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Metabolic turnover rates of carbon and nitrogen stable isotopes in captive juvenile snakes

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Metabolic turnover rates (*m*) of $\delta^{15}N$ and $\delta^{13}C$ were assessed in different tissues of newly hatched captive-raised corn snakes (*Elaphe guttata guttata*) fed maintenance diets consisting of earthworms (*Eisenia foetida*) that varied substantially in $\delta^{15}N$ (by 644‰) and $\delta^{13}C$ (by 5.0‰). Three treatments were used during this 144 day experiment that consisted of the same diet throughout (*control*), shifting from a depleted to an enriched stable isotope signature diet (*uptake*), and shifting from an enriched to depleted stable isotope signature diet (*elimination*). Values of $\delta^{13}C$ in the liver, blood, and muscle of the control snakes reached equilibrium with and were, respectively, 1.73, 2.25 and 2.29 greater than in their diet, this increase is called an isotopic discrimination factor ($\Delta\delta^{13}C = \delta^{13}C_{snake} - \delta^{13}C_{food}$). Values of $\delta^{15}N$ in snake tissues did not achieve equilibrium with the diets in any of the exposures and thus $\Delta^{15}N$ could not be estimated. Values of metabolic turnover rates (*m*) for $\delta^{13}C$ and $\delta^{15}N$ were greater in liver than in muscle and blood, which were similar, and relative results remained the same if the fraction of ^{15}N and ^{13}C were modeled. Although caution is warranted because equilibrium values of stable isotopes in the snakes were not achieved, values of *m* were greater for $\delta^{13}C$ than $\delta^{15}N$, resulting in shorter times to dietary equilibrium for $\delta^{13}C$ upon a diet shift, and for both stable isotopes in all tissues, greater during an *elimination* than in an *uptake* shift in diet stable isotope signature. Multiple explanations for the observed differences between uptake and elimination shifts raise new questions about the relationship between animal and diet stable isotope concentrations. Based on this study, interpretation of feeding ecology using stable isotopes is highly dependent on the kind of stable isotope, tissue, direction of diet switch (*uptake* versus *elimination*), and the growth rate of the animal. Copyright © 2009 John Wiley & Sons, Ltd.

Stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) have become a useful tool for assessing the feeding ecology of organisms. They can provide an integrated estimate of feeding behavior over days to years, depending on the tissue and species analyzed,[1] and offer additional benefits when studying rare, endangered, or threatened species because small, non-lethal tissue samples are adequate for stable isotope analysis.[2] However, there are a number of assumptions underlying the use of stable isotopes in ecology,[3,4] some of which are known[5–8] but often not incorporated in interpretation of stable isotope data. For example, isotopic discrimination factors ($\Delta\delta^{13}C = \delta^{13}C_{snake} - \delta^{13}C_{food}$; $\Delta\delta^{15}N = \delta^{15}N_{snake} - \delta^{15}N_{food}$) of $\delta^{13}C$ and $\delta^{15}N$ can vary among species,[4] tissues,[9] amino acids,[10] prey quality,[11] and within species under varying environmental conditions (e.g. temperature[12]). Other assumptions are not as well recognized in the scientific literature. For example, MacNeil *et al.*[13] recently demonstrated that the rate of change of $\delta^{15}N$ differed if an organism switched from a depleted ^{15}N to an enriched ^{15}N diet (called *uptake*) or was switched from an enriched ^{15}N to a depleted ^{15}N diet (called *elimination*). Such variation in stable isotope turnover rate has implications for animals that have varied or changing diets. Such species are unlikely to have stable isotope signatures that are near equilibrium with their diet and are more likely to have greater variability in stable isotope values.[14]

Due to the uncertainties with using stable isotopes in ecological studies, there is a need to carry out controlled laboratory studies on stable isotope turnover, particularly for unstudied species. Such studies have generally used mass-balance equations to estimate the contributions of growth (*k*) and metabolism (*m*) to model isotopic turnover.[13,15–18] To experimentally isolate proportion *m* from *k* requires a maintenance diet, whereby animals are provided enough food to live, but not enough to grow.[19] On a no-growth diet all isotopic turnover is directly attributable to *m*, and any confounding influence of growth rate (e.g. due to animal size

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or age) is controlled. Despite their utility, maintenance diets have rarely been used in stable isotope turnover experiments due to difficulties in limiting growth but maintaining high survival rates. In addition, despite the fact that specific isotopes are biased toward particular amino acids, few studies have controlled for the protein composition of their experimental diets, which can also influence protein assimilation and digestion energetics.[20]

Diet and food web information is critical to the management and monitoring of threatened or endangered animals; unfortunately, this information is generally lacking for snakes and other reptiles. Stable isotopes of nitrogen and carbon have the potential to provide a non-destructive method for assessing diet choice and trophic role of snakes that is confounded by limitations associated with stomach content analysis. Unfortunately, there have been few published reports of stable isotopes in snakes or other herpetofauna[1,21–23] and a single study that has quantified isotopic discrimination factors in reptiles, loggerhead turtles (*Caretta caretta*) under controlled conditions.[18] There is still a need to quantify their turnover rates in snakes, and reptiles in general, in controlled laboratory experiments.[1,3]

To quantify the metabolic turnover rate of stable isotopes of carbon and nitrogen in snakes we fed captive-raised, newborn, corn snakes (*Elaphe guttata guttata*) one of two earthworm (*Eisenia foetida*) diets that varied significantly in $\delta^{13}C$ (differed by ~5‰) and $\delta^{15}N$ (differed by ~644‰) values. Snakes fed the enriched diet (higher $\delta^{15}N$ and $\delta^{13}C$ signatures) were switched after 72 days to the non-enriched diet (lower $\delta^{15}N$ and $\delta^{13}C$ signatures) to capture both the rate of increase (*uptake*) and decrease (*elimination*) in stable isotope upon a diet switch. The use of the earthworms controlled for amino acid composition in the two diets, which can bias stable isotope turnover rates.[24] The snakes were fed a maintenance diet (i.e. no growth) to isolate the values of m from the influence of growth. The significance of these findings with regards to the use of stable isotopes in studying snake feeding ecology is discussed.

EXPERIMENTAL

Snakes and husbandry

Thirty-five captive-raised corn snakes (*Elaphe guttata guttata*) were acquired following hatching and prior to first feeding (initial weight (g) = 6.2 ± 0.2; initial length (cm) = 27.3 ± 0.3; mean ± 1 SE). Corn snakes were held individually in 6 quart Tupperware® containers with newspaper substrate, hiding places and air holes. The room temperature was maintained at 25°C with a 12 hour light/dark cycle, with access to sunlight through a window. The snakes were maintained on a diet of worms with a snake vitamin supplement (Reptivite, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA), fed every third day at 10% of body weight. Water was provided *ad libitum*.

Diets

To control for biases in amino acid composition between diets, we created isotopically distinct control and treatment diets with comparable amino-acid compositions using earthworms (*Eisenia foetida* (Savigny, 1826)).[13] The treatment diet was formed by adding 10 g of ^{15}N-NH_4Cl (99.9% ^{15}N) and 10 g 1-^{13}C-CH_3CO_2Na $3H_2O$ to 1 L of potato and water slurry that was incubated for 7 days to allow for absorption of the concentrates by local bacteria. The slurry was mixed with 40 L of soil and 4 kg of earthworms in a 100 L terrarium. The control diet consisted of approximately 4 kg of worms maintained in a second 100 L terrarium that had the same potato/water slurry but without the addition of the enriched stable isotope standards. After 8 days the worms were removed from the soil, thoroughly washed to remove all soil and debris, and homogenized to a consistent paste, then frozen until fed to the snakes. Final $\delta^{13}C$ and $\delta^{15}N$ values (n = 3) in the control worms were −20.78 ± 0.46 and 18.83 ± 0.40, respectively, and in the treatment worms were −15.80 ± 0.41 and 663.3 ± 38.45, respectively.

To check the potential influence of soil in the guts of the worms on stable isotope signatures in the food, a second experiment was carried out that was nearly identical to the protocol used above to enrich the worms. After 8 days in the soil, eight worms were collected from a control and enriched soil. Four worms had their guts removed prior to stable isotope analysis and four were processed with their gut contents.

Protocol

All snakes were placed on the control diet for three feedings to acclimatize to force feeding. Snakes were force fed by Drawing 10% of each snake's mass in worm paste into a 1 mL syringe, inserting the syringe into the mouth of the snake, past the glottis opening in the throat, and slowly pushing the paste into the throat of the snakes.

After the acclimation period, three snakes were randomly chosen, euthanized, and samples of blood, muscle, and liver were collected for stable isotope analysis; 23 snakes were switched to the treatment diet, and 9 snakes were left on the control diet. No effort was made to determine the sex of the snakes or to account for this effect since the snakes were very young and feeding rates were kept consistent for all snakes. On days 9, 18, 36 and 72, three snakes were euthanized from the treatment diet group and the same tissues were collected for stable isotope analysis. The remaining 11 snakes from the treatment diet were then switched to the control diet for 72 days, and on days 9, 18, 36 and 72 after the diet switch, 3 (2 on day 72) snakes were euthanized and the same tissues collected for stable isotope analysis. The 9 snakes fed the control diet throughout the experiment were sampled on days 36, 72 and 144, and 3 snakes were euthanized and the same tissues collected for stable isotope analysis. Snake length (snout-vent length) and weight were determined at each feeding for determination of an accurate food amount for that feeding.

Stable isotope analysis

Following the experiment, tissue samples were weighed and freeze-dried using a Labconco 4.5 freeze-dryer (Labconco Co., Kansas City, MO, USA). The dried samples were pulverized in a SPEX CertiPrep 8000-D ball milling unit (SPEX CertiPrep, Metuchen, NJ, USA), treated twice with 10 mL of 2:1 chloroform/methanol solution to remove lipids, dried in an oven at 60°C until dry, and divided into ~1.0 mg

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tin capsules for $\delta^{15}N$ and $\delta^{13}C$ analysis. Stable carbon and nitrogen isotope ratios were determined on Thermo Finnigan DeltaPlus isotope ratio mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an elemental analyzer (Costech, Valencia, CA, USA); the analytical precision was ± 0.15‰ (SD). Stable isotope abundances are expressed in δ notation as the deviation from standards in units of per mil (‰) according to the following equation:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000 \quad (1)$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

Modeling

To control for the potential bias of enriched samples on $\delta^{13}C$ and $\delta^{15}N$,[25] the fractions of the heavier isotopes, $F^{13}C$ and $F^{15}N$, respectively, were modeled in addition to $\delta^{13}C$ and $\delta^{15}N$. These values were calculated according to Fry:[25]

$$FX = (\delta + 1000)/[(\delta + 1000 + (1000/R_{standard}))] \quad (2)$$

where X is ^{13}C or ^{15}N, δ is $\delta^{13}C$ or $\delta^{15}N$ and $R_{standard}$ is ratio of heavy to light isotope of reference standard (for C Peedee Belemnite = 0.11180; for N is air = 0.0036765).

Hesselein's metabolic turnover model has been frequently used in diet-switch experiments to estimate rates of stable isotope turnover due to growth and metabolism,[15] primarily in the form of:

$$X_{tissue(t)} = X_{diet} + (X_{tissue(0)} - X_{diet})e^{-(k+m)t} \quad (3)$$

where X_{diet} is the stable isotope ($\delta^{15}N$ or $\delta^{13}C$) of F (^{15}N or ^{13}C) value for the new diet, $X_{tissue(0)}$ is the initial stable isotope or F value of a snake tissue, $X_{tissue(t)}$ is the stable isotope or F value of the tissue at sampling time t, k is the growth rate per day (here set to 0), and m is the metabolic (not-growth) stable isotope turnover rate constant per day. This model is concentration independent, meaning that the magnitude of the difference in stable isotope value between the animal and its diet does not influence the estimation of m.

We applied and assessed the performance of the Hesslein model using the non-linear least-squares (nls) routine in the statistical package R[26] to estimate m during uptake and elimination phases of the experiment. $X_{tissue(0)}$ was set to equal the mean stable isotope value of each tissue on the first day of a new diet. Because the Hesslein model is an asymptotic curve and our data did not reach steady state we made an important assumption in our model fitting to improve convergence of the nls algorithm. We chose to fix the value of X_{diet} in the Hesslein model to equal the current diet stable isotope signature plus a commonly used isotope discrimination factor of 3.4[4] for each tissue. While this would be a significant assumption to make on a conventional experimental diet, given the several orders of magnitude difference between our control and the treatment diet stable isotope signatures we believed it to be of minor importance.

To evaluate differences among tissues and quantify the uncertainty surrounding our estimates of m, we calculated model-based non-parametric bootstrap confidence intervals (R = 9999) for each tissue and treatment.[27] From estimates of m the times to 50 (T_{50}) and 95% (T_{95}) dietary equilibrium with a new diet were calculated using the equation:

$$T = [\log(1) - \log(1 - \alpha)]/m \quad (4)$$

where α is the target percentage of equilibrium (e.g. 95%).

RESULTS

All treatment and control snakes stayed at the same weight and length (initial mean mass = 6.2 ± 0.2 g; initial mean length = 28.0 ± 0.2 cm) during the 144 day experiment and most snakes shed their skin at least once. Snakes remained active and appeared healthy throughout the experiment.

In the second experiment, to check the influence of worm gut contents on stable isotope signatures in worms, worms grown in ^{13}C- and ^{15}N-enriched soil with gut contents removed prior to analysis had lower stable isotope signatures ($\delta^{15}N = 727 \pm 388$; $\delta^{13}C = -20.6 \pm 3.1$) than worms that were left with their gut contents ($\delta^{15}N = 3081 \pm 852$; $\delta^{13}C = -6.79 \pm 4.52$). In non-enriched soil, worms with gut contents removed prior to analysis had similar stable isotope signatures ($\delta^{15}N = 6.99 \pm 1.20$; $\delta^{13}C = -26.2 \pm 0.46$) to worms that were left with their gut contents ($\delta^{15}N = 4.87 \pm 1.00$; $\delta^{13}C = -26.1 \pm 0.15$).

Values of $\delta^{13}C$ in the control snake tissues initially became more positive (day 32) and reached an apparent steady state value by day 72 (Fig. 1). All tissues showed an enrichment in ^{13}C (i.e., more positive $\delta^{13}C$) compared with the food. Muscle and blood $\delta^{13}C$ values were similar throughout the experiment and were 2.29 and 2.25‰ more positive than the $\delta^{13}C$ of the food, respectively, on the last day of the experiment (i.e. after 144 days). Liver $\delta^{13}C$ values were depleted in ^{13}C compared with muscle and blood but were 1.73‰ more positive than the food after 144 days of feeding.

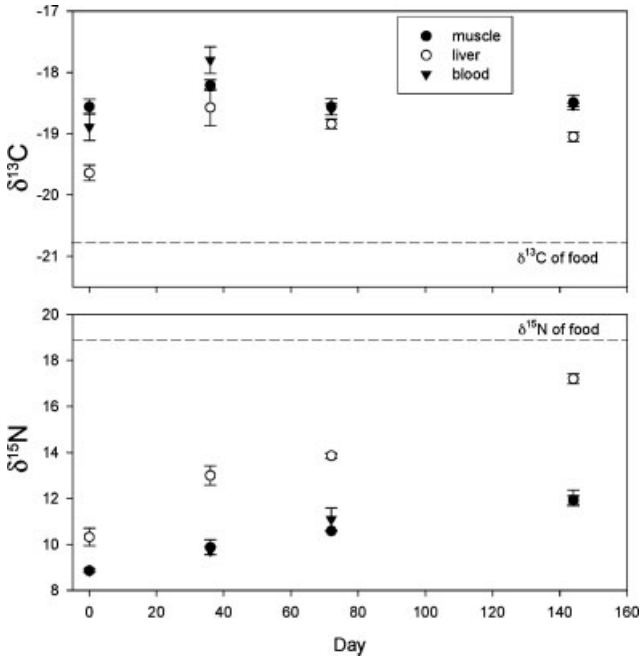

Figure 1. $\delta^{13}C$ and $\delta^{15}N$ values (mean ± 1 SE of 3 snakes) in the tissues of corn snakes fed the control food for 144 days. Dashed lines are $\delta^{13}C$ (−20.78 ± 0.46) and $\delta^{15}N$ (18.83 ± 0.40) mean values in the control food (n = 3).

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Values of $\delta^{15}N$ increased in the control snake tissues throughout the 144 day experiment, and did not appear to reach equilibrium with the diet by day 144 of the experiment (Fig. 1). This increase in $\delta^{15}N$ in the control snakes probably reflects the fact that the $\delta^{15}N$ of tissue and/or diet of the mothers were lower than the value in the control food; indeed the $\delta^{15}N$ values in snakes at the beginning of the experiments were more than 8‰ less than the control food. Muscle and blood had nearly identical $\delta^{15}N$ values on all days; liver $\delta^{15}N$ values were greater and increased at a more rapid rate.

The turnover rates of $\delta^{13}C$ and $F^{13}C$ during the uptake and elimination phases of the experiment fit the Hesslein model well (Eqn. (2), Fig. 2; $F^{13}C$ results not shown) and t-statistics for m were significant at $\alpha = 0.01$ except for elimination-phase $\delta^{13}C$ in muscle ($p = 0.071$; Table 1). The metabolic turnover (m) of C was slower in liver and blood of the snakes on a switch from a depleted (more negative $\delta^{13}C$ value) to an enriched (more positive $\delta^{13}C$ value) ^{13}C diet (*uptake*) than for a switch from an enriched to a depleted ^{13}C diet (*elimination*; Tables 1 and 2). Non-parametric bootstrap confidence intervals (CIs) confirmed significant differences among tissue turnover rates although bootstrap intervals were wider for m during elimination than uptake, largely due to the magnitude of differences between snake tissues and diet at the start of each phase. Again, the metabolic turnover from elimination-phase carbon in muscle was poorly estimated (CI included 0). The direction of the $\delta^{13}C$ diet switch did not appear to influence the turnover rate of $\delta^{13}C$ in the muscle tissue of the snakes.

Due to the slight but consistent increases in $\delta^{15}N$ values in the control snakes during the entire experiment (Fig. 1) we were able to estimate m in both the treatment and the control snakes. The turnover rates of $\delta^{15}N$ and $F^{15}N$ during uptake and elimination phases of the experiment fit the Hesslein model (Eqn. (2)) well, with good model fits for all treatments and tissues (Tables 1 and 2). In all cases, elimination estimates of m were greater than uptake estimates; metabolic elimination in liver was nearly 5 times higher than the estimated rate during uptake (Tables 1 and 2).

DISCUSSION

The metabolic turnover rate (m) of nitrogen and carbon in the captive corn snakes was dependent on tissue type and direction of diet change (i.e. from a depleted to an enriched, *uptake*, or an enriched to a depleted, *elimination*, switch in stable isotope signature of food) and could have a significant influence on observed stable isotope signatures in wild snakes, and probably in other reptiles. There was broad agreement between blood and muscle uptake and elimination rates for both carbon and nitrogen, although they varied between the elements, while liver turnover was more rapid than that of the other tissues in both experiment phases (i.e. *uptake* and *elimination*). Liver also showed the greatest sensitivity to the phase of diet switch present. There was also good agreement for values of m calculated for $\delta^{15}N$ in control and treatment snakes. The use of fraction of heavier isotopes ($F^{13}C$ or $F^{15}N$), instead of $\delta^{13}C$ and $\delta^{15}N$, in modeling resulted in m estimates that were very similar to those using the δ values.

An explanation and/or a potential confounding problem for the observed differences between uptake and elimination and between $\delta^{13}C$ and $\delta^{15}N$ m values relates to the use of an inaccurate value for X_{diet}, the stable isotope signature of the food. The X_{diet} value used in the uptake model may have been too high due to either: (1) soil left in the guts of the worms (an experimental issue); or (2) an inaccurate estimate of the true equilibrium value for the stable isotopes in the snakes (a theoretical issue).

Our second experiment showed that after 8 days, the stable isotope values in worms analyzed with guts that were reared in ^{15}N- and ^{13}C-enriched soil were nearly four times higher

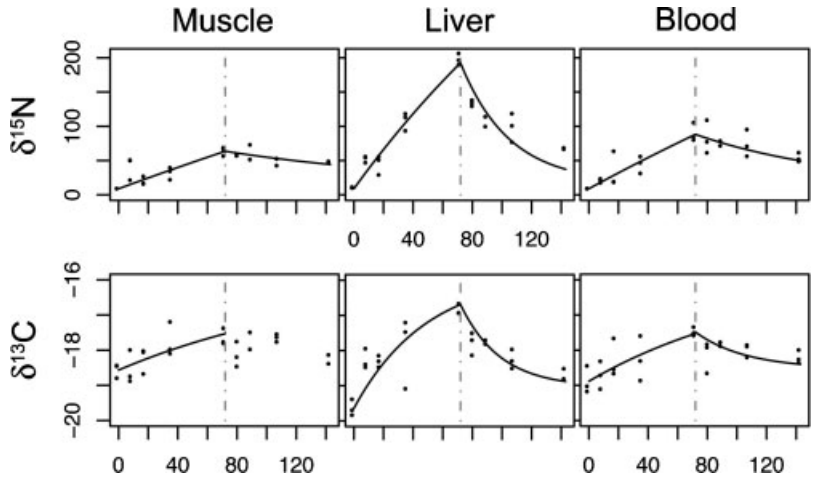

Figure 2. $\delta^{15}N$ and $\delta^{13}C$ trajectories of liver, blood, and muscle of corn snakes for days 0 to 144. Regression curves are least-squares fits for the model of Hesslein *et al.*[15] assuming no growth. Treatment diet $\delta^{15}N$ was switched from 663.3 ± 38.45 to 18.83 ± 0.40. Dashed grey line delimits uptake and elimination phases.

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Table 1. Tissue-specific estimates of metabolic turnover (*m*) of $\delta^{15}N$ and $\delta^{13}C$ per day; model-based, non-parametric bootstrap 95% confidence intervals (bCI); and estimated days to 50% isotopic change (T_{50} with 95% CI) and 95% isotopic change (T_{95} with 95% CI) for corn snake liver, blood, and muscle between uptake and elimination phases. Estimates of *m* derived from least-squares regressions (nls) from the mass-balance model of Hesslein *et al.*[15] in snakes on a maintenance (no growth) diet; *p*-values are calculated t-tests of the parameter estimate being zero

Tissue	m (day^{-1})	bCI	T_{50} (days)	T_{95} (days)	*p*-value
$\delta^{13}C$ Uptake					
Liver	0.0146	(0.0111, 0.0223)	47 (31, 62)	205 (134, 270)	<0.0001
Blood	0.0092	(0.0052, 0.0107)	75 (65, 133)	326 (280, 576)	<0.0001
Muscle	0.0105	(0.0041, 0.013)	66 (53, 169)	285 (230, 731)	0.0002
$\delta^{13}C$ Elimination					
Liver	0.0373	(0.0269, 0.0456)	19 (15, 26)	80 (66, 111)	<0.0001
Blood	0.0293	(0.0138, 0.0297)	24 (13, 50)	102 (91 – 217)	0.0066
Muscle					0.0710
$\delta^{15}N$ Uptake					
Liver	0.0046	(0.0042, 0.0049)	151 (141, 165)	651 (611, 713)	<0.0001
Blood	0.0018	(0.0015, 0.0021)	385 (330 462)	1,664 (1427, 1997)	<0.0001
Muscle	0.0012	(0.0007, 0.0013)	578 (533 990)	2,496 (2304, 4280)	<0.0001
$\delta^{15}N$ Elimination					
Liver	0.0305	(0.0256, 0.0356)	23 (19, 27)	98.2 (84, 117)	<0.0001
Blood	0.0100	(0.0051, 0.0144)	69 (48, 136)	300 (208, 587)	0.0013
Muscle	0.0066	(0.0031, 0.0088)	105 (79, 224)	454 (340, 966)	0.0008
$\delta^{15}N$ Uptake (Control)					
Liver	0.0058	(0.0052, 0.0064)	120 (108, 133)	517 (468, 576)	<0.0001
Blood	0.0020	(0.0017, 0.0023)	347 (301, 408)	1498 (1302, 1762)	<0.0001
Muscle	0.0019	(0.0017, 0.0020)	365 (347 408)	1577 (1498, 1762)	<0.0001

Table 2. Tissue-specific estimates of metabolic turnover (*m*) of $F^{15}N$ and $F^{13}C$ per day; model-based, non-parametric bootstrap 95% confidence intervals (bCI); and estimated days to 50% isotopic change (T_{50} with 95% CI) and 95% isotopic change (T_{95} with 95% CI) for corn snake liver, blood, and muscle between uptake and elimination phases. Estimates of *m* derived from least-squares regressions (nls) from the mass-balance model of Hesslein *et al.*[15] in snakes on a maintenance (no growth) diet; *p*-values are calculated t-tests of the parameter estimate being zero

Tissue	m (day^{-1})	bCI	T_{50} (days)	T_{95} (days)	*p*-value
$F^{13}C$ Uptake					
Liver	0.0203	(0.0143, 0.0284)	34 (48, 24)	148 (105, 210)	<0.0001
Blood	0.0080	(0.0051, 0.0121)	87 (57, 136)	375 (248, 587)	<0.0001
Muscle	0.0065	(0.0039, 0.0097)	107 (71, 178)	461 (309, 768)	<0.0001
$F^{13}C$ Elimination					
Liver	0.0123	(0.0086, 0.0167)	56 (42, 81)	244 (179, 348)	<0.0001
Blood	0.0051	(0.0030, 0.0076)	136 (91, 231)	587 (394, 999)	<0.0001
Muscle	0.0024	(0.0003, 0.0049)	289 (141, 2310)	1248 (611, 9986)	0.018
$F^{15}N$ Uptake					
Liver	0.0046	(0.0042, 0.0050)	151 (139, 165)	651 (599, 731)	<0.0001
Blood	0.0018	(0.0015, 0.0021)	385 (330, 462)	1664 (1427, 1997)	<0.0001
Muscle	0.0012	(0.0008, 0.0016)	578 (433, 866)	2496 (1872, 3745)	<0.0001
$F^{15}N$ Elimination					
Liver	0.0311	(0.0234, 0.0411)	22 (17, 30)	96 (73, 128)	<0.0001
Blood	0.0113	(0.0058, 0.0186)	61 (37, 120)	265 (161, 517)	<0.0001
Muscle	0.0079	(0.0042, 0.0124)	88 (56, 165)	379 (242, 713)	<0.0001
$F^{15}N$ Uptake (Control)					
Liver	0.0096	(0.0081, 0.0112)	72 (62, 86)	312 (267, 370)	<0.0001
Blood	0.0029	(0.0023, 0.0034)	239 (204, 301)	1033 (881, 1302)	<0.0001
Muscle	0.0026	(0.0023, 0.0029)	267 (239, 301)	1152 (1033, 1302)	<0.0001

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than in worms without gut contents; inclusion of the guts had no influence on stable isotope signature in worms that consumed soil that was not enriched in ^{15}N and ^{13}C. If this stable isotope-enriched soil in the worm guts was not assimilated in a similar manner as the worm tissue by the snakes, our estimate of X_{diet} is inaccurate and modeling results are confounded. However, the worms used in the snake experiment were given more than 4 h to defecate, which would reduce the amount and influence of the gut contents. There is also good evidence that the food given to the snakes, including the gut contents, was assimilated whole by the snakes. Although $\delta^{15}N$ values in the tissue of snakes did not approach values measured in the food, in a similar experiment with freshwater stingrays, $\delta^{15}N$ values in multiple tissues achieved a level that was similar to that in the worms used in this experiment (~200‰),[13] and these worms were not given time to defecate (i.e., had significant gut contents). Consistent results in estimates of m for $\delta^{15}N$ between the control and treatment groups in this study, where the magnitude of the difference in $\delta^{15}N$ between the snakes and diets were much different, were also observed. If the high $\delta^{15}N$ value in the treatment food was due to enriched nitrogen from the soil in the gut contents of the worms, and this was not assimilated by the snake, it would be highly unlikely that these estimates of m would agree so well between treatments. Thus, although enriched soil may have been present in the snake food, evidence would suggest that it was assimilated in a similar manner as worm tissue and that the X_{diet} and modeling results were not confounded by it.

Given our confidence in the assimilation of the enriched treatment diet, a second explanation for observed differences between uptake and elimination rates is that isotope discrimination factors may differ depending on the isotope concentration of the diet.[28] As we discuss below, the ultimate value for the isotope discrimination factor between an animal and its diet is thought to be a dynamic balance between the assimilation and catabolic breakdown of proteins in animal tissues.[29] If enriched diets have slower assimilation rates of ^{15}N relative to depleted diets (due to a greater proportion of molecules with higher bonding energies[30]) then we would expect a net reduction in the isotope discrimination factor of enriched diets. This would lead to lower estimated uptake rates as the X_{diet} value in the Hesslein model would be positively biased. Again, the consistent results between control and treatment estimates of m for $\delta^{15}N$ would suggest that the influence of this was minimal; however, it raises an important theoretical question about the physiology of isotope discrimination factors.

This is the first published report of which we are aware that has attempted to quantify metabolic turnover rates of stable isotopes in snakes; thus it is difficult to evaluate how representative the results are for snakes. However, a recent study examined $\delta^{13}C$ and $\delta^{15}N$ kinetics in neonate and juvenile loggerhead turtles, and this provides a good reference.[18] However, this turtle study only examined skin and blood components and no muscle or liver was analyzed. The snakes used in this experiment were neonatal and the metabolic turnover of stable isotopes may change with age. The maintenance diet also needs to be considered in interpreting these results because the metabolic routing of nitrogen and carbon between tissues may be different in a young growing snake from that in snakes in this experiment, and could influence the estimated turnover rates of $\delta^{13}C$ and $\delta^{15}N$.[31,32] Since the muscle tissue mass is much larger than that of the liver and blood, the effect of routing of stable isotopes between tissues is probably only significant from muscle to liver and blood. In addition, because the magnitude of the difference in $\delta^{15}N$ signatures of the diets was so great, the impact of tissue routing on observed metabolic turnover rates would be less and potentially insignificant.

Metabolic turnover rates of $\delta^{15}N$

Slower values of m, and thus longer times to dietary equilibrium for $\delta^{15}N$ observed in snake tissues during the uptake phase, than in the elimination phase, have also been reported in blood and liver from a similar experiment with a freshwater fish (*Potamotrygon motoro*).[13] However, turnover of $\delta^{15}N$ in muscle tissue of the freshwater rays was slower during the elimination than in the uptake phase, although the value for m for elimination was poorly estimated (i.e., $\delta^{15}N$ did not appreciably decline during the elimination phase).[13] Slower uptake than elimination of $\delta^{15}N$ has also been demonstrated in sockeye salmon (*Oncorhynchus nerka*), although this was not specifically tested or discussed.[17] Slower $\delta^{15}N$ turnover was also observed in loggerhead turtles during elimination versus an uptake feeding event, although these were not statistically compared in this study and were from separate groups of animals that varied in age.[18] Although these differences in turtles were not as large as those observed in this study, the authors concluded that growth by the turtles homogenized differences in stable isotope kinetic rates.[18]

The results of this study, and those from other studies,[13,17,18] demonstrate that the uptake-phase incorporation of ^{15}N-enriched compounds from the diet to a given tissue occurred at a considerably slower rate than the elimination-phase assimilation of ^{15}N-depleted compounds. In both phases (elimination and uptake), the stable isotope signatures of tissues are approaching a given equilibrium with the new diet. During uptake, where ^{15}N concentrations are higher in the food than in the animal, it may be that the assimilation of ^{15}N into tissues occurs at a slower rate than the assimilation of ^{14}N due to slower reaction kinetics. During elimination, where ^{15}N concentrations are lower in the food than in the animal, the situation could be reversed, and the assimilation of ^{14}N required to reach equilibrium with the new diet occurs more rapidly. The implication of these results is that the rate of non-growth nitrogen stable isotope turnover in a given tissue depends in part on the direction of a diet switch. The consistent results in m for $\delta^{15}N$ in the control and treatment groups, where the magnitudes of the difference in $\delta^{15}N$ between the snakes and diets were much different, support this contention. However, the aforementioned issues with depleted X_{diet} values (diet concentration dependence) present an alternative explanation that can only be distinguished through long-term controlled experiments run to steady state.

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The contributions of assimilation and metabolism are inherent in our estimate of *m* and the relationship between diet assimilation and metabolic processes has been suggested (theoretically) to explain why most animal tissues, when in steady state, are heavier in nitrogen than their diets.[33] While much of the debate surrounding isotope turnover in animal tissues has centered around the relative contributions of growth and metabolism,[13,18,34] the theoretical deductions of Ponsard and Averbuch[33] suggest that the mechanisms determining nitrogen ratios in tissues are primarily due to assimilation and metabolic processes. This point has been raised previously by Olive *et al*.[29] who stated that nitrogen enrichment in marine worms (*Neres virens*) reflected a dynamic equilibrium between assimilation and excretion (metabolism) factors. Olive *et al*.[29] also showed conceptually that the rate of nitrogen turnover is dependent on both the rate of isotopic excretion and the rate of kinetic isotope discrimination. Based on our own no-growth experiment in snakes, the relative importance of assimilation and metabolism versus growth in controlling the rate of change of δ^{15}N in an organism following a diet switch is highly dependent on the direction of the stable isotope change (i.e., elimination or uptake event to enriched or depleted diets) and tissue type, and on the growth rate of the organism, which can be much greater than *m*. Indeed, Reich *et al*.[18] concluded that growth was a major contributor to stable isotope change in growing turtles.

Metabolic turnover rates of δ^{13}C

Metabolic turnover rates for δ^{13}C in the snake tissues showed similar trends to those of δ^{15}N. Elimination rates were greater than uptake rates, with the exception of muscle for which values of *m* were similar for uptake and elimination. Values of *m* for δ^{13}C were greater than those estimated for δ^{15}N for all tissues, and thus the δ^{13}C value will reflect a change in diet by a snake more rapidly than δ^{15}N if growth is not significant. This is in contrast to studies with loggerhead turtles, where turnover rates of δ^{13}C and δ^{15}N were similar although not identical but were concluded to be homogenized by growth,[18] and thus metabolic differences between the isotopes might have been masked.

We are unaware of any studies that have examined both uptake and elimination of carbon stable isotopes in the same experiment with the same group of animals, which compromises any conclusions about how representative these results are for snakes or other organisms. In a study with loggerhead turtles, δ^{13}C turnover was found to be greater during elimination than during uptake but this varied with tissue type and the difference was not as large as seen in our study.[18] Further, in the loggerhead study the uptake and elimination experiments were in different groups of turtles of different ages. Ogden *et al*.[35] found that in birds the turnover rates of δ^{13}C and δ^{15}N were correlated and equal, although the birds were not growing, and the differences may relate to determinate versus indeterminate growth or metabolic differences between these groups of animals.

Differences in *m* between tissues

Liver tissues had the greatest values of *m* for both δ^{13}C and δ^{15}N, followed by blood and muscle. This is consistent with the metabolism of these tissues (i.e. liver is more metabolically active than blood or muscle) and other studies on stable isotope turnover in different tissues.[13,36,37]

Significance of varying metabolic turnover rates of stable isotopes

Upon a switch to a new diet, the δ^{13}C value in a snake would achieve equilibrium more quickly than δ^{15}N, and for both stable isotopes liver would achieve equilibrium before blood and muscle. Snakes would also achieve a tissue stable isotope value that was in equilibrium with food more quickly when switching to a diet that has a lower stable isotope signature, an *elimination* event, than to one that was higher, an *uptake* event. For example, assuming low growth (e.g. growth rates of adult rat snakes (*Elaphe obsoleta*) in Ontario were approximately 0.00003 day^{-1}),[38] it would take nearly 2500 days for δ^{15}N values in muscle tissue to come into equilibrium with a new diet that was higher in δ^{15}N, but liver would only take 651 days, and δ^{13}C in muscle and liver would take 285 and 205 days, respectively. Thus, as snakes get larger and begin to feed on new and larger items, at presumably higher trophic positions and δ^{15}N values, the δ^{15}N values in the muscle of snakes are unlikely to accurately reflect recent diet because of slow δ^{15}N turnover rates. In addition, snakes that eat a wide variety of items with varying stable isotope values are likely to have stable isotope values in their tissues that are skewed towards the lower δ^{15}N and more negative δ^{13}C items. MacAvoy *et al*.[39] also concluded that stable isotopes may underestimate a feeding shift in fish because of slow stable isotope turnover in tissues.

The significance of tissue type and direction of diet change on stable isotope kinetics in snakes could confound the use of stable isotopes to assess feeding ecology of wild snakes if not considered.[40,41] However, it is our contention, and that of others,[1,36,42] that this variability is more of an opportunity than a hindrance for understanding the feeding behavior of animals if these factors are considered. The most obvious and easily achieved application of this variability is stable isotope analysis of multiple tissues from individual organisms, which can indicate whether an organism is in equilibrium with its recent diet, has a fairly homogenous diet, and/or a recent shift in diet,[1,9,36,43] although differences in stable isotope signatures between tissues need to be considered.[8] If stable isotope values differ between tissues of an organism, this would suggest a recent diet shift or an omnivorous feeding strategy[40] and, depending on which tissues differed in which direction (an uptake or elimination event), estimates of the timing of recent diet change can be derived.

The variation in *m* across stable isotope and tissue type and diet switch direction has great potential to increase the sensitivity and precision of isotope source models, such as Isoconc and Isosource.[44–46] Such models, when properly validated for an organism, can provide a quantitative assessment of feeding ecology of an organism or population if some assumptions are met; for example, variation in stable isotope signatures between prey items. However, these models need to have stable isotope parameters, such as $\Delta\delta^{15}$N, that have been generated under controlled laboratory conditions.[1,3] In addition, the influence of environmental (e.g. temperature) and organismal (e.g. species) variation on

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these parameters, which can be significant,[47,48] has to be accounted for in these models.[49–51]

Enrichment of δ^{15}N and δ^{13}C from food to consumer

In most studies that utilize stable isotopes to assess feeding ecology there is a need to have isotopic discrimination factors ($\Delta\delta X = \delta X_{snake} - \delta X_{food}$, where X is either ^{13}C or ^{15}N) to assess trophic relationships between organisms in a food web. In most cases, a single discrimination factor for each stable isotope is used for all organisms, although it has been demonstrated that enrichment factors vary widely between species[4] and across changes in environmental conditions (e.g. feeding rate[51] and food quality[11]). Despite these potential pitfalls, isotopic discrimination factors can be used to quantitatively estimate feeding relationships in food webs. The isotope discrimination factor estimates for δ^{13}C snake tissues in this study (approximately 2‰ per trophic level) are the first reported for snakes, and could be assessed in the control study because they had achieved an apparent equilibrium or steady state with diet value during 144 days of feeding. Riech *et al*.[18] reported $\Delta\delta^{13}$C values of between 0.92 and 1.11 for skin and whole blood in growing loggerhead turtles, similar but lower values than those in this study, probably due to the influence of growth in the turtle experiment. Values of δ^{15}N in snake tissues did not achieve equilibrium with diet and thus enrichment factors could not be assessed.

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