Variable uptake and elimination of stable nitrogen isotopes between tissues in fish

M. Aaron MacNeil, Ken G. Drouillard, and Aaron T. Fisk

Abstract: We conducted a diet-switching experiment using freshwater ocellate river stingrays (*Potamotrygon motoro*) fed a novel earthworm (*Eisenia foetida*) diet to establish the relative contributions of growth and metabolism to $\delta^{15}N$ values in an elasmobranch species. We specifically controlled for the potential effects of protein composition of experimental diets on $\delta^{15}N$ turnover to determine whether $\delta^{15}N$ turnover after a low to high $\delta^{15}N$ diet switch (uptake) and a high to low $\delta^{15}N$ diet switch (elimination) will occur at the same rate within each consumer tissue. Our results showed that the turnover of $\delta^{15}N$ from metabolism and growth differed between uptake and elimination phases in the liver, blood, cartilage, and muscle of freshwater stingrays. During uptake, liver was found to track dietary $\delta^{15}N$ more closely than the other tissues, with the highest metabolic turnover rate of $\delta^{15}N$ (0.015 day^{-1}), whereas cartilage had the slowest rate of metabolic $\delta^{15}N$ turnover (0.0022 day^{-1}) relative to a constant rate of growth among tissues (0.003 day^{-1}). We propose that estimates of trophic position from muscle sampling alone have considerable uncertainty, particularly for scavenging or omnivorous species. We suggest that multitissue sampling can identify this problem and lead to a more robust evaluation of trophic dynamics for individual species.

Résumé : Nous avons mené une expérience de changement de régime alimentaire chez la pastenague d'eau douce ocellée (*Potamotrygon motoro*) nourrie d'un régime inusité de vers de terre (*Eisenia foetida*) afin d'établir les contributions relatives de la croissance et du métabolisme aux valeurs de $\delta^{15}N$ chez une espèce d'élasmobranches. Nous avons tenu compte de façon particulière des effets potentiels de la composition protéinique des régimes alimentaires expérimentaux sur le taux de remplacement de $\delta^{15}N$ afin de déterminer si le taux de remplacement de $\delta^{15}N$ est le même après un changement d'un régime de $\delta^{15}N$ bas à élevé (accumulation) et un changement d'un régime de $\delta^{15}N$ élevé à bas (élimination) dans chacun des tissus du consommateur. Nos résultats montrent que les taux de remplacement de $\delta^{15}N$ à partir du métabolisme et de la croissance diffèrent durant les phases d'accumulation et d'élimination dans le foie, le sang, le cartilage et le muscle chez les pastenagues d'eau douce ocellées. Durant l'accumulation, les valeurs mesurées dans le foie suivent de plus près le $\delta^{15}N$ alimentaire que celles des autres tissus, avec le taux de remplacement métabolique de $\delta^{15}N$ le plus élevé (0,015 $jour^{-1}$), alors que le cartilage a le taux de remplacement de $\delta^{15}N$ (0,0022 $jour^{-1}$) le plus lent relativement à un taux constant de croissance des tissus (0,003 $jour^{-1}$). Nous croyons que les estimations du rang trophique faites à partir d'un échantillonnage des muscles seuls comportent beaucoup d'incertitude, particulièrement chez les espèces détritivores ou omnivores. Nous suggérons d'échantillonner plusieurs tissus pour tenir compte de ce problème et pour obtenir une évaluation plus robuste de la trophodynamique des espèces individuelles.

[Traduit par la Rédaction]

Introduction

Stable nitrogen isotope analysis is a potentially powerful tool for providing quantitative information about energy flows in food webs (Hobson and Welch 1992; Jennings et al. 2002), temporal food web structure (Vander Zanden et al. 2003), and routes of migration (Hesslein et al. 1993). These kinds of analyses assume that nitrogen isotope fractionation (the tendency of ^{15}N to be enriched relative to ^{14}N with each trophic step in a food web) occurs within a specified ecosystem in a predictable way. If the level of isotope fractionation is consistent among individuals within a system, then consumer $\delta^{15}N$ values (the ratio of the heavier to lighter stable nitrogen isotopes relative to a standard) can be used to quantitatively estimate the trophic position of each species in the system (Minagawa and Wada 1984).

With the wide adoption of stable nitrogen isotope analysis to food web studies, some authors have raised questions about the potential for misuse (Gannes et al. 1997). For example, although fractionation estimates of 2.5‰–3.4‰ per trophic link have been observed in many aquatic systems (Post 2002; Vanderklift and Ponsard 2003), important differ-

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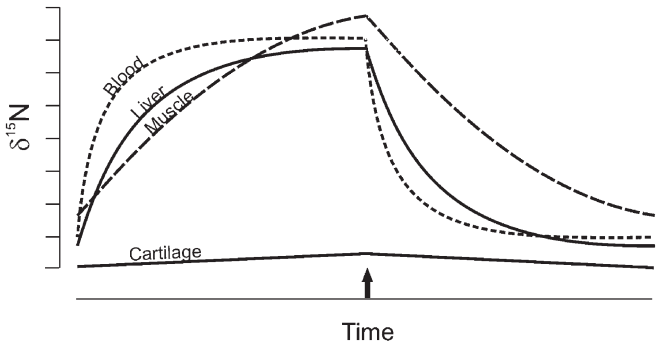
ences in fractionation values have been observed under certain conditions and for particular biological groups. Vander Zanden and Rasmussen (2001) reported a wide range of fractionation values for aquatic animals (–0.7‰–9.2‰), and both interspecies (Macko et al. 1982; Hobson and Clark 1992*a*) and intertissue (Pinnegar and Polunin 1999) variation has been reported. The deviations among species and tissues may be due to true differences in stable isotope fractionation between consumers and their prey, processes such as isotopic routing, or a failure of some organisms to achieve isotopic equilibrium with their diet at the time of sampling.

Hesslein et al. (1993) proposed that growth, rather than metabolism, determines $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$ values in fish muscle, and subsequent analyses determined that $\delta^{15}N$ signatures in larval and juvenile fish muscle are largely growth-dependent (Hesslein et al. 1993; MacAvoy et al. 2001; Bosley et al. 2002). Conversely, several authors found that high metabolic rates also appear capable of elevating muscular $\delta^{15}N$ signatures (Herzka and Holt 2000; Gaye-Siessegger et al. 2004). These results were achieved primarily in diet-switching experiments where treatment fish were fed an isotopically distinct diet and changes in their stable isotope profiles were recorded over time. With known growth rates, the percent stable isotope turnover owing to metabolism can be estimated from nonlinear regressions of the istotope turnover trajectories. These experiments focused solely on muscle tissue, however, and for most fishes, the roles of growth versus metabolism on tissue-specific $\delta^{15}N$ turnover rates have not been investigated. No diet-switching experiments have been conducted on elasmobranchs.

$\delta^{15}N$ values are conventionally regarded as average estimates of each organism's diet over its prior feeding period (Pinnegar and Polunin 1999), and consequently, the rate of isotope turnover determines each tissue's $\delta^{15}N$ at sampling — slower tissue turnover rates give $\delta^{15}N$ results representing longer feeding periods. Tieszen et al. (1983) suggested that more metabolically active tissues would show higher stable isotope turnover rates than less active tissues, which has been demonstrated for $\delta^{13}C$ turnover in birds (Hobson and Clark 1992*b*). If true in fish, this would imply that the $\delta^{15}N$ values in high-metabolism tissues such as liver would correspond to a shorter feeding period than the $\delta^{15}N$ values of lower-metabolism tissues such as muscle or cartilage. We extended this idea to suggest that differences in stable isotope turnover are also possible for each tissue depending on the relative stable nitrogen isotope signatures of the diet and consumer. In relating stable isotope turnover to ecological processes, an assumption has been made that tissue turnover rates are the same when switching from low to high $\delta^{15}N$ diets as when switching from high to low $\delta^{15}N$ diets, i.e., rates of ^{14}N and ^{15}N uptake equal rates of ^{14}N and ^{15}N decline. We define the turnover period after a low to high $\delta^{15}N$ diet switch until the animal reaches a new steady state as the uptake phase, and conversely, the period after a high to low $\delta^{15}N$ diet switch is defined as the elimination phase. Represented graphically (Fig. 1), the assumed equal rates of ^{15}N uptake and elimination give equivalent $\delta^{15}N$ uptake and elimination curves for each tissue on a given diet.

Multiple studies have shown that $\delta^{15}N$ values of a given tissue are dependent on its amino acid composition and that of the previously consumed diet because isotopic ratios often vary by amino acid (Gaebler et al. 1966; Gannes et al. 1998; Schmidt et al. 2004). Differences in amino acid composition among diets is certainly a natural phenomenon. Many animal species consume mixed diets, but in diet-switching experiments, these differences may confound laboratory results because experimental diets with distinct $\delta^{15}N$ values are apt to come from sources with unique amino acid profiles (e.g., Hesslein et al. 1993). Because the time to equilibrium may vary with diet among tissues and species, laboratory studies to determine tissue-specific $\delta^{15}N$ turnover rates on an amino acid consistent (protein-independent) diet are necessary to evaluate the relative effects of growth and metabolism.

Fig. 1. Hypothetical uptake and elimination trajectories of $\delta^{15}N$ after a diet switch. Equilibrium $\delta^{15}N$ values are tissue-specific in each direction, reflecting the different ultimate tissue fractionation values from Pinnegar and Polunin (1999), and therefore the curves approach unique asymptopes. It was predicted that equivalent values of $\delta^{15}N$ in cartilage for the high $\delta^{15}N$ diet would be reached slowly and well beyond the range of the *x* axis. The arrow indicates time at diet switch.



In this study, we used a novel enriched earthworm diet that controlled for protein composition and between-tissue growth rates to show that initial rates of $\delta^{15}N$ uptake and elimination vary within tissues of the freshwater stingray (*Potamotrygon motoro*) and that these differences primarily reflect the rates of metabolic $\delta^{15}N$ turnover for each tissue and their proximity to equilibrium. In addition, the potential for differences in $\delta^{15}N$ uptake and elimination are addressed. We discuss the research implications of this finding and suggest that several important assumptions regarding $\delta^{15}N$ turnover are poorly understood.

Methods

Diet design

To control for potential biases in amino acid composition between diets, we created isotopically distinct control and treatment diets with identical amino acid compositions using common earthworms (*Eisenia foetida*). The treatment diet was formed by adding 5 g of ammonium chloride ^{15}N concentrate (99.9% ^{15}N) to 1 L of potato and water slurry. This mixture was then incubated for 7 days to allow for absorption of the ammonium chloride by bacteria present in the mixture. The slurry was thoroughly mixed with approximately 40 L of loamy soil and 4 kg of live earthworms in a 100 L terrarium. After 8 days, 10 worms were randomly sampled and analyzed for $\delta^{15}N$ to be sure they had assimilated the isotope-enriched bacteria and attained $\delta^{15}N$ levels

greater than 200‰. Worms from the treatment terrarium were kept in a refrigerator at 7 °C and were used as the treatment diet for days 0 to 29 of the experiment. Ten worms were sampled on days 0, 2, 5, 10, 17, and 29 to monitor $\delta^{15}N$ levels. The control diet consisted of approximately 4 L of regular earthworms maintained in a second 100 L terrarium of loamy soil. Ten control worms were sampled on days 0, 2, 5, 10, 17, 29, 31, 34, 46, and 63 to verify consistent $\delta^{15}N$ values.

Experimental protocol

Thirty-nine freshwater stingrays averaging 106 ± 37 g were acquired from the Dallas World Aquarium (Dallas, Texas) and a private seller (Below Water Co., Montreal, Quebec, Canada) in November 2002. The stingrays were maintained at 26 °C and fed untreated earthworms for three months prior to the experiment to ensure that the fish had acclimated to their surroundings and were actively feeding on an earthworm diet. The stingrays were divided among three 3500 L closed-circuit filtration systems of two tanks each. For each pair of tanks on a single filtration system, one tank held nine treatment fish and the other tank held four control fish; filtration systems prevented the possibility of diet contamination between tanks. Three stingrays were sampled on day 0 to determine their initial isotope values. On day 1 of the experiment, fish in the treatment tanks were switched to the enriched earthworm diet. All animals were fed 2% of their body weight per day (chopped earthworms, averaged per total tank stingray mass), and all food items were consumed rapidly (stingrays were observed to feed vigorously and earthworms were not found in the filtration systems). Once the fish had ingested 58% of their body weight (29 days), the remaining treatment fish were returned to the control earthworm diet for 34 additional days. Rays were reweighed at four intervals during the experiment to adjust their feed rations for growth. Sampling was conducted by haphazardly sampling three treatment fish (one from each system) on days 2, 5, 10, 17, 29, 31, 34, 46, and 63. Dorsal muscle, liver, blood, and cartilage samples of 1–2 g each were taken from the same locations on each sacrificed animal and immediately frozen at –80 °C. Control fish were sampled, one from each tank pair, using the same protocol on days 2, 17, 29, and 63.

Following the experiment, tissue samples were divided into ~1 g batches and freeze-dried for at least 24 h using a Labconco 4.5 freeze-dryer (Labconco Co., Kansas City, Missouri). The dried samples were then pulverized in a SPEX CertiPrep 8000-D ball milling unit (SPEX CertiPrep, Metuchen, New Jersey) and divided into ~1 µg sealed lots in tin capsules for $\delta^{15}N$ analysis. Stable nitrogen isotope ratios were determined from gas chromatography in a Thermo Finnigan DeltaPlus mass-spectrometer (Thermo Finnigan, San Jose, California), and the precision of the isotopic analyses was ±0.15‰ $\delta^{15}N$. Two treatment samples (two muscle and two liver samples) were lost on days 10 and 29.

Statistical analyses

Comparisons of linear model log-likelihood ($\ell(\mathcal{L})$) were conducted in the analysis of variance (ANOVA) routine of the statistical package R (http://cran.r-project.org) to evaluate differences in $\delta^{15}N$ turnover between control and treatment diets and among tissues. Initially, a $\delta^{15}N$ by day model was evaluated for the entire dataset, and successive comparisons were made with the addition of treatment and tissue factors to the model. The significance of these additional factors was then determined based on their effects on $\ell(\mathcal{L})$, where increases in $\ell(\mathcal{L})$ indicate significant effects and statistical differences among factors. These results can be compared through Akaike's information criterion (AIC), the likelihood ratio ($\mathcal{L}$ ratio), and a between-model p value. Normality of the within-group errors were assessed with normal probability (QQ) plots of the standardized residuals, and departures from the linear models were examined in plots of the residuals versus fitted values.

Modelling

Mass-balance models have been most frequently used in diet-switching experiments to estimate rates of stable isotope turnover owing to growth and metabolism. These models are primarily of the form presented by Hesslein et al. (1993):

$$(1) \qquad \delta X_{\text{tissue}(t)} = \delta X_{\text{diet}} + (\delta X_{\text{tissue}(0)} - \delta X_{\text{diet}})\, e^{-(k_g+m)t}$$

where $\delta X_{\text{tissue}(0)}$ is the initial δ value of a fish tissue, δX_{diet} is the δ value for the new diet, $\delta X_{\text{tissue}(t)}$ is the δ value of the tissue at sampling time t, k_g is the growth rate per day, and m is the metabolic $\delta^{15}N$ turnover rate constant per day. We estimated stingray growth rates using the individual growth rate calculation of Hesslein et al. (1993):

$$(2) \qquad k = [\ln(W/W_0)]/t$$

where W_0 is the initial fish weight, and W is the fish weight at time of sampling, t. While eq. 2 explicitly accounts for the effects of growth on $\delta X_{\text{tissue}(t)}$, any other factors affecting $\delta X_{\text{tissue}(t)}$ are lumped into m and assumed to be the result of metabolic turnover.

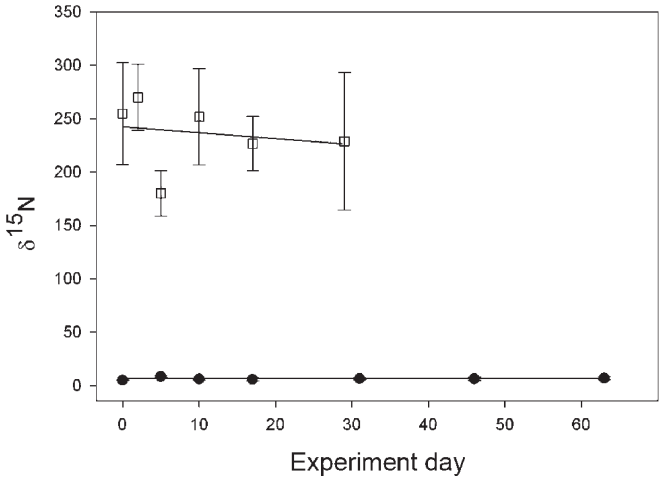
We applied and assessed the performance of the Hesslein model using the nonlinear mixed-effects (nlme) routine in the statistical package R to estimate the relative contributions of growth and metabolism during uptake and elimination phases of the experiment.

Results

Control and treatment diets maintained relative $\delta^{15}N$ differences of more than 200‰ during the experiment (Fig. 2); control diet $\delta^{15}N$ values were consistent over the experiment (6.71 ± 0.46‰; mean ± standard error, SE). Treatment worm $\delta^{15}N$ was 236.22 ± 17.87‰ and showed a slight decline during the experiment that was indistinguishable from 0 decline (Fig. 2; linear regression, $p > 0.75$). Importantly, the trend was inconsequential relative to the $\delta^{15}N$ differences between diets, and the variance of worm $\delta^{15}N$ represented would have been higher than in the actual ray diets because they reflected individual worm values, whereas stingrays were fed a homogeneous mixture of chopped worms.

Stingray stable isotope results were pooled by sex as we observed no differences in feeding behavior between sexes and assumed no differences would be present for sexually immature fishes. Initial (day 0) soft-tissue $\delta^{15}N$ values were enriched above the control diet (liver, 2.4‰; muscle, 1.6‰; blood, 0.69‰), and cartilage was depleted (–0.15‰) relative to the control diet at day 0.

Fig. 2. Control (□) and treatment (●) diet $\delta^{15}N$ trajectories with daily means (± standard error) for control days 0 to 63 and treatment days 0 to 29. Linear regression slopes were not significantly different from 0; $\alpha = 0.05$; $p > 0.75$.

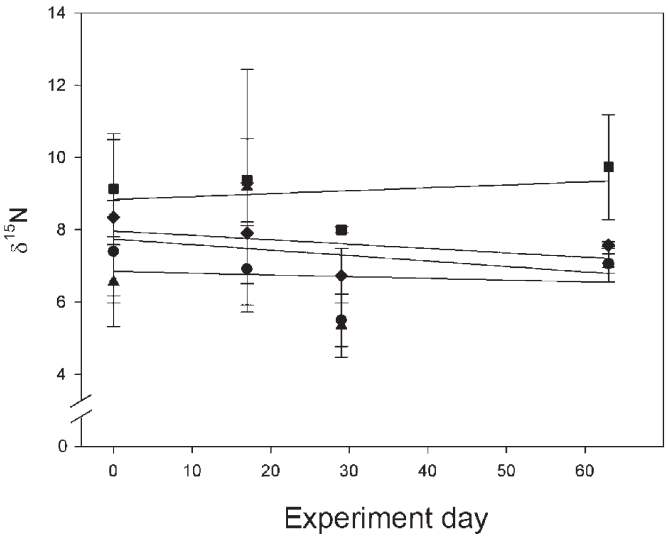


Control fish tissues showed little change in $\delta^{15}N$ signatures (Fig. 3), with relatively little variation for the duration of the experiment, suggesting that all of the rays were initially near isotopic equilibrium with the control worm diet. ANOVA results showed that the inclusion of treatment and tissue factors in the initial linear model (of both uptake and elimination) substantially increased $\ell(\mathbb{L})$, indicating that they have a significant effect on $\delta^{15}N$ values (Table 1). Although QQ plots of the data showed no departures from normally distributed errors, three of the treatment tissue samples had standardized residuals that were significant (>0.05) departures from the initial linear models. From these results and plots of the treatment data (Fig. 4), we concluded that treatment and control data were statistically different, as were the four treatment tissues.

Variation in $\delta^{15}N$ of control tissues (SE) remained <1‰ for the entire experiment. Variation in $\delta^{15}N$ of treatment tissue was <13.1‰, except liver tissue on day 31 (SE = 26.9‰). The high deviation on day 31 was due to a single ray liver sample that, at 162.9‰, had double the $\delta^{15}N$ signature of the other two replicate samples. The levels of control and treatment variation in $\delta^{15}N$ among stingray tissues reflected the variations observed in the control and treatment diets (0.46‰ and 17.9‰).

Because of difficulties arising from handling (the highly toxic) freshwater stingrays, our measurements of growth during the experiment were imprecise and unsatisfactory for estimating the individual growth rates, k, for each ray used in the Hesslein model. We were, however, able to estimate an average growth rate, $k_{\bar{x}}$, using eq. 2 and a nonparametric bootstrap of our original weight measurements. The resulting normal distribution provided a $k_{\bar{x}}$ of 0.0029 g·day^{-1} (±0.0012 SD), and using random draws from the N(0.0029, 0.0012) distribution, we simulated individual growth rates, k_b, for each fish. We were satisfied that this technique adequately described the stingray growth rates as the bootstrap estimate for k compared well with known growth rates for juvenile sharks (Cortés and Gruber 1994) and teleost fishes (MacAvoy et al. 2001). Because rays were sampled once and subsequently destroyed, no autocorrelation was present in our dataset, confining the effects of any potentially abnormal stingray growth to a single datum in each tissue.

Fig. 3. Control tissue $\delta^{15}N$ trajectories with daily means (± standard error) for blood (●), liver (■), muscle (◆), and cartilage (▲) of ocellate stingrays (*Potamotrygon motoro*) for days 0 to 63. Overall means: liver, 9.06 ± 0.87‰; muscle, 7.64 ± 0.80‰; blood, 6.72 ± 0.75‰; and cartilage, 7.35 ± 0.75‰. Linear regression slopes were not significantly different from 0; $\alpha = 0.05$; $p > 0.66$.



To assess the effects of the bootstrap procedure on our m estimates from eq. 2, we compared tissue fits of eq. 1 during uptake using the imprecise original measurements, k', the simulated estimates, k_b, and the mean bootstrap estimate, $k_{\bar{x}}$. Although the resulting predicted values for m were consistent among k', k_b, and $k_{\bar{x}}$ fits, k_b and $k_{\bar{x}}$ had improved (lower) $\ell(\mathbb{L})$ values compared with k' and substantial (>10%) reductions in residual error. All estimates of k were normally distributed and there was little difference between k_b and $k_{\bar{x}}$ model fits. As the purpose of the bootstrapping procedure was to separate the underlying variability in growth among stingrays from our imprecise measurements, we chose to use the k_b distribution for our estimates of metabolic $\delta^{15}N$ turnover, m.

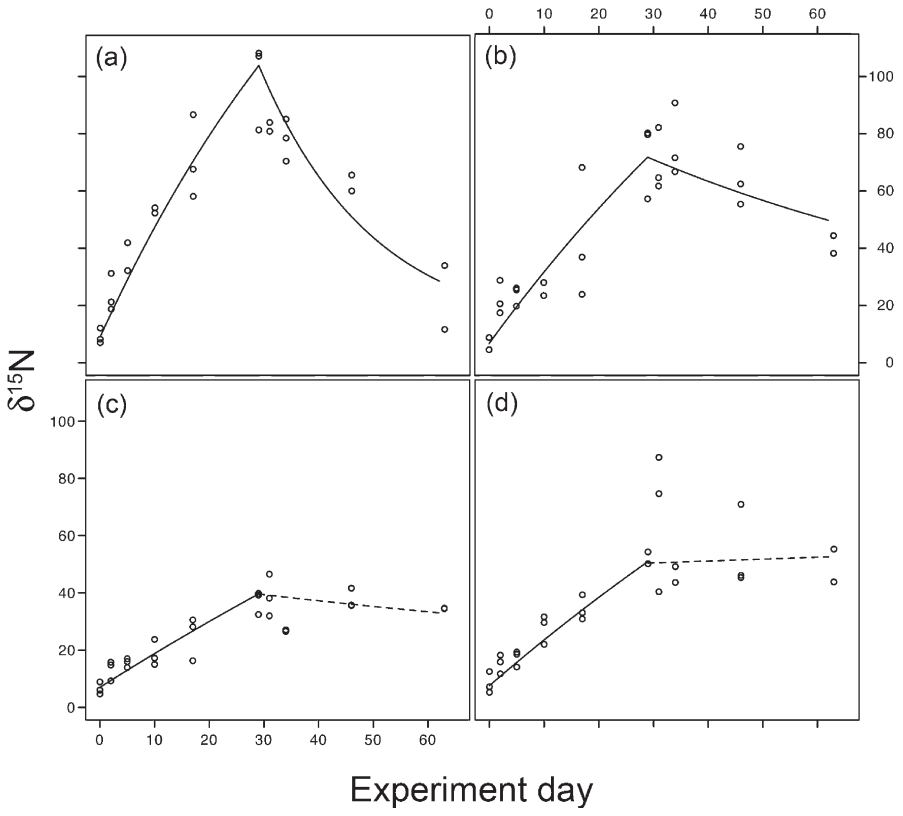
The Hesslein model described $\delta^{15}N$ turnover during the uptake phase of the experiment well, with good model fits among all tissues (Fig. 4). Turnover of $\delta^{15}N$ during uptake was greatest for liver, followed by blood, muscle, and cartilage. Parameter estimates for m during uptake differed among tissue types according to their perceived levels of metabolic activity (Table 2). Uptake-estimated times to 50% and 95% $\delta^{15}N$ equilibrium after the diet switch ($T50$ and $T95$) were long for muscle, indicating that it would take more than a year for the fish to reach equilibrium with a new, higher $\delta^{15}N$ diet in that tissue. Model fit was not as good for the elimination trajectories of $\delta^{15}N$ turnover among all tissues, where muscle samples were more variable on days 31 and 34, and estimates of m could not be distinguished from zero in either cartilage or muscle tissues. Muscle had approximately constant $\delta^{15}N$ values during the elimination phase. The parameter estimate for m during elimination in liver was three times its uptake value, confirming distinct rates of $\delta^{15}N$ turnover between the two phases

Table 1. Analysis of variance comparisons among linear models of ocellate stingray (*Potamotrygon motoro*) $\delta^{15}N$ trajectories over time for combined data, uptake, and elimination phases.

Model	df	AIC	$\ell(\mathbb{L})$	Test	$\mathbb{L}$ ratio	p value
Uptake + elimination						
1 $\delta^{15}N$ ~ day	4	1380.7	–686.3			
2 $\delta^{15}N$ ~ day | treatment	6	1362.2	–675.0	1 vs. 2	22.5	<0.0001
3 $\delta^{15}N$ ~ day | treatment, tissue	9	1342.1	–662.0	2 vs. 3	26.1	<0.0001
Uptake						
4 $\delta^{15}N$ ~ day	4	534.2	–263.1			
5 $\delta^{15}N$ ~ day | tissue	6	494.8	–241.4	4 vs. 5	43.4	<0.0001
Elimination						
6 $\delta^{15}N$ ~ day	4	334.4	–163.2			
7 $\delta^{15}N$ ~ day | tissue	6	326.5	–157.2	6 vs. 7	11.9	<0.0026

Note: Likelihood ratio ($\mathbb{L}$ ratio) tests show the importance of treatment and tissue type on observed $\delta^{15}N$ values; $\ell(\mathbb{L})$ indicates the log-likelihood estimate for each model.

Fig. 4. $\delta^{15}N$ trajectories of (*a*) liver, (*b*) blood, (*c*) cartilage, and (*d*) muscle of ocellate stingrays (*Potamotrygon motoro*) for days 0 to 63. Regression curves are least-squares fits for the model of Hesslein et al. (1993); broken lines represent poor model fits where the standard error range of the estimated parameter m included 0. Treatment diet $\delta^{15}N$ was switched from 239.6 to 6.7‰ on day 29. Parameter estimates are provided in Table 2.



in that tissue. The elimination $\delta^{15}N$ turnover estimates of m for blood and cartilage were similar to those calculated during uptake.

Discussion

$\delta^{15}N$ turnover in fish

Our results show the relative importance of growth versus metabolism in determining tissue-specific turnover rates of $\delta^{15}N$. This is the first experiment on an elasmobranch and the first to estimate metabolic $\delta^{15}N$ turnover in multiple tissues independent of experimental diet protein composition. The results show that the metabolic turnover of tissues will determine how closely the $\delta^{15}N$ in each tissue traces a diet switch. Specifically, liver appears to be the most metabolically active tissue sampled and has the greatest potential to indicate a recent change in dietary $\delta^{15}N$. Conversely, it appears that muscle $\delta^{15}N$ turnover times are slow to trace changes in diet, even with substantial $\delta^{15}N$ differences, and that it will take years to reach equilibrium with a new diet.

Table 2. Tissue specific estimates of metabolic turnover (m) of $\delta^{15}N$ per day and estimated days to 50% ($T50$) and 95% ($T95$) of new diet equilibrium for ocellate stingray (*Potamotrygon motoro*) liver, blood, muscle, and cartilage between uptake and elimination phases.

Tissue	k (day^{-1})	$m \pm$ SE (day^{-1})	$T50$ (days)	$T95$ (days)	p value
Uptake					
Liver	0.003	0.015±0.0011	39	166	<0.0001
Blood	0.003	0.0083±0.0011	61	265	<0.0001
Cartilage	0.003	0.0022±0.00040	134	576	<0.0001
Muscle	0.003	0.0041±0.00042	98	422	<0.0001
Elimination					
Liver	0.003	0.045±0.0079	14	62	0.0004
Blood	0.003	0.010±0.0042	53	230	0.047
Cartilage	0.003	0.0038±0.0043	—	—	0.40
Muscle	0.003	–0.0045±0.0066	—	—	0.51

Note: Estimates come from least-squares regressions from the mass-balance model of Hesslein et al. (1993) using constant growth rates (k) among tissues; SE denotes standard error estimates.

These results support the suggestion of MacAvoy et al. (2001) that, in fish, the often-assumed equilibrium of $\delta^{15}N$ values of muscle with a given diet may be frequently violated.

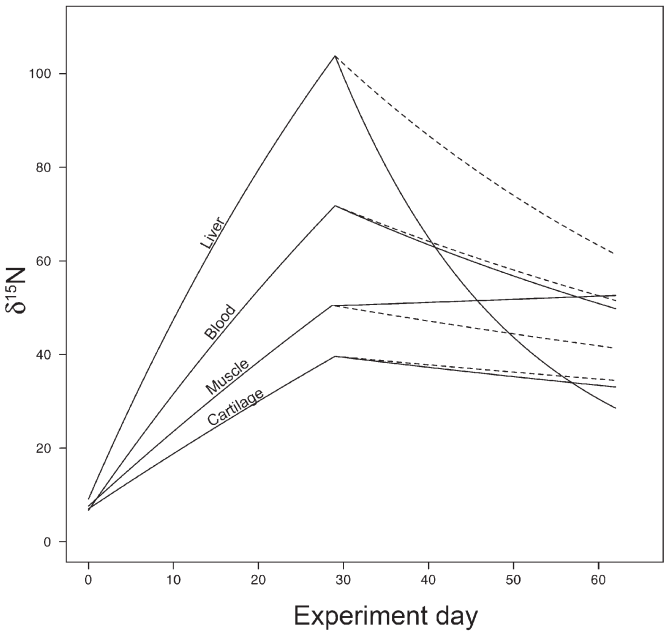
Turnover of nitrogen isotopes among fish tissues has not been well addressed empirically, as most studies have previously focused only on the $\delta^{15}N$ turnover of muscle. Hesslein et al. (1993) estimated metabolic $\delta^{15}N$ turnover, m, to be 0.0018 day^{-1} for muscle in growing broad whitefish (*Coregonus nasus*), but the results were confounded by the experimental food being only 1.9‰ different in $\delta^{15}N$ than the control food. Hesslein et al. (1993) subsequently reported difficulty in estimating a best fit for growth model from their $\delta^{15}N$ data. They concluded that metabolic replacement of stable isotopes in liver was similar to that in muscle, although the sensitivity of their results to changes in liver isotope composition was poor. In general, variability in the results appeared to overwhelm their estimates of metabolic $\delta^{15}N$ turnover. In a subsequent study, Vander Zanden et al. (1998) reported that growth accounted for 86% of $\delta^{15}N$ turnover in age-0 smallmouth bass (*Micropterus dolomieu*) and suggested that the remaining 14% of change was likely due to metabolic processes. These results were supported by Marcogliese (2001), who exploited a unique diet switch in migratory gobies (*Rhinogobius* spp.) to suggest that growth was responsible for 80% of $\delta^{15}N$ turnover in fish muscle. They estimated $T50$ to be from 30 to 100 days, corresponding to the initial and final growth rates of their age-0+ fishes. Marcogliese (2001) also suggested that the role of growth in $\delta^{15}N$ turnover diminished relative to metabolism as fishes aged and predicted that metabolic $\delta^{15}N$ turnover would dominate in slow-growing fishes. Both the Vander Zanden et al. (1998) and Marcogliese (2001) studies were, however, conducted on larval fishes in field conditions.

MacAvoy et al. (2001) sampled muscle and blood in blue catfish (*Ictalurus furcatus*) and, using a range of k values between 0.00218 and 0.009 day^{-1}, concluded that m contributed little to $\delta^{15}N$ turnover in muscle. Finally, Harvey et al. (2002) estimated m in juvenile lake trout (*Salvelinus namaycush*) muscle to be 0.0005 day^{-1} (k values not reported) and concluded that such results were critical for interpreting trophic pathways from stable isotope data. Muscle-based studies, then, have broadly found that 70%–90% of $\delta^{15}N$ turnover in juvenile fishes is due to growth and the rest is attributable to metabolism. Our results, in which 65%–75% of muscle $\delta^{15}N$ turnover was attributed to growth, agreed well with these results, suggesting a measure of agreement between teleost and elasmobranch fishes.

A prominent feature of our diet-switching experiment was that instantaneous rates of uptake and elimination $\delta^{15}N$ turnover were dramatically different among tissues. While initially confusing given the assumed equal rates of uptake and elimination, this real pattern in the plotted data was partly due to the higher-turnover liver and blood tissues having reached a greater percentage of equilibrium $\delta^{15}N$ at the end of the uptake phase than the lower-turnover muscle and cartilage. This meant that these tissues were closer to the depleted diet $\delta^{15}N$ equilibrium at the start of the elimination phase because the lower-metabolism muscle and cartilage tissues had not progressed as far toward the enriched-diet $\delta^{15}N$ equilibrium during uptake. This lead to an experimental artefact whereby the magnitude of difference between the plotted uptake and elimination phases appeared greater than it actually was. However, differences between uptake and elimination $\delta^{15}N$ turnover rates were detected. When we simulated the expected elimination phase using the Hesslein model uptake regression results and overlaid the simulations with our elimination data, the projections were a poor fit to the observed elimination results in liver and muscle tissues (Fig. 5). The projections had shallower than observed trajectories for liver and steeper than observed trajectories for muscle, demonstrating that, according to the Hesslein model, the $\delta^{15}N$ turnover rates of those tissues differed between uptake and elimination phases.

The elimination metabolic $\delta^{15}N$ turnover estimate for liver was three times the uptake estimate. This reflects two important points. Firstly, the metabolic $\delta^{15}N$ turnover estimate, m, is actually the $\delta^{15}N$ turnover that is not attributable to growth. This "not-growth" value encompasses all other processes leading to stable isotope turnover, such as intertissue recycling of nutrients and amino-acid effects, and these processes may operate differently during uptake versus elimination. Secondly, analysis of the relative fates of ^{15}N is lacking for all fishes, and without research on such specific details, we cannot know the mixture of biological processes leading to our estimate of m. Our results demonstrate, however, that

Fig. 5. Observed (solid) uptake and projected (broken) versus observed (solid) elimination trajectories of liver, blood, muscle, and cartilage tissues of ocellate stingrays (*Potamotrygon motoro*). A switch from a δ^{15}N-enriched to δ^{15}N-depleted diet occurred on day 29. Projected elimination trajectories were calculated from uptake nonlinear regressions of uptake data using the model of Hesslein et al. (1993).



for liver tissue, there are different processes acting upon ^{14}N and ^{15}N isotopes during uptake and elimination phases.

Blood differed from liver in showing consistent *m* estimates between uptake and elimination phases. This was not surprising given the role of blood in transporting nutrients that have previously been processed by the liver to other tissues around the body. An increase in muscle δ^{15}N was apparent during the elimination phase of the experiment, although the muscular parameter estimate for *m* was indistinguishable from 0 (no metabolic δ^{15}N turnover). This reflected the slow metabolic δ^{15}N turnover of muscle leading to gradual reductions in δ^{15}N relative to additions from growth. During uptake these processes are thought to act in concert to enrich muscle tissue, whereas during elimination they would be in relative opposition, with elimination growth of additional tissue diluting the high δ^{15}N present and metabolism enriching the tissue in δ^{15}N. Although our estimates of *m* during elimination were imprecise, the possibility remains that uptake and elimination δ^{15}N turnover rates differ in fish muscle and δ^{15}N may have an enrichment bias as a result. The interplay among growth, actual metabolism (as opposed to the estimated *m*), and processes such as internal recycling must be better understood to clarify how uptake and elimination phases differ among tissues.

Given the level of agreement between our estimate of uptake muscle *T*50 with that of Hesslein et al. (1993), we have some measure of confidence in our uptake estimates of *T*50 suggesting that an uptake diet switch would be detectable in liver tissue in just over a month and in muscle tissue by six months. The *T*95 estimates indicated that it would take six months for liver δ^{15}N to reach equilibrium with a new diet and that muscle tissue δ^{15}N would directly reflect equilibrium only after nearly two years of continuous feeding. Given a degree of periodicity in a fish's diet during a given year, we expect liver and blood tissues to reflect a diet switch within two or three months, whereas muscle tissue would represent an annual mean of diet δ^{15}N. Such a comparison has proved useful in the Northwest Atlantic, where multitissue analysis reflected a known annual diet switch in mako shark (*Isurus oxyrinchus*) (MacNeil et al. 2005). The uptake *T*95 suggested that as fishes grow and begin to exploit higher trophic level prey, it may take several years for muscle tissue to adequately reflect a permanent dietary shift. Tissue δ^{15}N could not be used to determine the diet trophic position until the animal reached equilibrium. If animals switch diets at intervals less than their *T*95 values, the animal will never be in equilibrium and estimates of trophic position will be influenced by the previous diet. Again, sampling of liver or blood tissue in addition to muscle should aid in observation of such dietary changes.

Our analysis assumed, as have previous stable isotope studies, that δ^{15}N enrichment was concentration-independent. The slower δ^{15}N turnover trajectories observed during elimination support this assumption whereby, under concentration independence, tissues returning to the lower equilibrium value had shallower slopes than those moving towards the highly enriched diet. A second assumption of our analysis was that the growth rates of individual tissues were consistent over the entire experiment. Although individual tissues are known to grow at different rates over the development cycle of fishes, the short duration of our experiment made changes among the relative growth rates of each tissue highly unlikely and our constant estimate of *k* among tissues appropriate. The ability to conduct the experiment over such a short time period was due to the highly enriched experimental diet.

Earthworm diet

Our results were improved by our use of a δ^{15}N-enriched earthworm diet that greatly amplified the isotopic δ^{15}N turnover of stingray tissues. The variability within the earthworm control and treatment diets was relatively low compared with the vast differences between their δ^{15}N values over the course of the experiment and this clarified interpretation of the δ^{15}N turnover trajectories. A considerable difficulty in studying stable nitrogen isotope dynamics has been in selecting suitable control and treatment diets that differ substantially in δ^{15}N. Differences in δ^{15}N of 3‰ are, in actual terms, differences at the 5th to 6th decimal place when converted to actual concentrations (Pinnegar and Polunin 1999), and delta notation is used to make such differences obvious. Most studies of ^{15}N turnover have not achieved differences in δ^{15}N of more than 3‰ between diets. Some studies recognized the computational advantages of radically different diet δ^{15}N signatures and attempted to incorporate such diets into their experiments. Hobson and Clark (1992*a*) fed American crows (*Corvus brachyrhynchos*) a diet based on perch (*Perca flavescens*) (δ^{15}N = 14.2‰) and compared the results with those from domestic chickens (*Gallus gallus*) that had been fed a relatively δ^{15}N-depleted grain-based feed (δ^{15}N = 4.7‰). As noted by Hobson and Clark (1992*a*), the problem with interpreting their results was that the two diets had different

protein compositions. With many authors having suggested a strong association between amino acid composition and tissue $\delta^{15}N$ enrichment (e.g., McClelland et al. 2003), studies of stable nitrogen isotopes must consider the effects of diet protein composition. The greatest difference we have found between diets in studies that controlled to some degree for protein composition was 6.7‰ (Olive et al. 2003). The consistency of our control and treatment diets, combined with their exceedingly large $\delta^{15}N$ differences, has introduced a new kind of experimental diet that is highly applicable to the study of stable nitrogen isotope dynamics. We suggest that earthworms are an ideal food source for experimental manipulation of dietary $\delta^{15}N$ in animals which will accept worms or worm-based food.

Implications for field applications of $\delta^{15}N$

Discrete uptake and elimination trajectories, particularly for muscle tissue, have not been observed previously for any species. This result has implications for much of the ecological research using stable nitrogen isotopes to estimate trophic position. Specifically, muscle tissue $\delta^{15}N$ values may be biased from actual diet $\delta^{15}N$, which will lead to spurious conclusions if not identified. It has been demonstrated that stable nitrogen isotopes are informative predictors of trophic position over broad levels of analysis (Post 2002), partly because system-wide analyses tend to reduce overall variance. Researchers are, however, increasingly applying stable nitrogen isotope analysis to the species level of inference (e.g., Estrada et al. 2003) by comparing the $\delta^{15}N$ signatures from a few species in the target ecosystem. This kind of problem has been emphasized previously where stable isotope results have been discredited by chemical tracers (Fisk et al. 2002). Without a complete understanding of nitrogen isotope dynamics in those few organisms, individual tissue- and species-level differences may easily confound results. This problem would be particularly acute for species with periodic, heterogeneous diets where, for instance, an unrecognized delay in muscle ^{15}N turnover will lead to a biased estimate of trophic position. A muscle bias may not appear for species consuming continuous, homogeneous diets, but a large-scale change in diet composition may not be evident for almost a year in any given species. We hypothesize that discrete feeding events on a higher trophic level diet would not be evident in muscle tissue $\delta^{15}N$ signatures alone and that multitissue sampling may be necessary to capture such events.

The multitissue field study of Lorrain (2002) found that $\delta^{15}N$ in scallop (*Pecten maximus*) digestive gland varied by up to 3‰ annually, whereas muscle $\delta^{15}N$ varied by only 1‰. They proposed several explanations for the results, including unaccounted additions to the scallop diet, selective assimilation of high $\delta^{15}N$ particulate organic matter (POM) components, differential dietary protein assimilation among scallop tissues, isotopic routing among tissues, and general metabolite flux. If applicable, our results provide another explanation for the scallop data: because of the high degree of metabolic $\delta^{15}N$ turnover, digestive gland tissue reflected diet variations more readily than muscle tissue. Field sampling of tissues across many species levels are required to determine the veracity of such a relationship among animal classes and systems.

Although many authors have discussed the need for laboratory experimentation to characterize stable isotope dynamics prior to their use in ecological studies, relatively little validation has been done. Dozens of stable isotope food web studies were published 2003, yet many did not address the potential for species- or tissue-specific differences in stable isotope turnover. The attractiveness of stable isotopes is obvious — researchers can, with minimum sampling effort, quantify trophic position for every species in a study area. We suggest, however, that a clear understanding of $\delta^{15}N$ dynamics in targeted species and tissues will allow ecologists to pursue more detailed research questions. If well understood, individual tissue turnover rates can bring greater resolution to stable nitrogen isotope analyses, where specific tissues reflect distinct aspects of an animal's life history that provide a more complete picture than was previously possible.

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