

## Trophic Discrimination Factors and Incorporation Rates of Carbon- and Nitrogen-Stable Isotopes in Adult Green Frogs, *Lithobates clamitans*

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### ABSTRACT

Stable isotope analysis is an increasingly useful ecological tool, but its accuracy depends on quantifying the tissue-specific trophic discrimination factors (TDFs) and isotopic incorporation rates for focal taxa. Despite the technique's ubiquity, most laboratory experiments determining TDFs and incorporation rates have focused on birds, mammals, and fish; we know little about terrestrial ectotherms, and amphibians in particular are understudied. In this study we used two controlled feeding experiments to determine carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) isotope TDFs for skin, whole blood, and bone collagen and incorporation rates for skin and whole blood in adult green frogs, *Lithobates clamitans*. The mean ( $\pm$ SD) TDFs for  $\delta^{13}\text{C}$  were 0.1‰ ( $\pm$ 0.4‰) for skin, 0.5‰ ( $\pm$ 0.5‰) for whole blood, and 1.6‰ (0.6‰) for bone collagen. The mean ( $\pm$ SD) TDFs for  $\delta^{15}\text{N}$  were 2.3‰ ( $\pm$ 0.5‰) for skin, 2.3‰ ( $\pm$ 0.4‰) for whole blood, and 3.1‰ ( $\pm$ 0.6‰) for bone collagen. A combination of different isotopic incorporation models was best supported by our data. Carbon in skin was the only tissue in which incorporation was best explained by two compartments, which had half-lives of 89 and 8 d. The half-life of carbon in whole blood was 69 d. Half-lives for nitrogen were 75 d for skin and 71 d for whole blood. Our results help fill a taxonomic gap in our knowledge of stable isotope dynamics and provide ecologists with a method to measure anuran diets.

**Keywords:** trophic discrimination factor, stable isotopes, isotopic incorporation, Anura, amphibian.

### Introduction

The use of stable isotopes as natural resource tracers in ecological studies has exploded over the past 2 decades (Fry 2006; Newsome et al. 2007; Martínez del Rio et al. 2009b; Layman et al. 2012). Although stable isotope analysis is an informative tool, taxon-specific knowledge of two important isotope properties is required to use them accurately in diet analyses. The first property is the degree of isotopic discrimination that occurs between trophic levels (Vanderklift and Ponsard 2003; Fry 2006), creating an offset in the isotope value of the consumer's tissues relative to that of its prey. Such systematic and predictable offsets are often called trophic discrimination factors (TDFs; Tieszen et al. 1983; Hobson and Clark 1992b; Stegall et al. 2008), and knowing their values is essential to quantifying diet composition via the use of mixing models (Phillips et al. 2005; Moore and Semmens 2008; Parnell et al. 2010). Across vertebrate taxa, these TDFs typically range from  $-0.4\text{‰}$  to  $7.9\text{‰}$  for carbon and from  $-0.1\text{‰}$  to  $4.0\text{‰}$  for nitrogen (Caut et al. 2009), and they often differ among tissues, a phenomenon known as tissue-specific discrimination (Martínez del Rio et al. 2009b).

The second property is the rate of isotopic incorporation and how it differs among metabolically active tissues within a species (DeNiro and Epstein 1978; Tieszen et al. 1983; Martínez del Rio et al. 2009a) and among species with different metabolic demands (e.g., ectotherms versus endotherms; Bauchinger and McWilliams 2009; Warne et al. 2010). Some tissues, such as skin and blood plasma, have relatively fast isotopic incorporation rates and thus provide diet information integrated over short timescales (days to weeks) before collection (Martínez del Rio et al. 2009b). Other metabolically active tissues, such as bone collagen, incorporate isotopes very slowly (Martínez del Rio et al. 2009b) and thus provide diet information integrated over much longer timescales (years). Incorporation rates, however, can vary among species (Martínez del Rio et al. 2009b), and body size, growth rate, and protein turnover have been shown to affect isotopic incorporation rates (Carleton and Martínez del Rio 2005; MacAvoy et al. 2006; Martínez del Rio et al. 2009b; Murray and Wolf 2013). Thus, isotope-based ecological studies require species- and tissue-specific incorporation rates to estimate the time period to which the ecological information provided by stable isotope analysis pertains. With this infor-

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mation, stable isotope analysis becomes a valuable tool to make ecological inferences and to enable the use of different tissues to quantify dietary and/or habitat switches over time (Tieszen et al. 1983; Hobson and Clark 1992a; Martínez del Rio et al. 2009a).

Trophic discrimination factors and isotopic incorporation rates vary greatly among taxonomic groups (Vander Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003; Caut et al. 2009; Martínez del Rio et al. 2009b). While it is essential to evaluate stable isotope dynamics across diverse species, there is a taxonomic bias in research on TDFs and isotopic incorporation rates within the vertebrates. Most studies have focused on fish, birds, and mammals (fish: Bosley et al. 2002; Logan et al. 2006; Suring and Wing 2009; Carleton and Martínez del Rio 2010; Hussey et al. 2010; Logan and Lutcavage 2010; Nelson et al. 2011; Kim et al. 2012; Heady and Moore 2013; birds: Hobson and Clark 1992a, 1992b; Bearhop et al. 2002; Ogden et al. 2004; Chérel et al. 2005; Hobson and Yohannes 2007; Bauchinger and McWilliams 2009; Connan et al. 2014; mammals: Tieszen et al. 1983; Roth and Hobson 2000; Lesage et al. 2002; MacAvoy et al. 2006; Stegall et al. 2008; Florin et al. 2011; Browning et al. 2014). Recent studies have also investigated TDFs and incorporation rates in reptiles (Seminoff et al. 2007, 2009; Reich et al. 2008; Fisk et al. 2009; Warne et al. 2010; Murrar and Wolf 2013). However, amphibians have largely been ignored (Dalerum and Angerbjörn 2005; Trakimas et al. 2011). No studies have determined these values for adult anurans, and only one study has investigated these values in tadpoles (Caut et al. 2013). However, Caut et al. (2013) used whole tadpoles for their experiment and thus did not determine TDFs or incorporation rates for different tissues.

Here we determine TDFs of skin, whole blood, and bone collagen and isotopic incorporation rates of skin and whole blood in adult green frogs, *Lithobates clamitans*, using two controlled feeding experiments in which we fed captive frogs a diet of known isotopic composition. In the first experiment, we fed frogs this diet for a long enough period to be able to determine TDFs for the three tissues. In the second experiment, we tracked isotopic incorporation rates after frogs began eating the laboratory (cricket) diet. With the information provided by these experiments, we not only broaden the general knowledge of stable isotope dynamics in animals but also provide researchers with a method to determine diet at multiple time frames without lethal harm to anurans. Anurans are a globally threatened taxon (Stuart et al. 2004) that is stressed by habitat loss and degradation (Bonk and Pabijan 2010; Campos et al. 2013), climate change (Barrionuevo and Ponsa 2008; McCallum 2010; McCaffery et al. 2012; Murray et al. 2013), and disease (Kilpatrick et al. 2010). A nonlethal method to determine diets and potential diet and/or habitat shifts of frogs and toads brought about by environmental changes will likely prove to be a useful tool for ecologists.

## Material and Methods

### Frog Capture and Housing

We used hand nets to capture frogs in September 2011 and July 2013 at Horner Biological Reserve in Oldham County, Kentucky. We immediately placed the frogs inside plastic containers,

20 cm × 14 cm × 12 cm, with shredded coconut husk as substrate and a small bowl of distilled water treated with 0.75 g/gal of aquarium salt. We captured 33 frogs in 2011 and 27 frogs in 2013. At the University of Louisville we kept the frogs in a Precision Scientific low-temperature incubator 815 at 23°C with an average relative humidity of 55%. The light:dark cycle in the incubator was 16L:8D, which mimicked summer in Kentucky. We added more treated distilled water to frogs' water bowls when they were less than half full. The bowls were cleaned and refilled twice a month, and the entire container was cleaned and fresh substrate was provided once a month. All frogs were captured, handled, and housed under the approval of the Institutional Animal Care and Use Committee (UL-IACUC-11015).

### Sampling Procedure

Frogs captured on the first trip in 2011 were used in the first controlled feeding experiment to determine the TDFs of whole blood and skin. These frogs were fed one cricket per day for 470 d. Crickets were maintained in the lab on the same proprietary diet they were fed at the company from which we purchased them. The mean ( $\pm$  SD)  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of these crickets were  $-21.0\text{‰}$  ( $\pm 1.0\text{‰}$ ) and  $3.5\text{‰}$  ( $\pm 0.2\text{‰}$ ), respectively ( $N = 10$ ). We randomly selected 10 frogs and used them to determine the TDFs of whole blood collected from the lingual vein and skin collected from a toe clip. When performing these toe clips, we used the third digit on the front limbs, as this toe does not serve a specific purpose, such as digging or amplexus. Additionally, to alleviate stress and reduce pain, frogs were injected intracoelomically with the amphibian-safe analgesia flunixin meglumine.

Frogs from the second trip in 2013 were used in the second controlled feeding experiment to determine the isotopic turnover rates of whole blood, skin, and bone collagen and to estimate the TDFs for bone collagen (see "Statistical Analysis"). These frogs were fed two crickets a day that had mean ( $\pm$  SD)  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of  $-20.1\text{‰}$  ( $\pm 0.5\text{‰}$ ) and  $4.6\text{‰}$  ( $\pm 0.4\text{‰}$ ), respectively ( $N = 15$ ). The stable isotope values of the crickets were measured at the beginning ( $N = 10$ ) and near the end of the 256-d experiment ( $N = 5$ ), and these values showed that the isotope values remained constant over the course of the experiment ( $\delta^{13}\text{C}$ ,  $t = -0.223$ ,  $df = 12.63$ ,  $P = 0.83$ ;  $\delta^{15}\text{N}$ ,  $t = -0.973$ ,  $df = 12.63$ ,  $P = 0.35$ ). Because our sampling procedure included a toe clip, we could not sample individual frogs more than twice. We accordingly designed an experimental setup that allowed us to sample each frog twice, once in the first four of the eight sampling days and once in the latter four. Frogs were randomly divided into four groups of six, with three frogs remaining to replace any frogs that died during the experiment. On a sampling day, all six frogs in a particular group were sampled, except on day 32 when only four frogs were sampled because of high mortality likely associated with acclimating to the laboratory and incubator. Frogs from the first group were sampled on day 0 (the day they were captured) and day 32 after the switch to the laboratory diet. Frogs from the second group were sampled on days 4 and 64 after switching to

the laboratory diet. Frogs from the third group were sampled on days 8 and 128 and frogs from the fourth group on days 16 and 256. In total, frogs were sampled on days 0, 4, 8, 16, 32, 64, 128, and 256 after the diet switch.

#### Stable Isotope Analysis

Whole blood and clipped toes were dried in the oven at 60°C for ~48 h. We removed the skin manually and then separated ligaments and tendons from the bone. Bone samples from the toe clips were demineralized in 0.5 N hydrochloric acid in a refrigerator for ~24 h, after which they were dried in an oven for 48 h at 60°C. We lipid extracted bone collagen via three 24-h soaks in a 2:1 chloroform:methanol solution, after which the bone collagen was thoroughly rinsed in distilled water and dried in the oven for ~48 h at 60°C. We weighed subsamples of whole blood, skin, and bone collagen to ~0.5 mg on a Mettler Toledo AG245 microscale and placed them in 5 × 3.5-mm tin capsules.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  isotope values were measured at the University of New Mexico Center for Stable Isotopes (Albuquerque). The samples were combusted in a Costech 4010 elemental analyzer (Costech, Valencia, CA) coupled to a Thermo Scientific Delta V mass spectrometer (Thermo Scientific, Bremen, Germany). Stable isotope values are expressed using delta notation ( $\delta$ ) in parts per thousand (‰), where  $\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1,000$ , with  $R_{\text{sample}}$  and  $R_{\text{standard}}$  indicating the molar ratios of  $\text{C}^{13}/\text{C}^{12}$  and  $\text{N}^{15}/\text{N}^{14}$  of the sample and the standard reference material, respectively. The reference material was Vienna–Pee Dee belemnite for carbon and atmospheric  $\text{N}_2$  for nitrogen. Repeated analysis of in-house reference materials of similar composition as the tissue we analyzed showed that precision (SD) for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values was  $\pm 0.2\text{‰}$ .

#### Statistical Analysis

We calculated TDFs as the difference between the  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  value of the consumer's tissues and that of its diet, for example,  $\delta^{13}\text{C}_{\text{tissue}} - \delta^{13}\text{C}_{\text{diet}}$ , which is also commonly denoted as  $\Delta^{13}\text{C}_{\text{tissue} - \text{diet}}$ . We used an ANOVA and a post hoc Tukey's honest significant differences test to examine differences between the TDFs of each tissue type. The TDF of bone collagen was estimated from the second controlled feeding experiment. To derive this estimate, we first had to determine the isotopic values of the wild frog diet. The isotopic values of wild green frogs varied little over time ( $\delta^{13}\text{C}$  season,  $t = 1.27$ ,  $\text{df} = 63$ ,  $P = 0.21$ ;  $\delta^{13}\text{C}$  year,  $t = -0.14$ ,  $\text{df} = 63$ ,  $P = 0.89$ ;  $\delta^{15}\text{N}$  season,  $t = 1.67$ ,  $\text{df} = 63$ ,  $P = 0.10$ ;  $\delta^{15}\text{N}$  year,  $t = -1.66$ ,  $\text{df} = 63$ ,  $P = 0.10$ ;  $N = 75$  frogs), and we assumed that any differences in isotopic values across tissue types should thus have resulted from tissue differences in TDFs. Accordingly, to determine the wild frog diet, we subtracted the TDFs of C and N for skin and blood from the isotope values of these tissues in group 1 on day 0 (i.e., the tissue values based on wild diet); we averaged these two values for C and N to obtain the baseline isotopic values for wild frogs' diet. We then determined the TDF of bone collagen by finding the difference be-

tween the bone collagen isotope values of frogs from group 1 on day 0 and the calculated resource baselines. We used those TDFs to estimate the final stable isotope value for bone collagen by adding the TDFs of bone collagen to the stable isotope values of the laboratory cricket diet ( $\delta^{13}\text{C} = -20.1\text{‰}$  and  $\delta^{15}\text{N} = 4.6\text{‰}$ ).

To model isotopic incorporation we followed the procedure of Cerling et al. (2007) and Martínez del Rio and Anderson-Sprecher (2008). We first calculated the reaction progress variable, which can help determine what type of model best fits the incorporation data. We also used the slopes of these reaction progress variables as starting values of  $k$  and  $f$  (see equations below) in iterative, nonlinear fitting routines. We constructed three models per tissue for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . The first was a one-compartment model,

$$\delta X_t = \delta X_\infty - (\delta X_\infty - \delta X_0)e^{-kt}, \quad (1)$$

where  $\delta X_t$  is the isotopic composition at time  $t$ ,  $\delta X_\infty$  is the isotopic composition at equilibrium on the new diet,  $\delta X_0$  is the initial stable isotope composition before the switch to a captive diet, and  $k$  is the fractional rate of isotopic incorporation. The second was a two-compartment model,

$$\delta X_t = \delta X_\infty - (\delta X_\infty - \delta X_0)\{pe^{-kt} + [(1-p)e^{-ft}]\}, \quad (2)$$

where  $p$  is the fractional contribution to the first compartment and  $f$  is the fractional rate of isotopic incorporation for the second compartment. The third was a delayed-response model, in which there is a measurable delay  $d$  in days between the diet switch and the incorporation of new isotopes into the tissue,

$$\delta X_t = \delta X_\infty - (\delta X_\infty - \delta X_0)e^{-k(t-d)}. \quad (3)$$

To determine which of the three models best fitted the data, we used Akaike information criterion corrected for small sample size ( $\text{AIC}_c$ ; Burnham and Anderson 2002). The model that produced the smallest  $\text{AIC}_c$  value was considered the model of best fit. We used a linear model to test whether frogs grew during our experiment and found that frogs did not increase in weight between the day they were captured and the second day on which they were sampled ( $F = 0.0004$ ,  $\text{df} = 1, 20$ ,  $P = 0.98$ ). We therefore did not consider growth in our models and assumed that all change was due to tissue maintenance. We calculated the half-life values of each tissue as  $\ln(2)/k$  (Cerling et al. 2007). To test for differences in isotopic incorporation rate between tissues, we ran two linear models in which  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were the response variables and sample day, tissue type, and a sample day/tissue type interaction were the explanatory variables. All statistical tests and calculations were performed in R (R Core Development Team 2013).

## Results

### Trophic Discrimination Factors: Controlled Feeding Experiment 1

For  $\delta^{13}\text{C}$ , skin had the lowest TDF and bone collagen the highest (table 1). There was a significant overall difference in

Table 1: Isotopic incorporation curve of best fit (lowest AIC<sub>c</sub>), half-lives, and trophic discrimination factors ( $\Delta$ ) for skin, whole blood, and bone collagen in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$

	Equation of best fit	$t_{1/2}$ (d)	$\Delta$ ( $\pm$ SD)
$\Delta^{13}\text{C}$ :			
Skin	$-19.9 - 4.9[(.82e^{-.007816t}) + (.18e^{-.087446t})]$	88.7, <sup>a</sup> 7.6 <sup>b</sup>	.1 (.4)
Whole blood	$-19.7 - 4.7e^{-.009998(t-4)}$	69.3	.5 (.5)
Bone collagen			1.6 (.6) <sup>c</sup>
$\Delta^{15}\text{N}$ :			
Skin	$6.9 - 2.6e^{-.009285t}$	74.65	2.3 (.5)
Whole blood	$6.3 - 2.2e^{-.00979(t-8)}$	70.8	2.3 (.4)
Bone collagen			3.1 (.6) <sup>c</sup>

Note. AIC<sub>c</sub> = Akaike information criterion corrected for small sample size.

<sup>a</sup>First compartment.

<sup>b</sup>Second compartment.

<sup>c</sup> $\Delta$  for bone collagen was estimated by establishing a resource baseline before capture with isotope values of skin and whole blood and finding the difference between those baselines and the day 0 samples for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in bone collagen.

TDFs among tissues for  $\delta^{13}\text{C}$  ( $F = 18.58$ ,  $df = 2, 22$ ,  $P < 0.001$ ). The TDFs for  $\delta^{13}\text{C}$  differed significantly between whole blood and bone collagen ( $P < 0.001$ ) and between skin and bone collagen ( $P < 0.001$ ). There was no significant difference in TDFs between whole blood and skin ( $P = 0.199$ ). For  $\delta^{15}\text{N}$ , blood and skin had similar TDFs, which were lower than the TDF for bone collagen (table 1). There was a significant overall difference among TDFs for  $\delta^{15}\text{N}$  ( $F = 6.218$ ,  $df = 2, 22$ ,  $P = 0.007$ ). The TDFs were significantly higher in bone collagen than in whole blood ( $P = 0.009$ ) or skin ( $P = 0.012$ ) but did not differ significantly between whole blood and skin ( $P = 0.992$ ).

*Isotopic Incorporation Rates: Controlled Feeding Experiment 2*

For  $\delta^{13}\text{C}$ , whole blood had the fastest incorporation rate followed by skin (tables 1, 2; fig. 1). For skin and whole blood,  $\delta^{13}\text{C}$  values of the samples collected on day 256 were within a standard deviation's length of the mean TDFs from the resource (tables 1, 2), indicating that they were approaching their equilibrium values. A two-compartment model best described isotopic incorporation in skin (table 3). A delayed-response model best described whole blood (table 3), where the delay was 4 d (table 1). The global linear model that tested for differences in  $\delta^{13}\text{C}$  values across tissues and

over time was significant ( $F = 286.5$ ,  $df = 2, 84$ ,  $P < 0.001$ ,  $R^2 = 0.869$ ). Values of  $\delta^{13}\text{C}$  changed significantly over the course of the experiment ( $t = 23.937$ ,  $P < 0.001$ ). Carbon isotopes in whole blood were not incorporated significantly faster than in skin ( $t = -0.319$ ,  $P = 0.751$ ).

Similar to  $\delta^{13}\text{C}$  values,  $\delta^{15}\text{N}$  values of whole blood had the fastest incorporation rate followed by skin (tables 1, 2; fig. 2). The final  $\delta^{15}\text{N}$  values for skin and whole blood were within or close to 1 SD of the mean TDF from the resource. One-compartment models best described nitrogen isotope incorporation for skin (table 3), while a delayed-response model best fitted the data for whole blood. However, the difference in  $\Delta\text{AIC}_c$  between the delayed-response model and the one-compartment model for  $\delta^{15}\text{N}$  in all tissues was less than 2 (table 3). The global linear model for  $\delta^{15}\text{N}$  was significant ( $F = 108.5$ ,  $df = 2, 84$ ,  $P < 0.001$ ,  $R^2 = 0.714$ ), and  $\delta^{15}\text{N}$  changed significantly over the course of the experiment ( $t = 14.28$ ,  $P < 0.001$ ). Nitrogen isotopes were not incorporated significantly faster in whole blood than in skin ( $t = 0.443$ ,  $P = 0.659$ ).

**Discussion**

We found that diet could be traced via  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in adult anurans. The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  TDFs for bone collagen were sig-

Table 2: Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in skin and whole blood over the course of the feeding experiment

Sample day	Skin $\delta^{13}\text{C}$ mean ( $\pm$ SD)	Whole blood $\delta^{13}\text{C}$ mean ( $\pm$ SD)	Skin $\delta^{15}\text{N}$ mean ( $\pm$ SD)	Whole blood $\delta^{15}\text{N}$ mean ( $\pm$ SD)
0	-24.8 (.5)	-24.5 (.5)	4.3 (.7)	4.1 (.6)
4	-24.8 (.6)	-24.6 (.4)	4.2 (.2)	4.2 (.5)
8	-24.0 (.2)	-24.2 (.2)	4.7 (.6)	4.2 (.7)
16	-23.4 (.3)	-23.9 (.1)	4.8 (.3)	4.5 (.4)
32	-23.2 (.6)	-23.7 (.5)	4.7 (.5)	4.1 (.3)
64	-22.4 (.4)	-22.3 (.6)	5.0 (.3)	4.8 (.8)
128	-21.7 (.5)	-21.0 (.3)	6.4 (.8)	6.0 (.3)
256	-19.9 (.2)	-19.7 (.4)	6.9 (.4)	6.3 (.3)



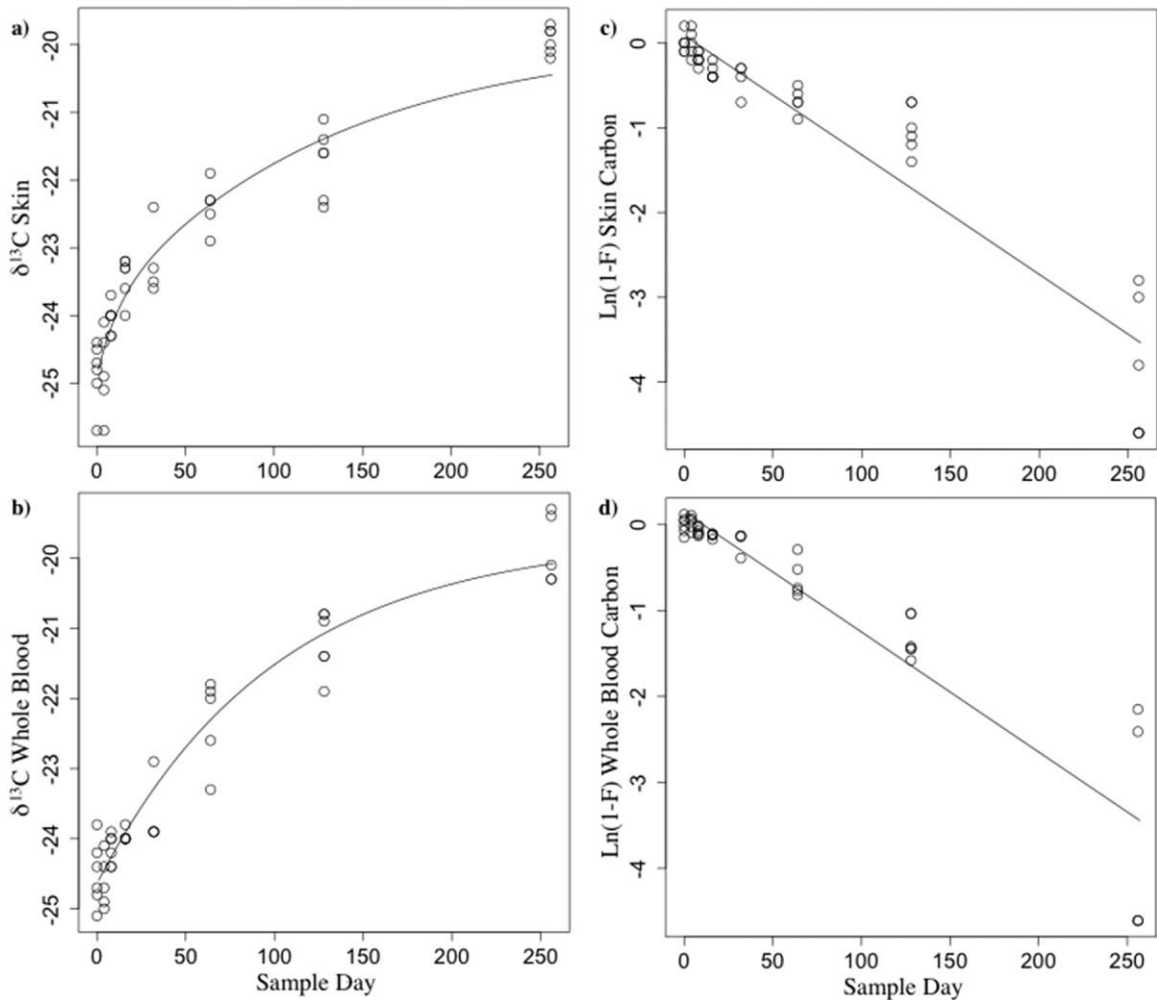


Figure 1. Isotopic incorporation curves for  $\delta^{13}\text{C}$  in skin (a; two-compartment model) and whole blood (b; delayed-response model). Diagnostic reaction progress variable,  $\ln(1 - F)$ , for skin (c) and whole blood (d). See table 1 for regression equations and half-lives.

nificantly greater than those for skin and whole blood (table 1). The latter two tissues did not differ significantly from each other for either  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  TDFs (table 1). Isotopic incorporation rates did not differ statistically between skin and whole blood of frogs for either  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$ , although isotopic incorporation rates were slightly faster in whole blood than in skin (table 1; figs. 1, 2). In our study species, we estimated that whole blood and skin integrate diet information from  $\sim 2\text{--}3$  mo before capture (table 1; figs. 1, 2). Thus,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  can be used to measure diet of wild-caught anurans.

#### Trophic Discrimination Factors

Trophic discrimination factors can vary greatly among taxonomic groups and even closely related species (Vander Zanden and Rasmussen 2001; Vanderklift and Ponsard 2003). In our experiment, TDFs for  $\delta^{13}\text{C}$  in all tissues were within the range of those for other freshwater and predator species, but these values

vary widely (Vander Zanden and Rasmussen 2001). Compared to other terrestrial ectotherms, our estimated  $\delta^{13}\text{C}$  TDF for skin was higher than that of the lizard species *Sceloporus undulatus* and *Crotaphytus collaris* (Warne et al. 2010) but lower than that of corn snakes, *Elaphe guttata* (Fisk et al. 2009). Our  $\delta^{13}\text{C}$  TDF for skin is much lower than that of two species of marine turtles, *Caretta caretta* (Reich et al. 2008) and *Dermochelys coriacea* (Seminoff et al. 2009). Our TDFs for  $\delta^{13}\text{C}$  in whole blood were lower than the TDFs for this tissue in corn snakes (Fisk et al. 2009), but they are similar to those of the marine turtles (Reich et al. 2008; Seminoff et al. 2009).

The primary reason TDFs for  $\delta^{13}\text{C}$  differ among tissues is variation in tissue amino acid composition (Vander Zanden and Rasmussen 2001; O'Brien et al. 2002, 2005; Howland et al. 2003; McMahon et al. 2010; Newsome et al. 2014). Essential amino acids undergo little, if any, alteration during assimilation and metabolism and therefore exhibit small differences in  $\delta^{13}\text{C}$  between consumers and resources (O'Brien et al. 2002; Howland et al. 2003;

Table 3: AIC<sub>c</sub> and ΔAIC<sub>c</sub> values for each model across both isotope types and all tissues types

Tissue and model	AIC <sub>c</sub>	ΔAIC <sub>c</sub>
<b>δ<sup>13</sup>C:</b>		
Skin:		
One compartment	72.62	6.48
<b>Two compartment</b>	<b>66.14</b>	<b>0</b>
Delayed response	80.54	14.4
Whole blood:		
One compartment	55.75	6.76
Two compartment	58.37	9.38
<b>Delayed response</b>	<b>48.99</b>	<b>0</b>
<b>δ<sup>15</sup>N:</b>		
Skin:		
<b>One compartment</b>	<b>73.35</b>	<b>0</b>
Two compartment	75.66	2.31
Delayed response	73.69	<u>.34</u>
Whole blood:		
One compartment	82.71	<u>1.54</u>
Two compartment	85.03	3.86
<b>Delayed response</b>	<b>81.17</b>	<b>0</b>

Note. Rows that are in bold indicate best-fitting models for that tissue and isotope type. The ΔAIC<sub>c</sub> values underlined are close enough to the values of models with lowest AIC<sub>c</sub> that the two models may fit the data equally well. AIC<sub>c</sub> = Akaike information criterion corrected for small sample size.

McMahon et al. 2010). Nonessential amino acids, on the other hand, can be synthesized de novo by prokaryotic consumers, and isotopic fractionation associated with their alteration and synthesis results in large variation in δ<sup>13</sup>C values in both glucogenic and ketogenic amino acids (Hare et al. 1991; O'Brien et al. 2002; Howland et al. 2003; McMahon et al. 2010; Newsome et al. 2011, 2014). Thus, δ<sup>13</sup>C values of different tissues and their associated TDFs vary depending on the relative contribution of essential and nonessential amino acids in each tissue type. In mammals, proteinaceous tissues are composed of a greater percentage of nonessential (60%–72%) than essential (28%–40%) amino acids (Newsome et al. 2014). As a result, the isotopic composition of nonessential amino acids likely plays a stronger role in determining TDFs, particularly for carbon isotopes.

We do not have a good understanding of the amino acid composition of frog tissues. However, two studies showed that skin peptides of two ranids, *Lithobates palustris* and *Rana dybowskii*, were rich in the essential amino acids arginine and leucine (Basir et al. 2000; Jin et al. 2009) and contained several other essential amino acids including threonine and valine (Basir et al. 2000). The presence of many of these essential amino acids in frog skin provides a possible mechanism for this tissue's small TDF. Similarly, in whole blood a greater concentration of the essential amino acids compared to the nonessential could explain why the whole-blood TDF in our frogs was small, and future studies that investigate the amino acid composition of frog blood across taxa would be useful. Bone collagen in frogs has a relatively greater amount of nonessential amino acids, such as alanine, proline,

serine, and glutamate (Dohi et al. 2004). The greater proportion of nonessential amino acids in bone is consistent with the larger TDF in bone in our frogs.

The TDFs for δ<sup>15</sup>N in *Lithobates clamitans* were similar to those of other ureotelic species (Vanderklift and Ponsard 2003). Most of the previous studies on terrestrial ectotherms and marine reptiles did not examine nitrogen isotopes, making comparisons difficult. However, TDFs for δ<sup>15</sup>N in whole blood of our green frogs were nearly identical to those of freshwater turtles (Seminoff et al. 2007). In our study, there was a greater similarity in nitrogen TDFs than in carbon TDFs when comparing across tissues, which is a common trend in many vertebrates (Bearhop et al. 2002; Lesage et al. 2002; Seminoff et al. 2009). Consistent with other species (Vanderklift and Ponsard 2003; Caut et al. 2009), the TDFs for bone collagen were higher than those for skin and whole blood for both δ<sup>15</sup>N and δ<sup>13</sup>C, but the difference between bone collagen and the other tissues was much less for nitrogen than for carbon isotopes (table 1). The similarity of the TDFs for δ<sup>15</sup>N among ureotelic species is consistent with the idea that the mode of excretion of nitrogenous waste drives these TDFs (Vanderklift and Ponsard 2003); however, mode of excretion does not explain differences in TDFs across tissue types, which may instead result from differences among amino acids.

For δ<sup>15</sup>N analysis, individual amino acids are typically grouped into two categories, trophic and source amino acids (McClelland and Montoya 2002; Schmidt et al. 2004; Popp et al. 2007; Lorrain et al. 2009). Similar to the nonessential and essential amino acid categories often associated with δ<sup>13</sup>C analysis, trophic amino acids often have large discrimination values, and source amino acids have little to no discrimination (McClelland and Montoya 2002; Schmidt et al. 2004; Lorrain et al. 2009). Trophic and source amino acids can vary among species (Bloomfield et al. 2011; Hoen et al. 2014), and in order to determine which amino acids are trophic and which are source, compound-specific stable isotope analysis will need to be performed on *L. clamitans*. Such analysis may illuminate why TDFs for δ<sup>15</sup>N differ little among tissues relative to those for δ<sup>13</sup>C.

#### Isotopic Incorporation Rates

We found that whole blood had the fastest incorporation rates for both δ<sup>13</sup>C and δ<sup>15</sup>N, but they were similar to the rates in skin for both isotope systems (tables 1, 2; figs. 1, 2). Given that frogs are indeterminate growers and that healthy, wild frogs will grow slowly over their lifetime, isotopic incorporation would occur during that growth. However, since frogs did not grow appreciably during our experiment, we hypothesize that all of the observed isotopic incorporation was associated with tissue maintenance. Protein turnover rate, and not basal metabolic rate, has been shown to be the principal factor in controlling isotopic incorporation among species (Bearhop et al. 2002; Carleton et al. 2008; Martínez del Río et al. 2009b). Our results corroborate others' findings that adult and slow-growing ectotherms have lower incorporation rates than similarly sized adult endotherms (Hobson and Clark 1992a; Bearhop et al. 2002;

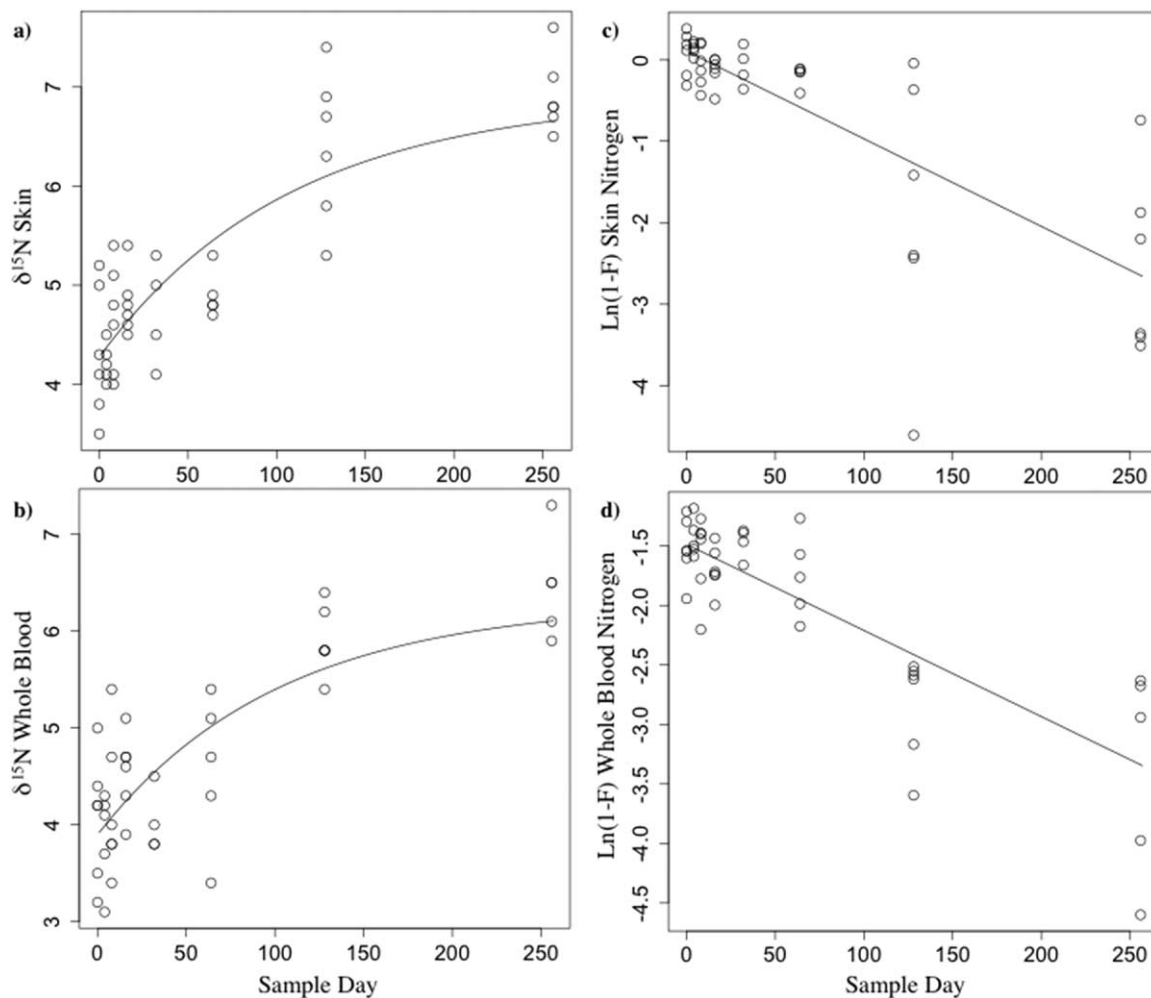


Figure 2. Isotopic incorporation curves for  $\delta^{15}\text{N}$  in skin (a; one-compartment model) and whole blood (b; delayed-response model). Diagnostic reaction progress variable,  $\ln(1 - F)$ , for skin (c) and whole blood (d). See table 1 for regression equations and half-lives.

Seminoff et al. 2007; Fisk et al. 2009; Murray and Wolf 2013; Browning et al. 2014).

Many studies have shown similar incorporation rates for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , including studies of loggerhead turtles, *C. caretta* (Reich et al. 2009); great skuas, *Catharacta skua* (Bearhop et al. 2002); and winter flounder, *Pseudopleuronectes americanus* (Bosley et al. 2002). In our study, isotopic incorporation rates of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were similar in skin and whole blood. Others have found that  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  can be incorporated at different rates. In juvenile corn snakes, *E. guttata*, for example,  $\delta^{15}\text{N}$  was incorporated at one-third the rate of  $\delta^{13}\text{C}$  in liver, one-fifth the rate in blood, and one-tenth the rate in muscle (Fisk et al. 2009). Natterjack toad, *Bufo calamita*, tadpoles incorporated  $\delta^{15}\text{N}$  at half the rate of  $\delta^{13}\text{C}$ , while western spadefoot, *Pelobates cultripes*, tadpoles had different incorporation rates of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  depending on diet composition (Caut et al. 2013). On a diet of zooplankton, *P. cultripes* tadpoles also incorporated  $\delta^{15}\text{N}$  at two-thirds the rate of  $\delta^{13}\text{C}$ , but when fed macrophytes and algae, they incorporated  $\delta^{13}\text{C}$  at half the rate and  $\delta^{15}\text{N}$  at one-fifth the rate, respectively (Caut et al. 2013). These

results suggest that  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  may not be incorporated in the same way by all species and that diet may influence the relative incorporation rates of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in different tissues.

Few studies have used the reaction progress variable and  $\text{AIC}_c$  to determine best-fitting models to quantify isotopic incorporation rates. While these studies frequently find that one-compartment-type models best fit the data, it has become clear that more than one type of model is often needed to best explain isotopic incorporation across several tissues even in a single species (Carleton et al. 2008; Kurle 2009; Warne et al. 2010; Heady and Moore 2013; Murray and Wolf 2013). No one type of model consistently fitted our data best (table 3), although one-compartment and delayed-response models generally performed better than two-compartment models. The incorporation rate of  $\delta^{13}\text{C}$  for skin was the only rate that was best fitted by a two-compartment model (table 3; fig. 1). A delayed-response model best fitted  $\delta^{13}\text{C}$  trends in whole blood. For  $\delta^{15}\text{N}$ , a delayed-response model again best fitted the data for whole blood, while a one-compartment model best fitted skin; however, the  $\Delta\text{AIC}_c$  values

were less than 2 between these models (table 3), suggesting that for  $\delta^{15}\text{N}$  the one-compartment and delayed-response models may explain the data equally well. Overall, the  $\Delta\text{AIC}_c$  values are much smaller for  $\delta^{15}\text{N}$  than for  $\delta^{13}\text{C}$ , a difference that may be driven by a larger variance in the  $\delta^{15}\text{N}$  data (table 2; fig. 2). This larger variance suggests that individual variation may be greater for  $\delta^{15}\text{N}$  than for  $\delta^{13}\text{C}$  incorporation rates.

While laboratory studies have recently used multicompartment models in describing isotopic incorporation, less is known about how to interpret these models in ecological field studies. Interpreting single-compartment models is intuitive, but how are the two half-lives of two-compartment models combined so the ecologists can draw meaningful conclusions regarding a relevant time frame? Carleton et al. (2008) suggested weighting the half-lives, such that each half-life is multiplied by  $p$  or  $1 - p$  from equation (3) before being summed. Applying the delayed-response models should be similar to the one-compartment models, where the half-life is applied directly. In the delayed-response models, the fractional rate of isotopic incorporation,  $k$ , is higher than in respective one-compartment models, suggesting that the delay increases  $k$ , and the delay does not need to be added to the half-life to obtain a suitable time frame for the stable isotope data.

Our examination of TDFs and isotopic incorporation rates in adult anurans provides useful information for understanding stable isotope dynamics. We help fill a taxonomic gap in our knowledge of TDFs and isotopic incorporation rates. Our results also highlight the complicated process of isotopic incorporation even within one species, where different tissues and isotope types can be best described by different models. Future studies on the stable isotope dynamics in anurans can test for differences in TDFs and incorporation rates between tadpoles and adults, for differences among seasons or climates, and for differences between the sexes, particularly when females are preparing to lay eggs. Our study has provided a method for ecologists to measure and monitor anuran diets. Globally, anurans face many problems and are recognized as one of the most imperiled taxa on the planet (Stuart et al. 2004). Anurans are especially vulnerable to novel diseases (Kilpatrick et al. 2010), habitat degradation/loss, and climate change (Barrionuevo and Ponssa 2008; Bonk and Pabijan 2010; McCallum 2010; McCaffery et al. 2012; Campos et al. 2013; Murray et al. 2013). Using stable isotope analyses on skin and whole blood will allow researchers to identify any dietary response to changes such as habitat loss if the frogs are repeatedly sampled at appropriate times. Researchers could also use this technique to characterize any habitat shifts that occur in response to habitat change or loss. Furthermore, our results show that researchers can get all this information from a simple toe clip and do not need to obtain any blood, which can be difficult for many small species.

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