) and also between tissues of the same species (Tieszen et al. 1983; Hobson and Clark 1992).

In this study, we estimate isotopic turnover rates of nitrogen in individual mummichogs that have been switched from a natural diet (baseline $\delta^{15}N \approx 8$ ‰) to a laboratory diet of tuna ($\delta^{15}N \approx 15$ ‰). The growth of each fish was tracked so that the total isotopic change could be separated into growth and metabolic turnover, the two components that contribute to a change in tissue isotopic values. Both liver and muscle tissues were measured to examine turnover rates in multiple tissues of a single organism.

Materials and methods

Fish collection and husbandry

Mummichogs (n = 65) were collected from a single salt marsh creek on the Rowley River, Plum Island estuary, Rowley, Massachusetts, USA on May 1, 2003. In

order to maximize growth rates for the experiment, the smallest abundant size class was selected (40-51 mm). After allowing 24 hours for gut clearance, individuals were blotted dry, weighed (g ± 0.01), measured (Total length (TL) ± 0.1 mm), and individually (n = 65) marked via subcutaneous injection of dorsal lateral bands of acrylic paint (Lotrich and Meredith 1974) following anesthesia with seltzer water.

All individuals were transported to the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts, USA on May 2, 2003. The initial $\delta^{15}N$ value of liver and muscle was determined by sacrificing five individuals. The fish were held (n = 20 per tank) in three heated (18ºC) 75.8 liter tanks with flow through ambient seawater for up to 102 days. Box filters with carbon inserts and daily siphoning of excess food and detritus maintained water quality under reduced flow conditions. Ground frozen tuna was fed daily to the mummichogs *ad libitum.* To ensure isotopic homogeneity of the food source, all of the tuna was homogenized, stored frozen, and thawed in aliquots prior to use.

Mummichogs (n = 2) were sampled approximately weekly to biweekly initially when isotopic change was greatest (6, 13, 19, 23, 27, 38, 46, 56 days after diet switch), and a final sample was collected after 102 days to estimate diet-tissue discrimination. Fish were placed in a separate tank for 24 hours to allow gut evacuation, then were anesthetized, measured (TL ± 0.1 mm), blotted dry, weighed (± 0.01 g), and sacrificed. The remaining fish (n = 42) either died in captivity or were sacrificed and archived for other studies.

Isotopic sample preparation

Liver and muscle tissue from 23 mummichogs were analyzed for $\delta^{15}N$. Whole liver and dorsal white muscle were sampled from each fish. Following sacrifice, liver

and muscle tissues were quickly rinsed with deionized water and dried in glass scintillation vials at 66ºC for at least 24 hours. Dried samples were ground to a homogeneous 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. Three samples of tuna were removed from the homogenized food supply and dried in the same manner as the tissue samples. Two tuna samples were soaked in deionized water for 10 to 15 minutes prior to drying to remove dissolved components that might be released from the food before consumption.

Sample analysis

Isotopic analyses were performed on individual liver and muscle tissue samples at the Stable Isotope Laboratory, Marine Biological Laboratory. Measurement of $\delta^{15}N$ was performed using a dual-inlet Finnigan MAT Delta S isotope ratio mass spectrometer with a Heraeus elemental analyzer - cryogenic "trapping box" preparation system. Analytical precision was ± 0.1 ‰ (http://ecosystems.mbl.edu/SILAB/). Atmospheric nitrogen gas was used as the standard. Stable isotope ratios are expressed as parts per thousand differences from this standard in the following equation (Peterson and Fry 1987):

$\delta^{15}N = [(R_{sample}/R_{standard})-1] \times 10^3$, where R is the ratio of heavy and light isotopes in a sample, $^{15}N/^{14}N$.

Growth rate and turnover rate estimation

An equation developed by Fry and Arnold (1982) was fitted to liver and muscle isotope data. The Fry-Arnold equation predicts tissue isotopic signature as a function of growth:

$y = a + b*M_R^c$, where $y = \delta^{15}N$, $a = \delta^{15}N$ value in equilibrium with lab diet, b = initial $\delta^{15}N$ value – $\delta^{15}N$ in equilibrium with lab diet, M_R = mass ratio = final mass/initial mass, and c = curve-fitted turnover rate.

A c-value of -1 indicates turnover due only to growth (simple dilution) while c-values less than -1 represent proportionately greater contributions of metabolic turnover to overall isotopic change (Fry and Arnold 1982).

Values of c were determined by fitting each equation using iterative, non-linear least squares regression. All equation curve fitting and statistical analyses were performed using SYSTAT version 10 (© SPSS Inc. 2000). Tissue-specific turnover rates of $\delta^{15}N$ were compared by an overall test for coincidental regressions (Zar 1984). This F-test compares the sum of squares error for curve-fitted individual tissue data with sum of squares error for combined data. Curve-fitted c-values with their associated asymptotic standard errors were statistically compared using a one-tailed t-test to a c value of -1. Specific growth rate (SGR = 100(ln initial mass – ln final mass)/t) where t = time since diet switch, and mass ratio (M_f/M_i) were calculated for individual fish.

Since variation in $\delta^{15}N$ for tissues sampled during the latter portion of this experiment was minimal, equilibrium with the lab diet was derived from fish collected 102 days after the diet switch (n=2). The mean $\delta^{15}N$ value of tuna or water soaked tuna was subtracted from this 102-day mean for liver or muscle tissues to estimate discrimination. All mean values are presented ± one standard error.

Results

Individual specific growth rates (SGR) ranged from 0.66 to 1.96 % change in grams per day (1.28 ± 0.11 % d^{-1}; n = 18). Starting (pre-diet switch) fish weight was 0.84

± 0.03 g (n = 23) with a final weight of 1.75 ± 0.02 g (n = 2) after 102 days (Fig. 1). Mass ratio (M_r) varied approximately linearly with time, with a maximum mass ratio of 2.50 ± 0.09 (n = 2) at 102 days.

Metabolic turnover contributed significantly to isotopic change for both liver and muscle ($P < 0.0001$; Fig. 2), and isotopic turnover rates varied significantly between liver and muscle tissues ($P < 0.0001$; Table 1). The estimated c-values for the Fry-Arnold equation were -5.85 ± 0.61 for liver and -2.33 ± 0.25 for muscle. Both c-values differed significantly from -1 ($P < 0.0001$; Table 1).

Discrimination estimates were low and varied according to calculated diet $\delta^{15}N$ values. Liver (15.6 ± 0.0 ‰; n = 2) and muscle (14.6 ± 0.1 ‰; n = 2) had different equilibrium values at the end of the experiment. Liver (8.8 ± 0.4; n = 5) and muscle (8.2 ± 0.4; n = 5) also differed slightly at the start of the experiment. Discrimination was estimated at 0.0 ‰ for liver and -1.0 ‰ for muscle when estimated final equilibrium values were compared to water-soaked tuna (15.6 ± 0.2 ‰) and were higher (1.2 ‰ for liver; 0.2 ‰ for muscle) when compared to fresh (not water-leached) tuna (14.4 ± 0.1 ‰).

Discussion

This is the first study to demonstrate a significant variation in isotopic turnover between liver and muscle tissues in fish, and one of the few studies to demonstrate a significant metabolic contribution to nitrogen isotopic turnover in fish. The mechanisms influencing significant metabolic contribution to $\delta^{15}N$ change in liver and muscle tissues cannot be determined from this study, but hypotheses regarding protein turnover, metabolic rate, and temperature are discussed in the following paragraphs.

Measurements of protein synthesis and turnover in fish indicate variation in protein turnover rates between tissues (Jackim and LaRoche 1973; Fauconneau and Arnal 1985). If nitrogen isotope turnover and protein turnover are related, observed variation in turnover rates between mummichog tissues would match variation observed in other fish species. Protein turnover in rainbow trout, *Oncorhynchus mykiss*, (Fauconneau and Arnal 1985) and mummichogs (Jackim and LaRoche 1973) is significantly higher in liver than white muscle tissue.

Differences in turnover observed between liver and muscle tissues could partly be explained by temperature effects. Mummichogs used for the current study were maintained in warm conditions (≈18ºC) typical of water temperatures encountered in their natural environment (Abraham 1985), which could partially explain the high metabolic turnover in liver tissue. A significant increase in liver protein turnover efficiency but not muscle protein was observed for rainbow trout raised at 18°C compared to 10ºC, indicating a significant increase in protein synthesis for liver at higher temperatures (Fauconneau and Arnal 1985). Elevated temperatures should have similar differential tissue turnover effects in other ectothermic species (Millward 1989). Previously observed similarity between liver and muscle isotopic turnover rates in broad whitefish, *Coregonus nasus*, (Hesslein et al. 1993) and in lake trout, *Salvelinus namaycush*, (Johnson et al. 2002) could have been related to cold water conditions. Juvenile broad whitefish were maintained at 10ºC (Hesslein et al. 1993) while lake trout were collected from cold water reservoirs (Johnson et al. 2002), and typically occupy colder waters with ideal temperatures near 10ºC (Scarola 1987).

The high metabolic contribution to nitrogen isotopic turnover in mummichog muscle relative to most other studied fish species is probably not related to temperature effects. Despite a significant increase in liver protein turnover at higher temperatures, little variation in whole body or white muscle protein turnover was observed in rainbow trout held at 10ºC and 18ºC (Fauconneau and Arnal 1985). Differences in metabolic turnover in whole larval red drum in field and lab conditions also could not be linked to variation in temperature (Herzka et al. 2001).

Discrimination estimates for $\delta^{15}N$ of mummichog liver (0.0 to 1.2 ‰) and muscle (-1.0 to 0.2 ‰) tissues were substantially lower than the 3 ‰ enrichment initially proposed as a general trophic level discrimination (Minagawa and Wada 1984). Recent meta-analyses of field and lab isotope data indicate a range in $\delta^{15}N$ discrimination from 2.3 ± 0.18 to 3.4 ± 0.13 (Vander Zanden and Rasmussen 2001; Post 2002; McCutchan Jr et al. 2003; Vanderklift and Ponsard 2003). The absolute value of our discrimination estimate depends on the estimate of $\delta^{15}N$ for the tuna diet (water soaked versus fresh). Fish may also not have fully equilibrated with the tuna diet during the time period of this experiment, although similar $\delta^{15}N$ values for mummichogs maintained under comparable conditions but not included in this experiment for 104 and 174 days following a diet switch (15.9 ± 0.2 ‰ (n = 4) for liver and 14.7 ± 0.3 ‰ (n = 4) for muscle) further suggest complete equilibration (Logan et al. unpublished data). Despite this uncertainty in diet $\delta^{15}N$, these discrimination values fall on the lower end within the range of values included in recent meta-analyses.

Results from this study demonstrate significant differences in turnover rates amongst tissue types and significant metabolic contributions to mummichog isotopic

turnover. These results suggest the potential of using multiple tissues to investigate fish movement and trophic position over different time scales. However, determination of species and tissue-specific turnover rate estimates are needed.

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References

Abraham BJ (1985) Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Mid-Atlantic) -- mummichog and striped killifish. In: Rep. USFWSB, vol. 82(11.40). U.S. Army Corps of Engineers, p 23

Bosley KL, Witting DA, Chambers RC, Wainright SC (2002) Estimating turnover rates of carbon and nitrogen in recently metamorphosed winter flounder *Pseudopleuronectes americanus* with stable isotopes. Mar Ecol Prog Ser 236:233-240

Currin CA, Wainright SC, Able KW, Weinstein MP, Fuller CM (2003) Determination of food web support and trophic position of the mummichog, *Fundulus heteroclitus*, in New Jersey smooth cordgrass (*Spartina alterniflora*), common reed (*Phragmites australis*), and restored salt marshes. Estuaries 26:495-510

Deegan LA, Garritt RH (1997) Evidence for spatial variability in estuarine food webs. Mar Ecol Prog Ser 147:31-47

DeNiro MJ, Epstein S (1981) Influence of diet on the distribution of nitrogen isotopes in animals. Geochim Cosmochim Acta 45:341-351

Fauconneau B, Arnal M (1985) *In vivo* protein synthesis in different tissues and the whole body of rainbow trout (*Salmo gairdnerii* R.). Influence of environmental temperature. Comp Biochem Physiol A Comp Physiol 82:179-187

Fry B, Arnold C (1982) Rapid $^{13}C/^{12}C$ turnover during growth of brown shrimp (*Penaeus aztecus*). Oecologia 54:200-204

Fry B, Baltz DM, Benfield MC, Fleeger JW, Gace A, Haas HL, Quinones-Rivera ZJ (2003) Stable isotope indicators of movement and residency for brown shrimp (*Farfantepenaeus aztecus*) in coastal Louisiana marshscapes. Estuaries 26:82-97

Fry B, Sherr EB (1984) $\delta^{13}C$ measurements as indicators of carbon flow in marine and freshwater ecosystems. Contrib Mar Sci 27:13-47

Gannes LZ, Martínez del Rio C, Koch P (1998) Natural abundance variations in stable isotopes and their potential uses in animal physiological ecology. Comp Biochem Physiol A Comp Physiol 119:725-737

Herzka SZ, Holt GJ (2000) Changes in isotopic composition of red drum (*Sciaenops ocellatus*) larvae in response to dietary shifts: potential applications to settlement studies. Can J Fish Aquat Sci 57:137-147

Herzka SZ, Holt SA, Holt GJ (2001) Documenting the settlement history of individual fish larvae using stable isotope ratios: model development and validation. J Exp Mar Biol Ecol 265:49-74

Hesslein RH, Hallard KA, Ramlal P (1993) Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$. Can J Fish Aquat Sci 50:2071-2076

Hobson KA (1999) Tracing origins and migration of wildlife using stable isotopes: a review. Oecologia 120:314-326

Hobson KA, Clark RG (1992) Assessing avian diets using stable isotopes I: turnover of ^{13}C in tissues. Condor 94:181-188

Jackim E, LaRoche G (1973) Protein synthesis in *Fundulus heteroclitus* muscle. Comp Biochem Physiol A Comp Physiol 44:851-866

Johnson BM, Martinez PJ, Stockwell JD (2002) Tracking trophic interactions in coldwater reservoirs using naturally occurring stable isotopes. Trans Am Fish Soc 131:1-13

Kurle CM, Worthy GAJ (2002) Stable nitrogen and carbon isotope ratios in multiple tissues of the northern fur seal *Callorhinus ursinus*: implications for dietary and migratory reconstructions. Mar Ecol Prog Ser 236:289-300

Lotrich VA, Meredith WH (1974) A technique and the effectiveness of various acrylic colors for subcutaneous marking of fish. Trans Am Fish Soc 103:140-142

MacAvoy SE, Macko SA, Garman GC (2001) Isotopic turnover in aquatic predators: quantifying the exploitation of migratory prey. Can J Fish Aquat Sci 58:923-932

Martínez del Rio C, Wolf BO (2004) Mass-balance models for animal isotopic ecology. In: Starck JM, Wang T (eds) Physiological consequences of feeding. Springer, Berlin Heidelberg New York

Maruyama A, Yamada Y, Rusuwa B, Yuma M (2001) Change in stable nitrogen isotope ratio in the muscle tissue of a migratory goby, *Rhinogobius* sp., in a natural setting. Can J Fish Aquat Sci 58:2125-2128

McCutchan Jr JH, Lewis Jr WM, Kendall C, McGrath CC (2003) Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. Oikos 102:378-390

Millward DJ (1989) The nutritional regulation of muscle growth and protein turnover. Aquaculture 79:1-28

Minagawa M, Wada E (1984) Stepwise enrichment of $\delta^{15}N$ along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. Geochim Cosmochim Acta 48:1135-1140

Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Ann Rev Ecol Syst 18:293-320

Post DM (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83:703-718

Robins CR, Ray GC (1986) A field guide to Atlantic coast fishes of North America. Houghton Mifflin Company, Boston

Scarola JF (1987) Freshwater fishes of New Hampshire. New Hampshire Fish and Game Department

Tieszen LL, Boutton TW, Tesdahl KG, Slade NA (1983) Fractionation and turnover of stable carbon isotopes in animal tissues: implications for $\delta^{13}C$ analysis of diet. Oecologia 57:32-37

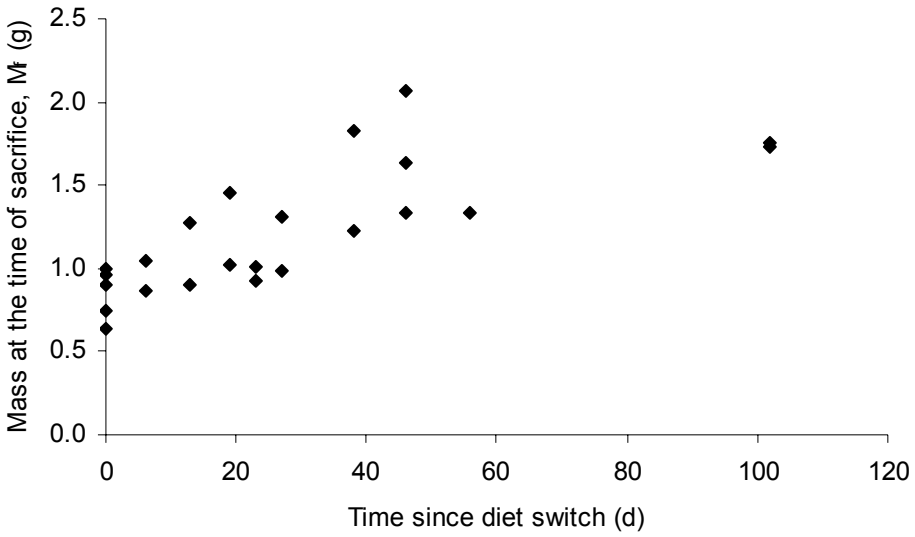
Tominaga O, Uno N, Seikai T (2003) Influence of diet shift from formulated feed to live mysids on the carbon and nitrogen stable isotope ratio $\delta^{13}C$ and $\delta^{15}N$ in dorsal muscles of juvenile Japanese flounders, *Paralichthys olivaceus*. Aquaculture 218:265-276

Vander Zanden MJ, Hulshof M, Ridgway MS, Rasmussen JB (1998) Applications of stable isotope techniques to trophic studies of age-0 smallmouth bass. Trans Am Fish Soc 127:729-739

Vander Zanden MJ, Rasmussen JB (2001) Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: implications for aquatic food web studies. Limnol Oceanogr 46:2061-2066

Vanderklift MA, Ponsard S (2003) Sources of variation in consumer-diet δ15N enrichment: a meta-analysis. Oecologia 136:169-182

Zar JH (1984) Biostatistical analysis, 2nd edn. Prentice Hall, New Jersey


Mass at the time of sacrifice, Mf (g)
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Time since diet switch (d)

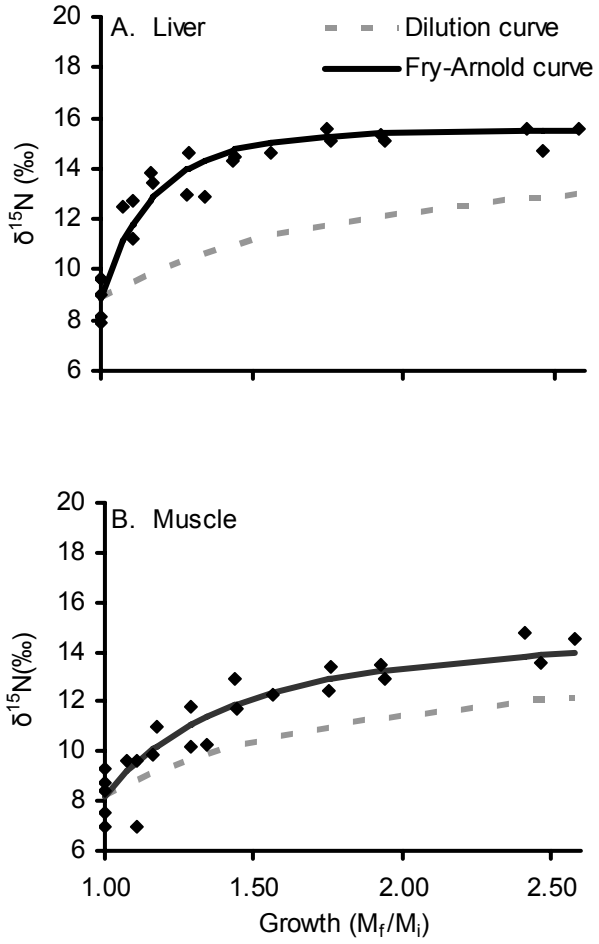

A. Liver
Dilution curve
Fry-Arnold curve
δ15N (‰)
20
18
16
14
12
10
8
6
B. Muscle
δ15N(‰)
1.00
1.50
2.00
2.50
Growth (Mf/Mi)

Table 1 Values of c for mummichog (*Fundulus heteroclitus*) liver and muscle tissue. Iterative non-linear least squares regression best fits for the Fry-Arnold model were used to generate c-values. A c-value of -1 represents isotopic change due solely to growth.

Tissue	**Fry-Arnold Equation**	**c (± SE)**	**df**	**R^2**
Liver	$Y = 15.6 - 6.8 * M_r^{-5.85}$	-5.85 ± 0.61	22	0.91
Muscle	$Y = 14.6 - 6.4 * M_r^{-2.33}$	-2.33 ± 0.25	22	0.87

Fig. 1 Mummichog (*Fundulus heteroclitus*) growth following diet switch. Growth is represented as mass at time of sacrifice (M_f)

Fig. 2 a,b $\delta^{15}N$ in mummichog A. liver and B. muscle relative to growth defined as mass at time of sacrifice (M_f) divided by initial mass (M_i). Dilution curve (dashed line) represents $\delta^{15}N$ change resulting only from growth (c = -1) (see Methods). Fry-Arnold curve (solid line) incorporates growth and metabolic turnover and represents best fit of data. Tuna diet $\delta^{15}N$ = 15.6 ± 0.2 ‰ (n = 2)